Interaction of Hepatitis C Virus-Like Particles and Cells: a Model System for Studying Viral Binding and Entry


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Hepatitis C virus-like particles (HCV-LPs) containing the structural proteins of HCV H77 strain (1a genotype) was used as a model for HCV virion to study virus-cell interaction. HCV-LPs showed a buoyant density of 1.17 to 1.22 g/cm³ in a sucrose gradient and formed double-shelled particles 35 to 49 nm in diameter. Flow cytometry analysis by an indirect method (detection with anti-E2 antibody) and a direct method (use of dye-labeled HCV-LPs) showed that HCV-LPs binds to several human hepatic (primary hepatocytes, HepG2, HuH7, and NKNT-3) and T-cell (Molt-4) lines. HCV-LPs binding to cells occurred in a dose- and calcium-dependent manner and was not mediated by CD81. Scatchard plot analysis suggests the presence of two binding sites for HCV-LPs with high (Kd ~1 µg/ml) and low (Kd ~50 to 60 µg/ml) affinities of binding. Anti-E1 and -E2 antibodies inhibited HCV-LPs binding to cells. While preincubation of HCV-LPs with very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), or high-density lipoprotein (HDL) blocked its binding to cells, preincubation of cells with VLDL, LDL, HDL, or anti-LDL-R antibody did not. Confocal microscopy analysis showed that, after binding to cells, dye-labeled HCV-LPs were internalized into the cytoplasms. This process could be inhibited with anti-E1 or anti-E2 antibodies, suggesting that E1 and E2 proteins mediate HCV-LPs binding and, subsequently, their entry into cells. Altogether, our results indicate that HCV-LPs can be used to further characterize the mechanisms involved in the early steps of HCV infection.
that by use of the truncated E2 protein alone may not accurately reflect interaction of HCV virion with cells. Both E1 and E2 glycoproteins are known to associate in two types of complexes: (i) heterodimers stabilized by noncovalent bonds, which presumably represents the prebinding form of the viral envelope, and (ii) high-molecular-mass disulfide-bonded aggregates representing the misfolded proteins (8, 11, 13). Indeed, using a pseudotype vesicular stomatitis virus (VSV) expressing either E1 or E2 protein, it has been shown that both proteins are required for efficient infection and fusion into target cells (29, 45). Furthermore, the HCV virion binds to mononuclear cell lines regardless of their CD81 expression, whereas recombinant E2 protein binds poorly because of the lack of CD81 (20).

In this study, we use hepatitis C virus-like particles (HCV-LPs) derived from H77 (1a genotype) (27) to determine whether HCV-LPs can be used as a model for studying the early events (binding and entry) of HCV infection. Our study demonstrates that HCV-LPs can gain entry into susceptible cells independent of CD81 and LDL receptor pathways.

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MATERIALS AND METHODS

Reagents and antibodies. Bovine serum albumin (BSA), very-low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) were obtained from Sigma (St. Louis, Mo.). Coomassie Plus protein assay reagent was from Pierce, Rockford, Ill. Lipophilic dye [CellTracker CM-Dil, a chloromethylene-benzamido derivative of Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine) and] SYTO 12 (SYTO) nucleic acid stain were from Molecular Probes (Eugene, Ore.), and sterile glass chamber slides (Lab-Tek II) were from Nalge Nunc International (Rochester, N.Y.). Soluble glutathione S-transferase fusion protein of the large extracellular loop of human CD81 (LEL-CD81) (17) was a gift from S. Levy (Stanford, Calif.). Flow cytometric analysis was performed on a FACS Calibur apparatus (Becton Dickinson). The peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgM were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, Md.). Rabbit anti-human LDL-R IgG was from Maine Laboratories (Hartford, Me.). Mouse anti-human CD81 IgG1 was a gift from S. Levy (Stanford, Calif.). Flow cytometric analysis was performed on a FACSCalibur apparatus (Becton Dickinson). The peroxidase-labeled goat anti-mouse IgG (4 μg/ml) for 30 min. Cell-bound HCV-LPs was analyzed by flow cytometry. Nonspecific fluorescence was measured by adding primary and secondary antibodies in the absence of HCV-LP to cells. The mean fluorescence intensity (MFI) of bound HCV-LP was determined after subtraction of the nonspecific fluorescence value.

In the direct labeling, HCV-LPs were labeled with SYTO (nucleic acid dye) or CM-Dil (lipophilic dye) according to the manufacturer’s protocol. HCV-LPs were incubated with a 5 μM concentration of SYTO or a 1 to 5 μM concentration of CM-Dil in TNC buffer at 4°C for 15 min and then repurified through a 30% sucrose cushion to remove the free dye. Cells were incubated with increasing concentrations of labeled HCV-LPs for 2 h at 4°C and washed twice, and then the cells were incubated with anti-E1/E2 antibodies (AP33 and ALP98) for 1 h, followed by FITC-labeled goat anti-mouse IgG (4 μg/ml) for 30 min. Cell-bound HCV-LPs was analyzed by flow cytometry. Nonspecific fluorescence was measured by adding primary and secondary antibodies in the absence of HCV-LP to cells. The mean fluorescence intensity (MFI) of bound HCV-LP was determined after subtraction of the nonspecific fluorescence value.

Effect of anti-E1/E2 antibodies, CD81, VLDL, LDL, and HDL on HCV-LP binding. SYTO-labeled HCV-LPs were preincubated with increasing amounts of anti-E2 (AP33 and ALP98), anti-E1 (A4), or isotype (control) IgG for 2 h at 4°C. The HCV-LP-antibody mixtures were then incubated with cells for 1 h. After a washing step, cell-bound HCV-LPs were analyzed as described above. The effects of VLDL, LDL, and HDL on HCV-LP binding were tested by preincubating cells with either VLDL, LDL, or anti-LDL-R IgG prior to the addition of HCV-LPs. Alternatively, HCV-LPs were preincubated with these lipoproteins before being added to the cells. Similarly, HCV-LPs or cells were preincubated with recombinant LEL-CD81 or with anti-CD81 MAbs.

Confocal microscopy. Huh7 and NKM-3 cells were incubated with CM-Dil or SYTO-labeled HCV-LPs for 45 min, followed by incubation at 37°C for various time periods. The specificity of the internalization process was determined by preincubating dye-labeled HCV-LPs with anti-E1 and anti-E2 antibodies before they were added to the cells. As a negative control, cells were incubated with CM Dil- or SYTO-labeled preparation from cells infected with Bac-GUS. Alternatively, cells were incubated with dye-labeled HCV-LPs. Cells were fixed with 4% paraformaldehyde, washed, and mounted with a DAPI (4′,6-diamidino-2-phenylindole)-antifade solution. Cells were imaged on a Leica TCS SP laser-scanning confocal microscope. Confocal images were captured using an epifluorescence microscope. SYTO and CM-Dil fluorescence dyes were excited by 499- and 553-nm laser lines, respectively, from a water-cooled argon laser (Coherent Laser). SYTO and CM-Dil fluorescence emissions were monitored at 519 and 570 nm, respectively.
RESULTS

Characterization of HCV-LPs. HCV-LPs 1a were harvested on day 3 postinfection by gentle permeabilization of cells with 0.25% digitonin. After fractionation of the HCV-LPs on an equilibrium sucrose gradient, each fraction was analyzed by ELISA and Western blotting for the presence of C, E1, and E2 proteins. The ELISA results showed that the peak of E2 reactivity was detected in fractions 6 to 8, which correspond to buoyant densities of 1.17 to 1.22 g/ml (Fig. 1A). Western blot analysis revealed that these fractions contain an E2 protein band at ~70 kDa, three major bands of E1 (~33, 32, and ~28 kDa), and a core protein band at ~21 kDa (Fig. 1B). The presence of three bands of E1 protein reflects the different extent of N-linked glycosylation. As analyzed by cryoelectron microscopy, HCV-LPs are vary in sizes (35 to 49 nm in diameter) (Fig. 1C). This size difference, in part, may be due to the difference in the amount of E1 or E2 proteins incorporated into each type of particle (data not shown).

FIG. 1. Characterization of HCV-LP 1a. (A) HCV-LPs 1a were harvested on day 3 postinfection and purified as described in Materials and Methods. Eleven fractions (1 ml) were collected from the top and tested for E2 reactivity by ELISA. (B) Western blot analysis of HCV-LPs. The similar fractions collected from panel A were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blot analysis with anti-E2 (ALP98), anti-E1 (A4), and anti-core (C1) MAbs. (C) Cryoelectron micrograph of HCV-LP 1a. Bar, 200 nm.

FIG. 2. HCV-LPs bind to human hepatic and T cells. Binding of HCV-LPs to human hepatic (primary human hepatocytes, HepG2, HuH7, and NKNT-3) and T (Molt-4) cells was detected by anti-E2 MAb, followed by FITC-labeled goat anti-mouse IgG (indirect method), as described in Materials and Methods; 2.5 µg of HCV-LPs was used for each binding study. Axes: x, MFI; y, number of cells. HCV-LPs did not bind to Aro, a human thyroid cell line.
Binding of HCV-LPs to human hepatic and lymphoid cell lines. Using HCV-LPs, we have developed a cell-based binding assay in two formats. The first is the indirect binding method, in which we use anti-E2 MAb to detect HCV-LP binding to cells. For the second format, HCV-LPs were labeled with a lipophilic (CM-DiI) or a nucleic acid (SYTO) dye and used for a direct binding assay. As a control for the direct binding assay, fraction prepared identically from control Bac-GUS-infected cells was labeled with the dye and used for the binding assay. The ability of HCV-LPs to bind various target cells was analyzed by flow cytometry first by the indirect method. As shown in Fig. 2, HCV-LPs bound to hepatic (PHH, HepG2, HuH7, and NKNT-3) and T-cell (Molt-4) lines, but not to thyroid cells (Aro). HCV-LPs also bound to human B-cell line (Daudi), but not to HeLa cells, mouse fibroblasts (3T3-L1), and mouse mastocytoma P815 cells (data not shown). Binding of HCV-LPs to target cells occurred in a dose-dependent manner and was saturable (Fig. 3A and B). HCV-LPs bound to Molt-4 and NKNT-3 cells with higher affinity than that to PHH and HepG2 cells.

Pretreatment of cells with 0.25% trypsin abolished HCV-LP binding (data not shown), suggesting that binding of HCV-LPs to cells is mediated by cellular surface protein(s). HCV-LPs binding to cells occurred, at least partially, in a calcium-dependent manner since the addition of 5 mM EGTA reduced this binding (Fig. 3C).

To estimate the affinity of HCV-LP binding to hepatic and lymphoid cells, Scatchard plot analysis was performed. Using the direct binding assay with SYTO-labeled HCV-LPs, we demonstrated the presence of a biphasic binding with high and low affinities to NKNT-3 and Molt-4 cells. The high-affinity binding site has a dissociation constants ($K_d$) of $\sim 1$ µg/ml, while the lower-affinity binding site has a $K_d$ of $\sim 50$ to 60 µg/ml (Fig. 3D and E).

Inhibition of HCV-LP binding by anti-E1 and anti-E2 MAbs. Binding of HCV-LPs to cells is most likely mediated through the envelope proteins E1 and E2. Preincubation of SYTO-labeled HCV-LPs with anti-E2 (AP33 or ALP98) or anti-E1 (A4) MAbs inhibited HCV-LP binding to cells in a dose-dependent manner. On the other hand, neither isotype
control IgG (Fig. 4) nor anti-core antibody (data not shown) had any effect.

**Effect of CD81 on HCV-LP binding.** Although HepG2, HuH7, NKNT-3, and Molt-4 cells all bound to HCV-LPs, significant differences in their CD81 expression existed. As assessed by reverse transcription-PCR, the strain of HepG2 cells used in the present study lacks CD81 expression, whereas others express CD81 (data not shown). Hence, HCV-LPs bound to HepG2 cells in a CD81-independent manner. Recombinant CD81 failed to inhibit HCV-LP binding to HuH7 cells, although it partially inhibited HCV-LPs binding to Molt-4 and NKNT-3 cells (Fig. 5A). Furthermore, anti-human CD81 MAb that had been shown to block truncated E2 binding to cells (17) did not have any significant effect on HCV-LP binding to HuH7 and Molt-4 cells (Fig. 5B).

**Effect of VLDL, LDL, and HDL on HCV-LP binding.** Molt-4 cells that express LDL-Rs and have been used previously to characterize HCV-cell interaction (54) were used here to evaluate the effect of VLDL, LDL, and HDL on HCV-LP binding. Using the indirect binding, we found that LDL inhibited HCV-LPs binding when added simultaneously to cells (Fig. 6A), whereas preincubation of HCV-LPs with LDL completely abolished their binding to cells (Fig. 6B). Previous study has proposed that association of HCV virions and β-lipoproteins in the plasma may mask the virions from circulating antibodies and, at the same time, represent one mechanism of HCV entry into cells, i.e., through the LDL receptor (33). There are two explanations for this finding. LDL may bind to the HCV-LPs and inhibit their binding to cells; alternatively, LDL binding to HCV-LPs may hinder the accessibility of HCV-LPs to anti-E2 MAb used in this indirect binding method. To distinguish between these two possibilities, the direct binding method was used. Cells were incubated with SYTO-labeled HCV-LPs. As shown in Fig. 6C, preincubation of labeled HCV-LPs with LDL reduced their binding to Molt-4 cells by >50%. A similar phenomenon was observed when HCV-LPs was preincubated with VLDL or HDL. However, when cells were preincubated either with VLDL, LDL, or HDL before the addition of HCV-LPs, HCV-LP binding was only slightly increased (Fig. 6C). Altogether, these results indicate that preincubation of HCV-LPs with VLDL, LDL, and HDL resulted in a lipoprotein–HCV-LP complex that inhibited HCV-LP binding to cell. Second, the slight increase in HCV-LP binding after preincubation of cells with these lipoproteins suggested that HCV-LPs might also interact with cell-bound VLDL, LDL, or HDL, in addition to other cell surface molecule(s). This view was confirmed by the inability of two anti-LDL-R antibodies to significantly block HCV-LP binding (Fig. 6C).

**Internalization of labeled-HCV-LPs by hepatic cells.** We further examined whether binding of HCV-LPs to cells can be followed by entry. HuH7 and NKNT-3 cells were incubated with CM-Dil- or SYTO-labeled HCV-LPs, respectively, for 30 min at 4°C and then at 37°C for various time points. Figure 7 showed the internalization of CM-Dil-labeled HCV-LPs by...
weak signal of SYTO-labeled HCV-LPs was found mostly surrounding the cell surface (Fig. 7D). The incorporation of dye into the cytoplasm increased when cells were incubated at 37°C for 30 min (Fig. 7E). We also observed that SYTO dye was found in the nucleoli, which is presumably due to the staining of the RNA-containing nucleoli by the dye released from HCV-LPs after entry. NKNT-3 cells reacted poorly with SYTO-labeled Bac-GUS preparation (Fig. 7F). To assess whether specific antibodies could inhibit HCV-LP entry into cells, labeled HCV-LPs were preincubated with anti-E1 or anti-E2 antibodies for 2 h at 4°C. HCV-LPs (in the absence of antibodies) and after preincubation with antibodies were then incubated with cells for 15 min at 37°C. Although the control HCV-LPs were internalized by cells (Fig. 7G), preincubation with antibodies significantly reduced the incorporation of labeled HCV-LPs (Fig. 7H). These data suggest that E1 and E2 protein mediate HCV-LP binding and, subsequently, their entry into cells.

**DISCUSSION**

One of the major impediments in studying the initial steps of HCV infection is the lack of a robust cell culture system for HCV propagation. In the absence of purified HCV particles, the baculovirus-derived HCV-LP (5) has provided a surrogate model for this purpose. Generation of virus-like particles by using a recombinant baculovirus expression system has been successfully reported for many viruses such as papillomavirus (30), rotavirus (23), and JC polyomavirus (19). These virus-like particles have been demonstrated to be a valuable tool for investigating virus-cell interaction of papillomavirus (10) and for identifying the candidate receptor for Norwalk virus (46).

In the present study, we generated HCV-LPs of 1a as a model for studying the mechanism of attachment and entry of HCV infection. The buoyant density of HCV-LPs (1.17 to 1.22 g/ml) resembles that of the high-density HCV particles in circulation (22, 24, 38, 54). It is believed that the low- and intermediate-density fractions represent HCV particles associated with β-lipoproteins (38, 47). Cryoelectron microscopy analysis featured HCV-LPs as double-shelled particles 35 to 50 nm in diameter. Of note, we also found that HCV-LP 1a is recognized by a panel of human and mouse anti-E1 and anti-E2 MAbs; some of these are conformation-sensitive anti-E2 MAbs, indicating that the E2 protein on HCV-LPs adopts a proper conformation (49).

HCV-LPs can bind to human hepatic and lymphoid cells that are believed to be the major sites of viral tropism in vivo. Binding of HCV-LPs to these cells was found to be dose dependent in saturated manner. Scatchard plot analysis of HCV-LP binding to hepatic and lymphoid cells indicates a biphasic binding with two different affinities, i.e., high- and low-affinity bindings. We estimated that the high-affinity binding site has a $K_d$ of $\sim$1 μg/ml with at least 150 to 200 sites/cell, whereas the lower affinity has $K_d$ of 50 to 60 μg/ml with $>1,500$ sites/cell.

Depletion of calcium by 5 mM EGTA partially inhibited HCV-LP binding to cells, suggesting that one of the cellular receptors involved is a calcium-dependent type receptor. The requirement of calcium for virus binding has also been reported for pseudotype VSV bearing HCV E1 and E2 proteins (32) and rotavirus (40). The infectivity of pseudotype VSV-
HCV E1 or E2 virus to HepG2 cells was reduced by up to ~50% in the presence of EGTA, suggesting that calcium is, in part, required for E1 or E2 binding to their receptor(s) (32). Many cellular receptors, including the LDL-R (3) and the ASGP-R (31), require calcium ions for their ligand-binding activity. Another possibility for the adverse effect of EGTA on HCV-LP binding maybe due to the importance of calcium ions for the conformation of E1 or E2 proteins. Calcium ion has been known to be important for particle assembly for many viruses, including herpesvirus (55), bovine papillomavirus (35), JC polyomavirus (19), rotavirus (12), and African swine fever virus (7).

Specific anti-E2 and anti-E1 antibodies can inhibit HCV-LP binding to cells in a dose-dependent manner, suggesting that both E1 and E2 proteins mediate binding of HCV-LPs to cells. Alternatively, the inhibitory effects exerted by these antibodies occurred through steric hindrance rather than via direct blocking of the receptor-binding site on the envelope proteins. Consistent with our findings, Garcia et al. (18) has recently identified six hepatocyte-binding sequences (HBSs) located on E1 and E2 by using a series of 20-mer, overlapping synthetic peptides corresponding to the entire length of E1 and E2 protein of HCV 1a genotype. Two of the HBSs in the E1 region were mapped at the N-terminal (aa 192 to 211) and central (aa 242 to 261) regions, whereas the remaining four HBSs are clustered within E2: aa 384 to 404 (HVR-1), aa 444 to 483, aa 505 to 543, and aa 564 to 613. Interestingly, the anti-E1 (A4) and anti-E2 (AP33 and ALP98) MAbs that blocked HCV-LP binding recognize epitopes within or near these HBSs. A4 recognizes aa 197 to 207 (14), whereas AP33 and ALP98 recognize aa 412 to 423 and aa 640 to 653, respectively (34).

Virus-cell interaction is a multistep process and frequently involves more than a single receptor. There are, at least, three ways employed by virus to bind its target cells. First, virus can harbor two receptor-binding sites that allow binding to alternative receptors expressed on different cell types (e.g., adeno-virus type 37) (53). Second, virus can bind to a “common” surface molecule that captures and concentrates virus at the cell surface, and this event is followed by binding to a high-affinity primary receptor (e.g., herpes simplex virus) (6). Third, virus binds to a high-affinity receptor, and this event induces conformational changes leading to the exposure of binding sites for a coreceptor (e.g., human immunodeficiency virus type 1) (16, 25). So far, little is known about which mechanism is adopted by HCV to bind and enter target cells. The association of CD81 and the LDL-R with E2 protein or HCV virion,

FIG. 6. Effect of VLDL, LDL, and HDL on HCV-LP binding to Molt-4 cells. Cell-bound HCV-LPs were analyzed by flow cytometry by the indirect method (A and B) or the direct method (C). (A) Increasing concentrations of HCV-LPs with or without LDL (0.5 mg/ml) were added simultaneously to the cells. (B) Alternatively, HCV-LPs were preincubated with LDL for 2 h at 4°C before being added to the cells. (C) SYTO-labeled HCV-LPs were incubated with cells for 1 h at 4°C, and cell-bound HCV-LPs were analyzed as described in Materials and Methods (open bars). Cells were preincubated with VLDL, LDL, or HDL (0.5 mg/ml) or anti-human LDL-R IgG (20 µg/ml) for 2 h at 4°C before the addition of SYTO-labeled HCV-LPs (hatched bars). Alternatively, SYTO-labeled HCV-LPs were preincubated with VLDL, LDL, or HDL at 4°C before being added to the cells (solid bar).
FIG. 7. Confocal microscopy analysis of labeled-HCV-LP internalization by cells. HuH7 cells were incubated with CM-DiI-labeled HCV-LPs at 4°C (A) and then at 37°C (B). As a negative control, cells were incubated with a CM-DiI-labeled control Bac-GUS preparation at 37°C (C). (D and E) NKNT-3 cells were incubated with SYTO-labeled HCV-LPs at 4°C (D) and then at 37°C for 30 min (E). (F) As a negative control, cells were incubated with SYTO-labeled Bac-GUS at 37°C for 30 min. (G) NKNT-3 cells were incubated with SYTO-labeled HCV-LPs for 15 min at 37°C. (H) Alternatively, cells were incubated with SYTO-labeled HCV-LPs that had been preincubated with anti-E1 or anti-E2 antibodies for 2 h. On each panel, six images representing the top to the bottom of the cells (left to right) are shown.
respectively, have led to the assumption that either one may represent the cellular receptor for HCV (1, 33, 37). Despite several reports demonstrating the E2 binding to CD81 (17, 21), the interaction between HCV virion with this molecule is less clear. CD81 molecule only inhibited the binding of truncated E2 protein, but not HCV virion, to Molt-4 cells (54), suggesting that the HCV virion may use other receptor(s) for entry into cells.

In the present study, we showed that HCV-LPs bind to various cell lines regardless of their CD81 expression. On CD81-positive cells (HuH7, NKNT-3, and Molt-4), blocking of cellular CD81 with anti-CD81 MAb did not inhibit HCV-LP binding. However, we found that recombinant CD81 partially reduced its binding to Molt-4 and NKNT-3 cells, but not to HuH7 cells. The different effects of CD81 on HCV-LP binding to different cell types is presumably due to the weak interaction observed between HCV-LPs and CD81 (49). It is possible that CD81-E2 interaction may serve some purpose other than viral attachment and/or entry. The poor ability of cellular CD81 to internalize bound ligands (36) also makes it unlikely as a sole determinant for viral attachment or entry. Recently, two independent studies demonstrated that engagement of CD81 with E2 protein led to the inhibition of natural killer cell activation, gamma interferon production and proliferation (9, 50). These results suggest that HCV may establish persistent infection through interaction of its E2 protein with the immune system.

The biological roles of lipoprotein-HCV interaction in the plasma of infected individuals remain unclear. In the present study we observed that the interaction between HCV-LPs and VLDL, LDL, or HDL might hinder HCV-LP recognition by the anti-E2 antibody. We also have preliminary data that LDL complexes with HCV-LPs by altering the sedimentation distribution of HCV-LPs in sucrose gradient centrifugation (not shown). These observations may have a biological relevance, i.e., lipoproteins could mask HCV virions from recognition by circulating antibodies. Our data corroborate the previous observation in which, after fractionation on a sucrose gradient, HCV-positive fractions with high infectivity in chimpanzee were found predominantly in low buoyant density (associated with lipoproteins) and could not be precipitated with an anti-HCV immunoglobulin antibody (22). In addition, we observed that preincubation of HCV-LPs with these lipoproteins inhibited HCV-LPs binding in a dose-dependent manner. Similar findings have also been reported for HCV virion (54) and pseudotype VSV expressing HCV E1 protein (32). The inhibition effect shown by these lipoproteins is more likely due to their interaction with HCV-LPs rather than competition for the cell surface receptor(s). This hypothesis is supported by the observation that preincubation of cells with VLDL, LDL, HDL, or anti-LDL-R did not inhibit HCV-LP binding. However, we cannot completely eliminate the possibility that the LDL/LDL-R pathway may play a minor role in mediating HCV-LP–cell interaction in this system.

As the mode of entry of HCV remains unknown, we sought to determine whether HCV-LPs could be used to elucidate this process. Incubation of cells with SYTO- or CM-Dil-labeled HCV-LPs at 37°C resulted in the incorporation of dye in the cytoplasm, suggesting the labeled-HCV-LPs were internalized. The internalization of labeled-HCV-LPs is shown to be specific for HuH7 and NKNT-3 cells and also HepG2 (data not shown). Although we could not be absolutely sure that the incorporated dye inside the cell represents specifically the internalized HCV-LPs, several lines of evidence indicate that this is the case. First, preincubation of HCV-LPs with anti-E1 or anti-E2 antibodies significantly reduced the uptake of dye-labeled HCV-LPs by the cells, suggesting that E1 and E2 proteins mediate the particle binding and, subsequently, its entry into cell. Second, both HuH7 and NKNT-3 cells did not internalize dye-labeled Bac-GUS control preparation. Finally, dye-labeled HCV-LPs was not internalized by Aro cells that also did not bind HCV-LPs (not shown). In line with this study, Wellnitz et al. (51) has recently reported that HCV-LPs derived from H77c strain bind to hepatic and lymphoid cell lines. However, detailed characteristics of HCV-LP binding, i.e., the effects of calcium, anti-E1 antibody, lipoproteins, binding affinities, and the internalization process were not analyzed in that study.

Taken together, we have shown that HCV-LPs can be used as a valuable tool to study the mechanism of binding and entry of HCV infection. Further characterization of HCV-LP and its interaction with cells will help us understand the early steps of HCV infection and will facilitate studies to identify other candidate receptor(s) for HCV.

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