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Detection of intrahepatic replication of hepatitis C virus RNA by *in situ* hybridization and comparison with histopathology

(chimpanzee animal model/viral morphogenesis/viral pathogenesis/digoxigenin-labeled oligonucleotide probe)

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ABSTRACT **A nonisotopic *in situ* hybridization (NISH) assay was used to detect hepatitis C virus (HCV) RNA. A synthetic oligonucleotide complementary to bases 252–301 of the highly conserved 5′ noncoding region of the HCV genome was end-labeled by terminal deoxynucleotidyltransferase using digoxigenin-conjugated dUTP. The hybridized oligomer was revealed by an immunohistochemical reaction after incubation with an alkaline phosphatase-conjugated anti-digoxigenin antibody and subsequent amplification with a complex of alkaline phosphatase and anti-alkaline phosphatase antibodies. The intracellular distribution of HCV RNA was monitored in the livers of two chimpanzees experimentally infected with the H strain of HCV and compared with the serum alanine aminotransferase activity, serum HCV RNA, and liver histopathology. Most cells were stained in the cytoplasm as early as 2 days after inoculation, 1 and 2 days, respectively, before the appearance of viral RNA in the serum. The time course of HCV RNA replication was correlated with increases in serum alanine aminotransferase. However, neither one paralleled the appearance of liver cell necrosis nor showed any correlation with the inflammatory response. The NISH signal was not found in liver biopsy specimens taken from these two animals before inoculation with HCV, from chimpanzees with acute hepatitis type A, B, or δ, or from two animals never experimentally infected with any hepatitis agent; moreover, it disappeared when the positive specimens were predigested with RNase and it was not observed after hybridization of positive controls with a labeled oligomer unrelated to HCV RNA. Thus, detection of liver HCV RNA by NISH is a sensitive and specific method for studying HCV replication at the cellular level. Intracellular replication of HCV did not appear to be associated with histopathologic changes in the liver, although the correlation with increases of liver enzyme activity in the serum suggested possible damage to the liver cell membrane.**

Hepatitis C virus (HCV), the agent responsible for the majority of human non-A, non-B hepatitis cases, has remained elusive for almost two decades. Its discovery and subsequent characterization have indeed followed unconventional routes, such as direct cloning and expression of cDNA prepared from the serum of infected humans (1) or chimpanzees (2). This was necessary because HCV replication in the host is limited (3–6) and the immune response to viral antigens is relatively poor (7–10).

Several groups of investigators have reported the partial or complete genome sequence of different isolates of HCV (1, 11–18). Sequence analyses of the HCV RNA show that HCV is distantly related to human flaviviruses, including yellow fever (YF) virus (another hepatotropic virus), as well as to animal pestiviruses.

Sensitive assays have been developed to detect HCV RNA by the polymerase chain reaction (4–6, 9, 19). Infected livers, however, must be processed for nucleic acid extraction, thus hindering morphological evaluation. On the other hand, direct assays for expression of viral antigen within infected tissue (20, 21) require potent antisera and thus have not been extensively evaluated. For the present study, we designed an assay for detecting HCV RNA by a nonisotopic *in situ* hybridization (NISH) procedure using a digoxigenin-tailed oligonucleotide complementary to the highly conserved 5′ noncoding region of the HCV genome (12, 14–18). The assay was performed on serial biopsy specimens taken from chimpanzees experimentally infected with HCV, in which all phases of viral infection and their histopathological correlates could be conveniently followed.

MATERIALS AND METHODS

Chimpanzees. Chimpanzees (*Pan troglodytes*) were housed in an approved breeding facility under contract with the National Institute of Allergy and Infectious Diseases and maintained under conditions meeting all requirements for research on primates. Two animals (1304 and 1313) were each inoculated with 0.5 ml of undiluted plasma obtained from a patient (H) with post-transfusion acute non-A, non-B hepatitis (3), as reported before (22). Serum and liver specimens were obtained from these two animals before inoculation, daily during the first week of infection, weekly thereafter for the first 12 weeks, and at 20 weeks after inoculation. Additional liver biopsy specimens were taken from other chimpanzees at the time of acute hepatitis that followed experimental infection with hepatitis A virus (animal A243), hepatitis B virus (animal A133) or hepatitis δ virus (animal 1241) (R.H.P., unpublished observations). Further liver samples were taken from two animals (A164 and A178) that had never been exposed to hepatitis agents.

Serum and Liver Assays. All serum samples were kept at −70°C until use. Sera were tested for alanine aminotransferase (ALT) activity and, for HCV RNA, by a reverse transcription–polymerase chain reaction assay (4). Liver biopsy specimens were fixed in formalin and embedded in paraffin. Serial 5-μm sections were processed for NISH (see below) or examined under code for routine histology after staining with hematoxylin and eosin (H&E) by one of us (G.B.).

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Abbreviations: ALT, alanine aminotransferase; CMV, cytomegalovirus; HCV, hepatitis C virus; H&E, hematoxylin and eosin; NISH, nonisotopic *in situ* hybridization; YF, yellow fever.

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NISH. The synthetic oligonucleotide probe (JBC-1) has the sequence 5'-CGGGGCACTCGCAAGCACCCTATCAG-GCAGTACCACAAGGCCTTTCGCGA-3' and is complementary to bases 252–301 of the 5' noncoding region of the HCV genome (15). The probe (25 pmol) was labeled at the 3' end by using terminal deoxynucleotidyltransferase (Promega) and digoxigenin-11-dUTP (Boehringer Mannheim) as detailed elsewhere (23). Sections (5 μm) of the liver biopsy specimens were layered onto microscope slides coated with 0.1% poly(L-lysine) (Sigma). Sections were dewaxed and hydrated to phosphate-buffered saline (PBS) through a series of ethanol dilutions. Specimens were then digested with proteinase K (Merck), 0.5 mg/ml in PBS, for 15 min at 37°C. After washing in PBS, sections were denatured for 5 min at 65°C in 70% formamide/0.1× standard saline citrate (SSC), washed again in PBS, and prehybridized for 1 hr at room temperature (RT) with a mixture containing 50% formamide, 5× SSC, 1× Denhardt's solution, sheared salmon sperm DNA (0.5 mg/ml), and *Escherichia coli* tRNA (0.25 mg/ml). Hybridization was carried out overnight (16 hr) at 37°C in this same mixture but containing the digoxigenin-labeled probe (100 nM). After hybridization, slides were washed for 1 hr at RT in 2× SSC, then for 1 hr at RT in 1× SSC, 30 min at 42°C in 0.5× SSC, and 1 hr at RT in 0.1× SSC. After a brief wash in 100 mM Tris·HCl, pH 7.5/150 mM NaCl (TN buffer), sections were incubated twice in TN buffer containing 2% normal sheep serum and 0.3% Triton X-100 (30 min at RT) and then TN buffer containing 1% normal sheep serum, 0.3% Triton X-100, and 0.2% sheep anti-digoxigenin antibody conjugated with calf intestinal alkaline phosphatase (Boehringer Mannheim) (2 hr at RT). Unbound antibody was washed off by immersing the slides for 5 min in PBS. Sections were incubated for 30 min at RT with a monoclonal mouse anti-alkaline phosphatase antibody (Zymed Laboratories) diluted 1:100 in PBS containing 0.1% bovine serum albumin (Sigma). Detection of the secondary antibody was then amplified as described (24). In brief, sections were first incubated for 30 min at RT with goat anti-mouse antibody (Techno Genetics-Recordati, Trezzano, Italy) diluted 1:50 in PBS and then for another 30 min at RT with calf intestinal alkaline phosphatase complexed to saturation with mouse monoclonal anti-alkaline phosphatase antibody (Dakopatts, Copenhagen) diluted 1:50 in PBS/10 mM $MgCl_2$. Both steps were repeated once to allow for amplification. Sections were washed twice for 15 min in TN buffer and finally for 2 min in 100 mM Tris·HCl, pH 9.5/100 mM NaCl/50 mM $MgCl_2$. The color was developed using nitroblue tetrazolium salt as the acceptor, as suggested by the manufacturer (Boehringer Mannheim). The development solution contained 0.024% tetramisole (Sigma). The reaction was stopped in 10 mM Tris·HCl, pH 8.0/1 mM EDTA and the sections were counterstained in 1% methyl green. Slides were then dehydrated and mounted. In some experiments, predigestion with RNase (Bethesda Research Laboratories), 400 μg/ml in PBS, was carried out for 1 hr at 37°C prior to the prehybridization step. In another experiment, hybridization by the above NISH protocol used a digoxigenin-labeled oligomer specific for region D of cytomegalovirus (CMV) DNA (probe 628; ref. 25).

RESULTS

Chimpanzee 1304. Fig. 1*A* summarizes the serum and NISH results. Biopsy samples taken before, at the time of, and 1 day after inoculation were negative by NISH. On day 2 a positive signal was found within the cytoplasm of most hepatocytes. It had a panlobular distribution, and Kupffer's cells were not stained (Fig. 2 *Left*). Positive NISH results coincided with the first ALT increase but preceded by 1 day the appearance of HCV RNA in serum (Fig. 1*A*). One week after inoculation, the NISH signal appeared to be concentrated under the perisinusoidal hepatocyte membranes (Fig. 2 *Right*), although occasionally some diffusely stained hepatocytes could still be seen. Subsequently, the staining decreased in both intensity and number of positive cells, becoming negative 3 weeks after inoculation. At this time, serum ALT was normal and HCV RNA was negative. One week later, the NISH positivity returned and then fluctuated, paralleling the serum ALT activity. Peak intensity was observed at week 8, with the cytoplasm of most hepatocytes stained. The staining became submembranous again 1 week later and disappeared at week 11. A liver sample taken 20 weeks after inoculation showed only a weak membranous positive reaction with the NISH assay. In contrast, serum HCV RNA, except for a single negative value 3 weeks after inoculation, remained positive throughout the follow-up period.

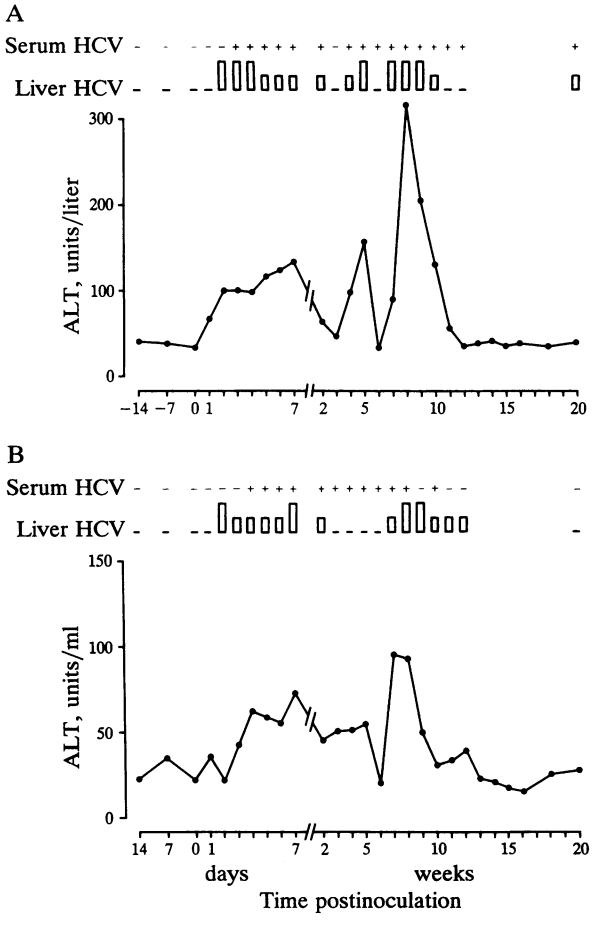

FIG. 1. Comparison of serum ALT activity and presence of HCV RNA in serum and liver of chimpanzees 1304 (*A*) and 1313 (*B*). Results of detection of serum HCV RNA are given qualitatively (either positive or negative). Results of liver HCV RNA by NISH are given semiquantitatively as follows: large open block, 50–95% of cells positive; small open block, <50% of cells positive; dash, negative.

Chimpanzee 1313. Fig. 1*B* shows the results of both serum and NISH assays. As with chimpanzee 1304, liver biopsy samples taken before, on the day of, and 1 day after inoculation were negative by NISH. Cytoplasmic staining was observed on day 2 and persisted through week 2 of infection. Positive results in the liver again coincided with the first increase in serum ALT and occurred 2 days before the appearance of HCV RNA in the serum. The signal could not be detected during weeks 3–6 of infection but recurred during week 7 in parallel with the second ALT increase. During

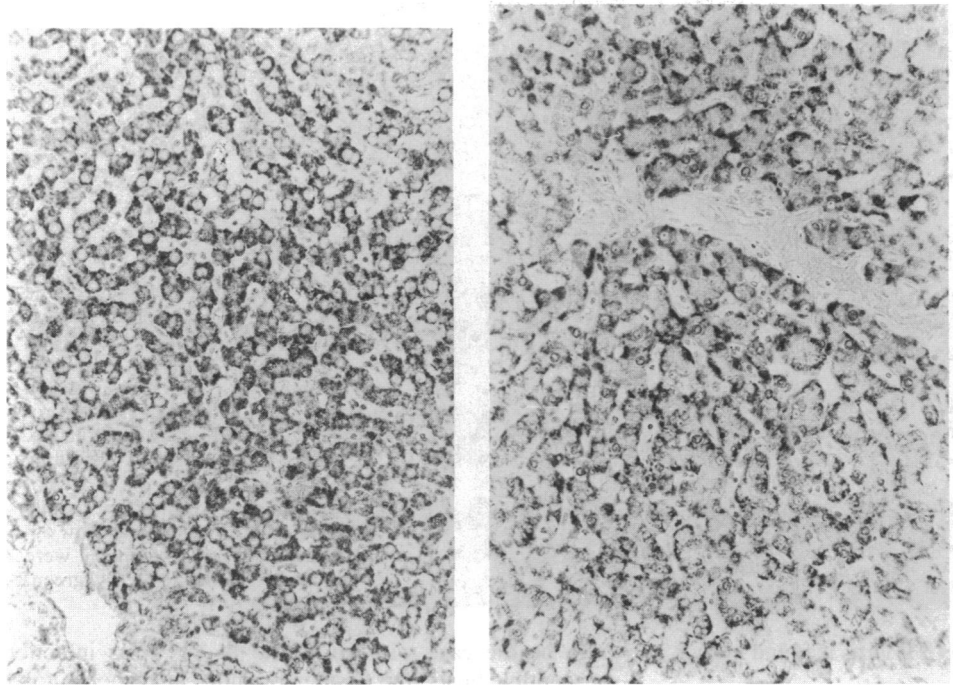

FIG. 2. Occurrence of diffuse cytoplasmic (*Left*) and perisinusoidal, submembranous (*Right*) HCV RNA as detected by NISH. Nuclei were counterstained with methyl green. (×40.)

week 12, some hepatocytes were still positive, but this was no longer detectable 20 weeks after inoculation. A predominantly submembranous, perisinusoidal signal was observed at 5 days and at 2, 11, and 12 weeks after inoculation. Serum HCV RNA was negative 20 weeks after inoculation.

Specificity Controls. The specificity of the NISH reaction was confirmed by the failure to detect a positive signal during experiments that involved (*i*) hybridization with the oligomer specific for HCV RNA to liver biopsy sections obtained from the animals with acute hepatitis type A, B, or δ or from the two animals never experimentally exposed to any hepatitis agent (Fig. 3); (*ii*) predigestion with RNase of a biopsy section taken from animal 1304 at the time of peak positivity for intrahepatic HCV RNA (week 9 after inoculation); (*iii*) hybridization of a section from the same liver biopsy with a digoxigenin-tailed oligomer having a sequence unrelated to HCV RNA (specific for CMV).

Histopathology. Examination of coded liver biopsy specimens taken from both animals before inoculation with HCV and routinely stained with H&E showed the presence of rare and small intralobular accumulations of inflammatory mononuclear cells, mainly lymphocytes and histiocytes. These were consistently detected throughout the whole period of observation, without gross variations in size or frequency, and were similar to those frequently seen in normal nonhuman primates. They were, however, absent at the end of the protocol—i.e., 20 weeks after the inoculation with HCV. Rare foci of liver cell necrosis were observed in both animals 1 week after inoculation, approximately when the serum ALT reached its first peak value. Intralobular necrosis reached its maximum 1 week later, when affected hepatocytes showed a pyknotic nucleus and pigment deposits within the cytoplasm. At that time, remarkable features also included the collapse of the reticulum and a mild lymphocytic and histiocytic infiltrate. Analysis of similar sections showed that areas corresponding to necrotic hepatocytes were negative for HCV RNA as detected by NISH (Fig. 4), whereas cells stained for the viral RNA appeared to have a normal morphology (Fig. 5). Three weeks after inoculation the histopathological findings had reverted to those seen before the challenge. Although both animals experienced a second and more significant ALT increase 8 and 7 weeks, respectively, after inoculation with HCV, and intrahepatic HCV RNA reappeared in both, these were not accompanied by recurrence of significant hepatocellular necrosis.

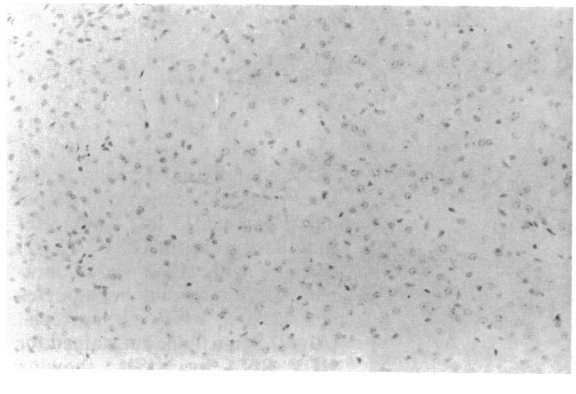

FIG. 3. Lack of staining after NISH for HCV RNA in a liver biopsy section of a chimpanzee never exposed to hepatitis agents. Original magnification ×60. Nuclei were counterstained with methyl green. (×40.)

DISCUSSION

We report here the use of NISH to detect intrahepatic HCV RNA replication. Our method was intended to detect genomic-strand HCV RNA and its specificity was established by appropriate control experiments. Interestingly, detection of HCV RNA required denaturation prior to hybridization. It is not known whether the viral genome is annealed to its complement (as in a replicative intermediate), to itself (because of internal base pairing), or to cellular components.

HCV RNA appeared in the liver very soon after inoculation (2 days in both animals), confirming that viral replication is an early event in experimental infection with HCV (22). The site of replication was the cytoplasm of the infected hepatocytes. Both findings are consistent with the data obtained *in vitro* as well as *in vivo* with flaviviruses (26–28). The cell tropism of HCV may be different from that of YF virus, a mainly hepatotropic flavivirus, since HCV RNA was not detected within Kupffer's cells, at least within the range

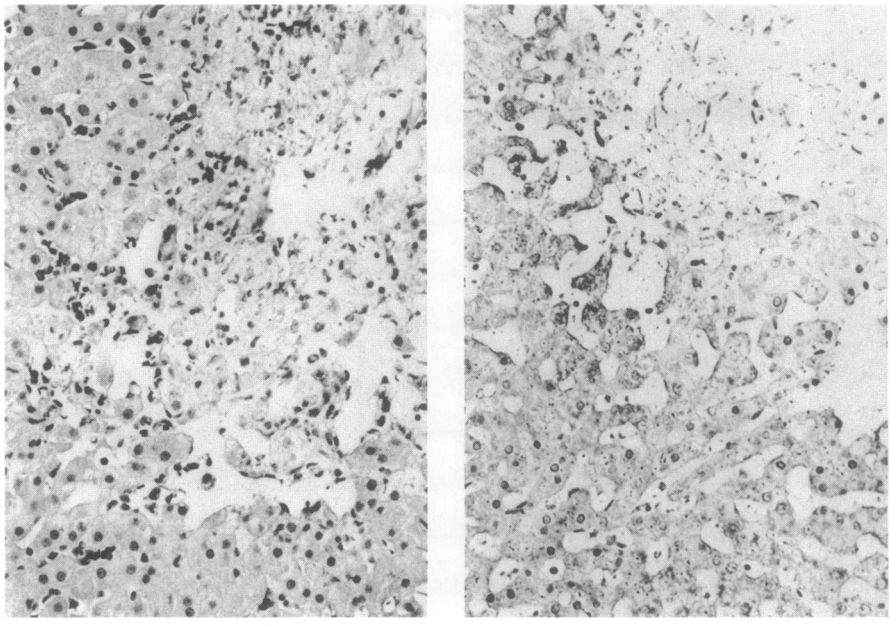

FIG. 4. Serial sections of the liver biopsy specimen taken from chimpanzee 1304 two weeks after inoculation with HCV. (*Left*) Staining with H&E, showing an area of focal hepatocellular necrosis with inflammatory infiltrate. (*Right*) Same field, but stained by NISH for HCV RNA. The area of focal necrosis is negative, while surrounding hepatocytes show cytoplasmic staining. Nuclei were counterstained with methyl green. (×170.)

of sensitivity of our assay, whereas YF virus is found in such cells.

The appearance in the liver of HCV RNA as detected by NISH paralleled the pattern of serum ALT activity. This close temporal relationship suggests a possible direct cytotoxicity caused by the replication of HCV in hepatocytes. However, histopathological evaluation of these same liver biopsy specimens routinely stained with H&E failed to suggest a correlation between ALT elevation and lobular necrosis. Moreover, the rare, small necrotic areas did not stain for HCV RNA, suggesting that viral replication and cell death are not directly related. Since most infected hepatocytes appeared normal, it is possible that HCV exerts its toxic effect by altering cell membrane permeability or some other physiological function, without gross alteration of liver cell morphology, at least as viewed by light microscopy. This is in contrast to the pathological findings observed in YF virus infections of humans and nonhuman primates, where necrosis may involve all hepatocytes in particularly severe disease (27). On the other hand, host resistance to development of the clinical syndrome of YF, despite high titers of viremia, has been described in some African primates (27), and this might also be the case with HCV. As in YF virus infection of susceptible hosts, the tissue mononuclear response to HCV infection in the chimpanzee is very weak.

On some occasions, as ALT decreased, there was a shift from diffuse cytoplasmic staining to a more submembranous appearance. This intracellular distribution might reflect the maturation stage of virus particles, preceding their release into the circulation. A similar pattern has been described during the morphogenesis of the alphaviruses, a family of viruses similar to, but distinct from, the flaviviruses (28), but this has not been conclusively shown for the flaviviruses. Alternatively, this accumulation could reflect impaired release of virions, induced, for instance, by the local production of interferon(s). This phenomenon has been reported for some murine tumor viruses (29, 30) and might also apply to HCV. In fact, both chimpanzees in the present study demonstrated intrahepatic expression of the 48-1 antigen within the cytoplasm of hepatocytes (22). This antigen is contained in typical microtubular structures (31), which have been shown to be induced by α-interferon (32). Such interferon-associated structures are found frequently in the livers of

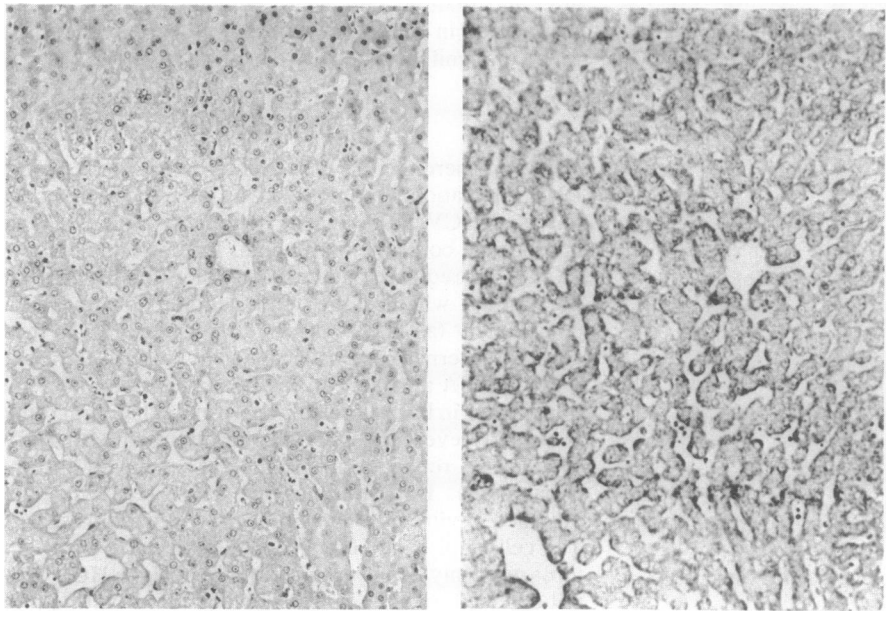

FIG. 5. Serial sections of the liver biopsy specimen taken from chimpanzee 1313 one week after inoculation with HCV. (*Left*) Staining with H&E shows morphologically normal hepatocytes and lack of liver cell necrosis. (*Right*) Same field, but stained for HCV RNA by NISH, showing that most hepatocytes are positive, with a mainly submembranous staining. Nuclei were counterstained with methyl green. (×140.)

HCV-infected chimpanzees but seldom in the livers of similarly infected humans.

The correlation between NISH staining and detection of serum HCV RNA is less evident. A striking difference was found between the number and intensity of stained hepatocytes and the low levels of viremia commonly seen even during the acute phase of HCV infection (3–6). However, neither assay used to detect HCV RNA is quantitative, and therefore a direct comparison of the results is difficult. Moreover, assembly of HCV virions within hepatocytes might not necessarily be followed by their release, as discussed above. On the other hand, occasional discrepancies may be explained by a slow clearance of the virus from the serum. Alternatively, an extrahepatic site of HCV replication, capable of supporting a continuous viremia, must be considered and, indeed, such is suggested by the finding (33) that certain lymphocytes appear to support the replication of HCV *in vitro*.

In conclusion, detection of HCV RNA by NISH appears to be a sensitive and specific method of monitoring intrahepatic viral replication. This study of experimental HCV infection in the chimpanzee animal model suggests that hepatocytes are permissive for viral replication with minor histopathological changes, at least at the light microscopy level. These observations need to be extended to infections in humans because of their potential for furthering our understanding of the pathogenesis of hepatitis C.

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