Characterization of Low- and Very-Low-Density Hepatitis C Virus RNA-Containing Particles

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Received 1 February 2002/Accepted 12 April 2002

The presence of hepatitis C virus (HCV) RNA-containing particles in the low-density fractions of plasma has been associated with high infectivity. However, the nature of circulating HCV particles and their association with immunoglobulins or lipoproteins as well as the characterization of cell entry have all been subject to conflicting reports. For a better analysis of HCV RNA-containing particles, we quantified HCV RNA in the low-density fractions of plasma corresponding to the very-low-density lipoprotein (VLDL), intermediate-density lipoprotein, and low-density lipoprotein (LDL) fractions from untreated chronically HCV-infected patients. HCV RNA was always found in at least one of these fractions and represented 8 to 95% of the total plasma HCV RNA. Surprisingly, immunoglobulins G and M were also found in the low-density fractions and could be used to purify the HCV RNA-containing particles (lipo-viro-particles [LVP]). Purified LVP were rich in triglycerides; contained at least apolipoprotein B, HCV RNA, and core protein; and appeared as large spherical particles with a diameter of more than 100 nm and with internal structures. Delipidation of these particles resulted in capsid-like structures recognized by anti-HCV core protein antibody. Purified LVP efficiently bind and enter hepatocyte cell lines, while serum or whole-density fractions do not. Binding of these particles was competed out by VLDL and LDL from noninfected donors and was blocked by anti-apolipoprotein B and E antibodies, whereas upregulation of the LDL receptor increased their internalization. These results suggest that the infectivity of LVP is mediated by endogenous proteins rather than by viral components providing a mechanism of escape from the humoral immune response.

Although hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide, the virus has not yet been cultured in vitro and little is known about its biological and physicochemical properties (16). A recent accumulation of data revealed the density heterogeneity of HCV RNA-containing particles (7, 19, 27). By use of nonquantitative PCR to detect HCV RNA in gradient fractions of infected human serum, HCV RNA-containing particles were found at densities of between 1.03 and 1.25 g/ml, with considerable variations from serum sample to serum sample (41, 42). Titration of infectivity in chimpanzees revealed a relationship between the density of particles and infectivity; the highest infectivity of HCV RNA found in higher-density fractions seemed to be poorly infectious (5, 15).

The unusually low density of some HCV RNA-containing particles suggested an association of the virus with plasma lipoproteins (41, 42). The main function of lipoproteins is to transport and deliver lipids and lipid-soluble materials throughout the body. Lipoproteins are 20- to 80-nm particles which consist of a hydrophobic core of neutral lipid surrounded by a monolayer of amphipathic phospholipids and free cholesterol in which apolipoproteins reside. Synthesis and secretion of these particles occur in hepatocytes in the form of very-low-density lipoprotein (VLDL) (density of <1.0063 g/ml) with apolipoproteins B and E (ApoB and ApoE, respectively). Transformation of VLDL in the circulation gives rise to particles of a smaller size, with intermediate to low density (intermediate-density lipoprotein [IDL] and low-density lipoprotein [LDL]), enriched in cholesteryl esters, depleted of triglycerides, and containing only ApoB (14, 39). The interaction of HCV with plasma lipoproteins was first confirmed when Thomssen et al. (41) found that low-density materials can be precipitated with anti-ApoB antibodies (41, 42). Several investigators have since extended this observation and shown that HCV envelope proteins bind to human lipoproteins of various densities (28). It is not known, however, whether HCV simply binds to circulating lipoproteins or whether an interaction occurs during lipoprotein synthesis by infected hepatocytes to form a hybrid virus-like particle.

Identification of the HCV receptor is a major challenge for the development of both cell culture systems and therapy. Two cell surface receptors, the LDL receptor and CD81, interact with HCV and HCV envelope protein E2, respectively, leading to the hypothesis that they both act as viral receptors (28, 30, 31, 40). Although several reports have shown a highly specific interaction between HCV E2 and cellular CD81 (31), most recent studies have indicated that putative lipoprotein-associated HCV particles may infect cells via the LDL receptor (1, 44). Characterization of this pathway has become challenging, especially since it appears to be preferentially active on hepatocytes.

The apparent heterogeneity of data concerning the various putative forms of HCV RNA-containing particles may be due...
to the lack of systematic and comparative analyses. Using a quantitative approach (22), we conducted an analysis of chronically HCV-infected patients to determine the representativeness and the precise nature and infectivity of HCV particles in low-density plasma fractions corresponding to VLDL, IDL, and LDL.

**MATERIALS AND METHODS**

**Samples.** Thirty-six volunteers attending the Liver Unit at Necker Hospital, Paris, France, were selected in accordance with hospital ethics committee statements; they were chronically HCV-infected patients with chronic active hepatitis and had not been given antiviral therapy for more than 6 months. Screening for hepatitis B virus or human immunodeficiency virus infection was negative. Blood was obtained by venous puncture. EDTA at 1 mM final concentration was added to 40 ml of blood, and samples were sent to the laboratory at an ambient temperature. Plasma and serum were immediately processed for density fraction separation, and aliquots were stored at −80°C. For control and competition experiments, plasma from noninfected blood donors was provided by the Etablissement de Transfusion Sanguine in Lyon, France.

**Preparation of low-density fractions.** Plasma from infected patients or from blood donors was separated by sequential centrifugation to obtain the low-density fractions whose densities corresponded to those of VLDL, IDL, and LDL (25). The lowest-density fraction, with a density of <1.0063 g/ml, was obtained by centrifugation of plasma for 4 h at 4°C and 543,000 × g with a TLA100.4 rotor and a TL100 ultracentrifuge (Beckman Instruments S. A., Courtaboeuf, France). After collection of the first fraction, the density of the remaining plasma was adjusted to 1.025 g/ml with NaBr (Sigma). The second fraction, with a density of between 1.006 and 1.025 g/ml, was collected after centrifugation under the same conditions. A last run at a density of 1.055 g/ml (by the addition of NaBr) allowed the collection of the third fraction, with a density of between 1.025 and 1.055 g/ml. All fractions were then extensively dialyzed at 4°C against 150 mM NaCl-0.2 mM EDTA (pH 7.4) buffer, filtered through 0.2-μm pore-size filters (Millipore S. A., Saint Quentin, France), and stored at 4°C in the dark.

**Immunopurification.** A 10-μl quantity of protein A-coated magnetic beads (Miltenyi Biotec, Paris, France) was incubated at room temperature with 1 ml of the low-density fractions in phosphate-buffered saline (PBS) with gentle rocking for 30 min. The beads were then washed through a magnetic column (Miltenyi Biotec), washed with PBS, and collected in 500 μl of PBS or Dulbecco modified Eagle medium (DMEM)-0.2% bovine serum albumin (BSA) (Gibco/BRL, Life Technologies, Cergy Pontoise, France). Immunoprecipitated particles were des- igned purified lipoviro-particles (LVP).

**Electron microscopy.** Dilution of HCV-infected low-density fractions or purified LVP were made in PBS. For some experiments, purified LVP were delipidated by ether-butanol extraction as described above. Formvar-carbon support film-coated 200-mesh copper grids were incubated (Electron Microscopy Sciences; Hatfield, Pa.) without fixation on drops of buffer at 3 min at room temperature and stained for 3 min by flotation on 4% (wt/vol) phosphotungstic acid buffered at pH 7.2 with NaOH. The grids were then dried and examined with a JEOL apparatus at the Centre Commun d’Imagerie de Laennec in Lyon, France.

For immunoelectron microscopy, Formvar-carbon support film-coated 200-mesh nickel grids were floated on LVP that had been diluted in 0.5% Tween 80–PBS for 30 min at room temperature with gentle rocking and were incubated for 1 h at room temperature by flotation on 0.05 M Tris-HCl (pH 7.4) containing 1% BSA and 0.1% cold fish gelatin (Sigma). The grids were then floated on a drop of monoclonal antibody 19D9D6 (18) or isotopic control immunoglobulin G1 (IgG1) (5 μg/ml) in Tris-HCl (pH 7.4) overnight in a moist chamber at 4°C. The grids were then washed by flotation on drops of Tris-HCl (pH 7.4, pH 8.2, and pH 8.2) with 1% NaCl. The second antibody was incubated by floating grids on a 1:40 dilution of goat anti-mouse IgG–colloidal gold particles (10-nm diameter; BioCell Research Laboratories, Cardiff, United Kingdom) in Tris-HCl (pH 8.2)–1% BSA for 1 h at room temperature. Washings were first performed with Tris-HCl at pH 8.2 and then at pH 7.4 and finally with water. The grids were negatively stained by flotation on 3% uranyl acetate.

**Protein, ApoB, and lipid quantitation.** Protein concentrations were determined according to the Lowry method as modified by Markwell (Sigma). Protein concentrations were calculated from a calibration curve by using BSA as a standard. ApoB concentrations in fractions and sera were determined by using an immunochemical kit according to the manufacturer’s protocol (ApoB kit; bioMérieux S. A., Marcy l’Etoile, France). The concentrations were determined from a calibration curve established with the ApoB kit standard. Total cholesteryl, phospholipid, and triglyceride concentrations were calculated from a calibration curve by using BSA as an internal standard for the lipoprotein compartment. Total cholesterol, phospholipid, and triglyceride concentrations were calculated from a calibration curve by using BSA as an internal standard for the lipoprotein compartment. Total cholesterol, phospholipid, and triglyceride concentrations were calculated from a calibration curve established with the ApoB kit standard. Total cholesterol, phospholipid, and triglyceride concentrations were calculated from a calibration curve by using BSA as an internal standard for the lipoprotein compartment.
Cell association, uptake, and kinetic assays. For the HCV RNA cell association assay, 5 x 10^4 PLC cells per well were cultured in 96-well plates for 24 h with culture medium. PLC cells were washed three times with PBS and incubated for 3 h with serum, whole-density fractions, or purified LVP and with or without competitors in FCS-free medium containing 0.2% (wt/vol) BSA. After washing, cells were harvested in 350 μl of RNeasy kit lysis buffer (Qiagen), and RNA was extracted. HCV RNA was quantified as described above. For inhibition experiments, the monoclonal antibodies against the receptor-binding site of ApoB were 4G3 and 5E11. The receptor-binding site of ApoE was blocked with monoclonal antibody 1D7 (Ottawa Heart Institute Research Corporation, Ottawa, Ontario, Canada).

For kinetic and uptake experiments, HCV RNA-containing particles bound to cell surface receptors after the incubation period and three washings with PBS–0.2% BSA were removed by incubation with 10 mM suramin (Sigma) in PBS at 4°C for 1 h. Cells were washed three times in PBS and harvested in 350 μl of RNeasy kit lysis buffer. Internalized HCV RNA was quantified as described above.

The cell protein content in the RNeasy lysis buffer was directly measured after RNA binding to the RNeasy mini spin column. Nonspecific binding to plastic wells was controlled by incubating HCV samples under identical conditions in wells containing no cells.

RESULTS

Analysis of very-low-, intermediate-, and low-density plasma fractions. Plasma from 27 chronically infected patients was fractionated into three fractions corresponding to the floating densities of VLDL (<1.0063 g/ml), IDL (1.0063 to 1.025 g/ml), and LDL (1.025 to 1.055 g/ml). The amount of HCV RNA in each fraction was measured by real-time PCR as previously described (22). Figure 1A shows that all patients had HCV RNA in at least one of the three fractions irrespective of the amount of HCV RNA in the serum or the HCV genotype. The index of the association of HCV RNA with the density fractions varied from 8 to 95%, depending on the patient, with a mean of 42%.

Some HCV particles with a high density have been reported to be associated with immunoglobulins (8, 15, 20, 42). To search for immunoglobulins, we subjected the three fractions corresponding to VLDL, IDL, and LDL to SDS–10% PAGE and transferred to PVDF membranes for IgG and IgM detection. A positive control experiment was performed by running 200 ng of purified IgG in the gel.
HCV RNA-containing particles can be the target of a humoral response, with more immunoglobulins being found in the very-low- and intermediate-density fractions. We found no correlation between the amount of immunoglobulins and the amount of HCV RNA.

**Purification and characterization of HCV RNA-containing particles.** To verify whether immunoglobulins were directed to HCV RNA-containing particles, immunoglobulins from various fractions were purified with protein A-coated magnetic beads, and the quantities of HCV RNA in the purified and unpurified materials were determined. HCV RNA always copurified with immunoglobulins. Table 1 shows four representative examples of copurification indicating that the association of HCV RNA-containing particles with immunoglobulins ranged from 11 to 85% irrespective of the density of the fraction. The association of HCV RNA-containing particles with immunoglobulins therefore depends on individual variations rather than on density.

The presence of immunoglobulins on HCV RNA-containing particles was then used for an additional step of purification with protein A-coated magnetic beads. This step allowed the separation of immunoglobulin-positive HCV RNA-containing particles from normal lipoproteins and from particles not covered by immunoglobulins. Such immunopurified material was referred to as purified LVP. Triglycerides, total cholesterol (free cholesterol and cholesteryl esters), and ApoB in the density fractions and in the corresponding purified LVP were quantified (Table 2). As expected, the triglyceride/cholesterol ratios of the density fractions before LVP purification were 3.4 ± 1.7 and 0.42 ± 0.2 for the very-low- and low-density fractions, respectively. Conversely, the triglyceride/cholesterol ratios of purified LVP from the very-low- and low-density fractions did not vary (3.1 ± 1.6 and 2.8 ± 1.9, respectively).

**TABLE 1.** Quantitation of low-density HCV RNA-containing particles associated with immunoglobulins (Ig)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Fraction density</th>
<th>HCV RNA copy no. (10^6) in the following fractiona</th>
<th>Yield (%)b</th>
<th>% HCV RNA associated with Ig^c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Whole</td>
<td>Ig^a</td>
<td>LVP</td>
</tr>
<tr>
<td>1</td>
<td>&lt;1.0063</td>
<td>9</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>&lt;1.0063</td>
<td>19.3</td>
<td>13.2</td>
<td>2.65</td>
</tr>
<tr>
<td>3</td>
<td>1.025–1.055</td>
<td>4.32</td>
<td>0.73</td>
<td>2.8</td>
</tr>
<tr>
<td>4</td>
<td>1.025–1.055</td>
<td>1.04</td>
<td>0.89</td>
<td>&lt;0.006</td>
</tr>
</tbody>
</table>

a Ig^a LVP^a, Ig positive LVP depleted; Ig^b, Ig negative.

b Expressed as the percentage of HCV RNA copies in the Ig^a and Ig^b fractions/HCV RNA copies in the whole fraction.

c Expressed as the percentage of HCV RNA copies in the Ig^b LVP^a fraction/HCV RNA copies in the whole fraction.

Although the triglyceride/cholesterol ratios of VLDL and of LVP purified from the very-low-density fraction appeared in the same range, the lipid contents of LDL and of LVP purified from the low-density fraction differed significantly (0.42 ± 0.2 and 2.8 ± 1.9, respectively; P ≤ 0.01). In addition, LVP purified from the very-low-density fraction contained more triglyceride per ApoB molecule than LVP purified from the low-density fraction, explaining the difference in density. LVP purified from both very-low- and low-density fractions contained more triglyceride per ApoB molecule than lipoproteins from the same fractions.

The lipid compositions of lipoproteins and purified LVP suggested that LVP are unusual particles and not just aggregates of lipoproteins and HCV virions. We found no correlation between the amount of ApoB and the amount of HCV RNA in purified LVP (data not shown).

**Electron microscopy of purified LVP.** Electron microscopy was performed with purified LVP obtained from the plasma fraction corresponding to LDL and with the same fraction depleted of LVP. The LDL fraction from infected plasma after LVP depletion displayed a homogeneous spherical structure with an average diameter of 25 nm, similar to that of normal LDL (Fig. 2A). In contrast, purified LVP (Fig. 2B) were unusually large spherical structures with an average diameter of 100 nm. Internal structures began to be visible when purified LVP were observed at the highest magnification (Fig. 2C). These structures could be better observed after delipidation of purified LVP with ether-butanol and appeared as capsid-like particles with some heterogeneity. The largest structures were 30 to 35 nm in diameter, and smaller particles might have represented defective or incomplete capsids (Fig. 2D). These structures were poorly recognized by immunoelectron microscopy with a monoclonal antibody to the basic and hydrophilic N-terminal region of the HCV core protein (18) (data not shown). However, after more drastic treatment with Tween 80, particles appeared smaller and partially disrupted, with irregular borders (Fig. 3A), and could be identified by immunoelectron microscopy with the same monoclonal antibody (Fig. 3B). The presence of HCV core protein in purified LVP after delipidation was further demonstrated by Western blotting with the same anti-HCV core protein monoclonal antibody (Fig. 3C). Under our experimental conditions, both monomers and dimers of HCV core protein were detected in LVP and in a core protein-expressing cell line. In contrast, we failed to detect any HCV envelope proteins in purified LVP by Western blotting despite the use of monoclonal antibodies recognizing...
FIG. 2. Electron microscopy of fractions corresponding to LDL from infected and noninfected patients. (A) LDL fraction depleted of immunoglobulin-positive LVP from an infected donor. (B) LVP purified from the LDL fraction of an infected donor. Protein A-coated magnetic beads are seen as dark granules 10 to 20 nm in diameter. Purified LVP appears as spherical particles whose average diameter is 100 nm (extremes, 50 to 150 nm). (C) Same fraction as in panel B but at a higher magnification (×250,000), showing the internal structures. (D) Ether-butanol-treated purified LVP adsorbed on grids and stained with phosphotungstic acid. Capsid-like particles 25 to 35 nm in diameter can be seen.
different conformational and linear HCV envelope epitopes (provided by J. Dubuisson, Institut Pasteur, Lille, France) (31).

Cellular binding and uptake of purified LVP. Purification of LVP combined with a previously described method for measuring HCV RNA allowed a precise analysis of the binding, association, and internalization of purified particles tested with hepatocyte cell lines.

In the first experiment, samples containing 50,000 copies of HCV RNA were incubated for 3 h with PLC cells at 37°C. Cells were then washed, and RNA was extracted. Cell-associated HCV RNA was quantified and represented the amount of bound or internalized virus during this time period. We used four sources of HCV RNA from the same patient: serum, whole-density fraction corresponding to VLDL, the same fraction depleted of LVP (and therefore containing immunoglobulin-negative HCV RNA-containing particles and normal lipoproteins), and purified LVP. Maximum association was obtained with purified LVP (Fig. 4A). No association or an extremely weak association was detected when serum or fractions were used as a viral source.

As nonpurified LVP contained in fractions could not associate with cells, it is likely that these fractions contained inhibitors of LVP binding. Lipoproteins were thus tested for their ability to inhibit the cell association of purified LVP. Competition experiments revealed that the cell association of purified LVP could be blocked in a dose-dependent manner by VLDL, the same fraction depleted of LVP (and therefore containing immunoglobulin-negative HCV RNA-containing particles and normal lipoproteins), and purified LVP. Maximum association was obtained with purified LVP (Fig. 4A). No association or an extremely weak association was detected when serum or fractions were used as a viral source.

Suramin is a polysulfonylnaphthylurea that has been extensively used to study receptor kinetics and specificity. In particular, it has been shown to separate lipoproteins from their cell surface receptors (37) and to inhibit HCV infection of HepG2 cells as well as the binding of human immunodeficiency virus to CD4 and galactosylceramide (12, 45). Suramin treatment was thus applied to distinguish surface binding of purified LVP from internalization. Quantitation of HCV RNA remaining associated with cells after suramin treatment provides an estimation of what has been internalized during incubation with LVP. When purified LVP were incubated at 37°C for 3 h, half of the HCV RNA was removed by subsequent suramin treatment (Fig. 5A). This result indicated that 50% of the purified LVP bound to hepatocarcinoma PLC cells were internalized during the 3-h incubation period.

Figure 5B and C show the amounts of purified LVP internalized by PLC cells as a function of time. Two examples are shown to illustrate representative data obtained with different amounts of purified LVP. For both viral preparations, the process of viral uptake was rapid and achieved saturation in 1 h. The rates of uptake did not differ but reached a higher plateau when more HCV RNA copies were provided during the incubation period.

Cell entry pathway for purified LVP. The activity of the LDL receptor is regulated by cholesterol. As a consequence, LDL receptor activity is upregulated when cells are grown in LPDS instead of FCS (37). To determine whether the internalization...
pathway for HCV is regulated by cholesterol, cells were grown in 10% LPDS for 24 h before incubation with purified LVP.

Figure 6A shows a twofold increase in the uptake of purified LVP when cells were grown in LPDS. This result is in agreement with the twofold increase in LDL receptor expression and activity usually obtained under these experimental conditions (data not shown).

N1 normal human fibroblasts were used to study the internalization of LVP in nonhepatocarcinoma cells. As shown in Fig. 6B, N1 fibroblasts expressing the LDL receptor could...
internalize substantial amounts of HCV RNA. However, the inoculum had to be more concentrated for N1 fibroblasts than for HepG2 cells to internalize similar amounts of purified LVP in 3 h. The uptake of purified LVP was also analyzed by using LDL receptor-negative fibroblasts (FH) derived from a patient with familial hypercholesterolemia (13). Purified LVP could still be internalized by FH fibroblasts, but the internalization was 10 times less efficient than that seen with fibroblasts expressing the LDL receptor.

These data suggest that the LDL receptor pathway is the main route of entry for purified LVP. The LDL receptor recognizes lipoproteins through the direct binding of ApoB on LDL and ApoB and ApoE on VLDL (14, 39). We therefore tested whether the binding of purified LVP to the LDL receptor could be blocked by antibodies directed to the receptor-binding sites of ApoB and ApoE (26, 43) (Fig. 7). Two anti-ApoB monoclonal antibodies recognizing different epitopes within the ApoB receptor-binding site and one anti-ApoE monoclonal antibody directed to the receptor-binding site of ApoE only slightly inhibited the cell association of purified LVP when used independently. In contrast, a strong blockade of purified LVP cell association (>85%; \( P < 0.02 \)) was obtained when all three antiapolipoprotein antibodies were used together. The participation of the anti-ApoE monoclonal antibody in the blockage of LVP binding also indicated that LVP contained ApoE.

**DISCUSSION**

HCV RNA is detected over a large range of densities in gradient fractions of infected human plasma, with variations from serum sample to serum sample (7, 15, 27, 42). The presence of low-density fractions containing HCV RNA and the possibility of immunoprecipitation of HCV RNA with anti-ApoB antibodies suggested an association of viral components with lipoproteins (32, 41, 42). To clarify this association, we performed sequential centrifugations to prepare fractions containing VLDL, IDL, and LDL from infected plasma (25). Blood samples were processed within a few hours after collection without freezing or storage at low temperature. Real-time PCR allowed accurate quantitation of HCV RNA in plasma. However, HCV RNA amplification from low-density fractions was impeded by inhibitors, most likely of lipid origin. These inhibitors were inactivated by the addition of normal human serum (data not shown). With this optimized procedure, we confirmed the presence of HCV RNA in low-density fractions and showed that low-density HCV RNA-containing particles are a constant feature of chronic HCV infection.

It has been suggested that high-density HCV RNA-containing particles are immune complexes whereas particles of lower density are free of immunoglobulins (8, 15, 21, 42). Surprisingly, IgG and IgM were detected in the low-density fractions from all chronically HCV-infected patients but not from controls. This result indicated that low-density HCV RNA-containing particles should contain large amounts of very-low-density material to allow flotation during centrifugation despite the presence of immunoglobulins. These particles could then be purified with protein A-coated magnetic beads and further characterized. In addition to HCV RNA and immunoglobulins, these purified particles contained the viral core protein and ApoB. They were very rich in triglycerides, explaining their low density. Variations in the quantities of triglycerides might also explain why we found these particles at densities of between 1.006 and 1.055 g/ml. Importantly, the differences in lipid compositions between LVP and their li-
protein counterparts strongly suggested that LVP are not just classical flavivirus-like virions attached to normal lipoproteins by classical flavivirus receptors, providing an efficient mechanism of escape from neutralizing antibodies directed to envelope proteins. As LDL receptor expression is ubiquitous, such an entry mechanism may not restrict HCV infection to hepatocytes and may lead to extrahepatic compartments of viral replication.

**ACKNOWLEDGMENTS**

This work was supported by INSERM, Agence Nationale de Recherche Contre le SIDA, and bioMérieux.

We thank Fabienne Chambrion, Hôpital Necker, Paris, France, for help with electron microscopy; and Geneviève Sibai, bioMérieux, for the preparation of human immunoglobulins.

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