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Hepatitis B Virus Infection of Adult Human Hepatocytes Cultured in the Presence of Dimethyl Sulfoxide

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We investigated the possibility of infecting normal adult human hepatocytes maintained in pure cultures or in cocultures with hepatitis B virus (HBV). Several assays with different infectious sera and hepatocyte populations from various donors identified only limited HBV replication, with significant variations from one cell preparation to another. The addition of 1.5% dimethyl sulfoxide to the culture medium markedly enhanced the infection process. Indeed, hepatitis B e antigen secretion, the appearance of both HBV DNA replicative forms and major HBV transcripts, and the release of complete HBV particles into the medium were demonstrated. It is possible that the significant increase in intracellular HBV DNA in dimethyl sulfoxide-treated cells was related to enhanced adsorption of the virus. When viral particles produced by a transfected $HepG_2$ cell line were used to infect normal hepatocytes, the same results were obtained. In addition, comparative assays with hepatocytes from three different donors showed that although high amounts of intracellular viral DNA were found in all cases, viral replicative intermediates were visualized in only one case. These findings suggest that this HBV-producing cell line could serve as a reproducible source of infectious virus and that primary culturing of human hepatocytes represents a unique tool for analyzing intracellular regulating factors which, in addition to the penetration step, modulate HBV replication.

Attempts to infect in vitro human liver cells with hepatitis B virus (HBV) have met with limited success over the last 10 years. This could be due to the difficulties in obtaining human adult hepatocytes and in maintaining their liver-specific functions for a prolonged period of cultures.

Recent rapid progress in both hepatitis B virology and molecular techniques has reopened the field and provided new impetus for further experimentation. The recognition that viruses structurally related to HBV exist naturally in ducks, ground squirrels, and woodchucks (26) has created new opportunities for the study of in vitro animal models. Thus, it was demonstrated that the biological activity of both duck HBV (33; I. Fourel, P. Gripon, O. Hantz, L. Cova, V. Lambert, C. Jacquet, C. Guguen-Guillouzo, and C. Trépo, *in* A. J. Zuckerman, ed., *Viral Hepatitis and Liver Diseases*, in press) and woodchuck HBV (29) could be maintained in duck and woodchuck hepatocytes in vitro and that important differences in the replication potencies existed between the two species in cultures. The most marked progress was obtained with the $HepG_2$ (22, 28) and Huh6-C15 (31) cell lines, both of which are derived from human hepatic tumors and produce replicative intermediates and mature virions after transfection with HBV DNA.

However, such models do not mimic normal adult human hepatocytes since they originated either from nonhuman species or from transformed cells (2–4, 12, 14, 19, 22, 24, 28, 31, 33, 34), which have generally undergone genetic and metabolic changes. Therefore, interest in similar systems with normal adult human hepatocytes is great, particularly for determining the specific factors involved in hepatocyte recognition and infection by HBV as well as for understanding the regulation processes of viral replication and integration in humans.

The definition of culture conditions that allow nonproliferative mammalian hepatocytes, including human ones, to survive for several weeks and to maintain their functional integrity in cocultures with liver epithelial cells (10) or in the presence of dimethyl sulfoxide (DMSO) in the culture medium (16) has brought new insights into the search for conditions suitable for the propagation of HBV. In this study we defined culture conditions which allow HBV replication in human adult hepatocytes. The use of the coculture system and the addition of DMSO to the medium greatly prolonged cell survival and enhanced the infectivity of parenchymal cells, as shown by the appearance of viral replicative intermediates in the cells as well as by the synthesis of virus-specific transcripts. In addition, viral particles produced by transfected $HepG_2$ cells were as infective as those from human infectious serum. Furthermore, a large number of infection assays with different infectious sera showed that human hepatocyte infectivity is subject to important variations which cannot be prevented by the use of conditioned medium from transfected $HepG_2$ cells instead of infectious serum.

MATERIALS AND METHODS

Cell isolation and culturing. Human hepatocytes were obtained from the livers of kidney transplantation donors. Cells were isolated by the procedure of Guguen-Guillouzo and Guillouzo (11). A portion of the left lobe of the liver was rapidly perfused with calcium-free *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer and then with a HEPES-buffered solution containing 0.05% collagenase and 5 mM $CaCl_2$. The cell suspension was filtered through gauze and washed three times by centrifugation at 50 × *g*, and the cells were counted.

Freshly isolated hepatocytes were seeded in 10 ml of culture medium at a density of 8×10^6 cells per 75-cm^2 flask. The medium was composed of 75% minimum essential medium and 25% medium 199 supplemented with 10 μg of

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insulin per ml, 0.2% bovine serum albumin, and 10% fetal calf serum. After 12 h the medium was renewed. Some of the cultures were set up as pure cultures, and some were set up as cocultures by adding nonparenchymal rat liver epithelial cells (RLECs) (6). The media for both pure cultures and cocultures were renewed daily or every 2 days and supplemented with 7×10^{-5} M hydrocortisone hemisuccinate. In some experiments the culture medium was deprived of fetal calf serum and supplemented with 1.5% DMSO.

Infection of cell cultures. Hepatocytes were infected with HBV-positive sera either in suspensions immediately after perfusion or in cultures 1 to 3 days after seeding. In the first case, 10^7 cells were pelleted, suspended in 1 ml of infectious serum, and incubated for 1 h at room temperature, with gentle agitation every 5 min. Cells were washed three times and seeded. In the second case, 8×10^6 hepatocytes in a 75-cm^2 flask were incubated overnight at 37°C with 1 ml of infectious serum diluted in 10 ml of culture medium. After incubation, cell monolayers were rinsed three times. Both conditions were efficient. However, since the latter conditions were more appropriate for modulating the experimental conditions of infection, we used them for analyzing the effect of DMSO.

Assays for HBV-specific proteins. Hepatitis B surface and e antigens (HBsAg and HBeAg, respectively) were identified by a radioimmunoassay kit obtained from Abbott Laboratories. The conditions were those recommended by the manufacturer. A signal/noise ratio (P/N ratio) of >2.1 was considered positive.

DNA extraction and analysis. Cells were lysed in 0.5% sodium dodecyl sulfate–10 mM Tris hydrochloride (pH 8)–10 mM EDTA–10 mM NaCl–200 μg of proteinase K per ml and incubated overnight at 37°C. To the lysate was added 1 M NaCl, and the mixture was stored overnight at 4°C, in accordance with the Hirt (13) procedure, allowing cellular DNA to precipitate. Cellular DNA was pelleted at 15,000 rpm for 1 h at 4°C in a JA20.1 rotor (Beckman Instruments, Inc.). The supernatant was deproteinized by two extractions with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) followed by two extractions with chloroform-isoamyl alcohol (24:1 [vol/vol]). To the aqueous phase was added 0.3 M sodium acetate (pH 5.5), and nucleic acids were precipitated with 2 volumes of ethanol. The pellet was dissolved in 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA.

To obtain viral DNA in the culture medium, we pelleted viruses through a cushion of 10 to 20% sucrose in 10 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–1 mM EDTA (TNE) in a Beckman SW50 rotor at 45,000 rpm for 5 h at 4°C. Nucleic acids were extracted as described above, except that the Hirt precipitation was omitted.

DNA was analyzed by electrophoresis in horizontal slab gels of 1.5% agarose in 40 mM Tris-acetate (pH 8)–2 mM EDTA. Gels were soaked in 0.25 N HCl for 15 min, and the DNA was denatured in situ, neutralized, and transferred to nitrocellulose filters by the method of Southern (25). Hybridization was performed with a ^{32}P-labeled HBV probe (5). Molecular weight markers were *Hin*dIII-digested bacteriophage λ and HBV DNA restriction fragments (3,182 and 1,504 base pairs).

RNA extraction and analysis. Cells were lysed in a solution containing 5 M guanidine thiocyanate, 0.1 M sodium acetate (pH 5.5), 1 mM EDTA (pH 7.4), 2% sarcosyl, and 5% β-mercaptoethanol by the method of Glisin et al. (9) as modified by Raymondjean et al. (20). The cell lysate was layered on a 4-ml cushion of CsCl (0.99 g/ml) in 0.1 M EDTA (pH 7.4) in a Beckman SW28.1 polyallomer tube and centrifuged at 25,000 rpm for 22 h at 20°C. The pellet was suspended in 10 mM Tris chloride (pH 7.45)–1 mM EDTA–1% sarcosyl, adjusted to 0.3 M with sodium acetate (pH 5.5), and precipitated with 2.5 volumes of ethanol. The precipitate was washed successively with 3 M sodium acetate (pH 5.5) and ethanol and suspended in water. RNA preparations were kept in solution in water at −70°C. RNA samples were electrophoresed through 1.5% agarose gels containing 1.1 M formaldehyde (17) and transferred to nitrocellulose filters for hybridization.

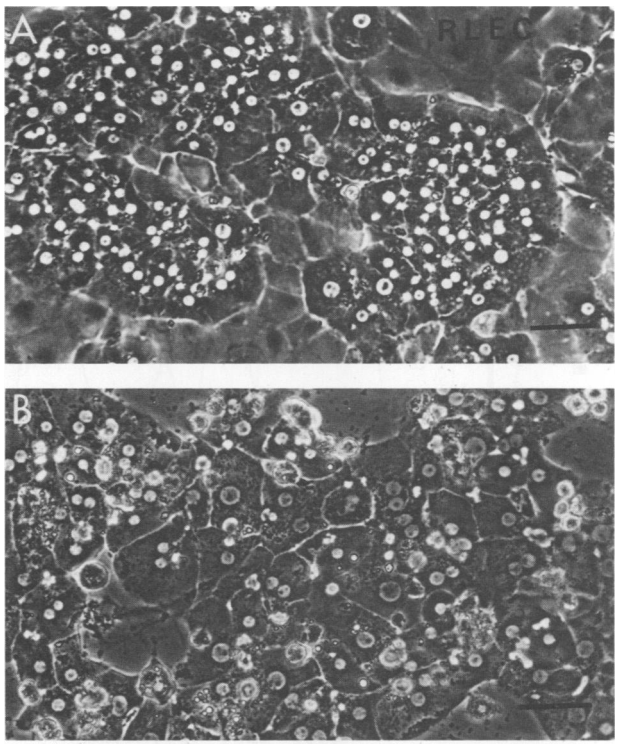

FIG. 1. Phase-contrast micrograph of human hepatocyte primary cultures. (A) Appearance of a 10-day-old coculture maintained in the absence of DMSO; hepatocytes are surrounded by RLECs. (B) Appearance of a 10-day-old pure culture maintained in the presence of 1.5% DMSO. Bars, 50 μm.

Isopycnic centrifugation in a cesium chloride gradient. CsCl (1.38 g) was added to 4.5 ml of culture medium obtained from infected hepatocytes. Centrifugation was performed in a Beckman SW50 rotor at 40,000 rpm for 60 h at 4°C. The gradient was collected from the top and fractionated into 375-μl samples. The density of each fraction was determined by weight measurement, and the samples were dialyzed against TNE. DNA from fractions 3 to 13 was extracted as described above.

RESULTS

The two-step collagenase perfusion of a portion of adult human liver yielded 10^8 to 10^9 hepatocytes with a viability which varied from 70 to 90%. When seeded in pure cultures, hepatocytes formed monolayers of granular epithelial cells which survived for around 2 weeks. In contrast, coculturing of these cells with RLECs prolonged their survival for up to 6 weeks and favored the maintenance of their liver-specific functions (Fig. 1A) (6). This stability in cocultures was

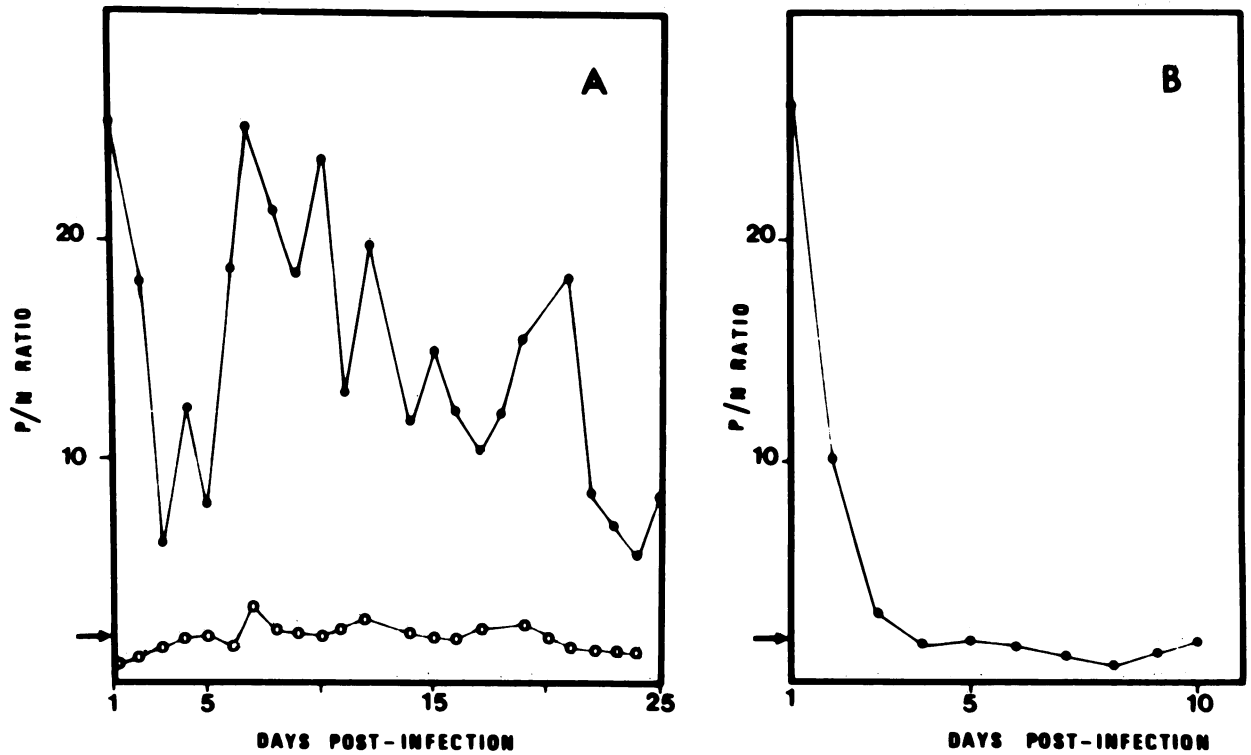

FIG. 2. Kinetics of HBsAg and HBeAg production by human hepatocytes experimentally infected with HBV and maintained in cocultures. Hepatocytes were prepared from different donors and infected with various HBV-positive human sera. (A) HBsAg (●) and HBeAg (○) secretion pattern observed in one experiment from the group of highly efficient infection assays (group 1). (B) Pattern of HBsAg secretion observed in one experiment from the group of negative infection assays (group 2). Both viral antigens were measured by a radioimmunoassay from Abbott Laboratories and expressed as P/N values (P, counts per minute measured in the sample; N, counts per minute measured in the negative control). A P/N higher than 2.1 (arrow) was considered positive.

correlated with the maintenance of higher levels of liver-specific mRNAs. Thus, the level of albumin mRNA in cocultured cells was maintained at 50% of its initial value in freshly isolated hepatocytes for at least 21 days (data not shown). A few cell divisions occurred in hepatocyte colonies between days 4 and 10.

The addition of 1.5% DMSO to the fetal calf serum-deprived culture medium slightly prolonged cell survival (Fig. 1B) and strongly improved the functional stability of human hepatocytes in pure cultures (unpublished results), as described for rat hepatocytes (15). In cocultures, DMSO did not modify the survival of hepatocytes, whereas it altered that of RLECs after 1 week of exposure. However, parenchymal cells remained highly differentiated for at least 2 weeks.

Detection of HBV proteins and DNA in media from cultured human hepatocytes experimentally infected with human sera. Human hepatocytes either freshly isolated or in primary cultures for 1 or 2 days were exposed to human serum containing viral particles. To determine if HBV was replicating in these cultures, we measured HBsAg and HBeAg production in the culture media collected daily from day 1 to 3 weeks after infection. A total of 77 infection assays were performed with hepatocyte preparations from 39 donors and 27 different HBeAg- and HBV DNA-positive infectious sera. The results were classified in three groups according to the level and the pattern of viral antigen secretion. In the first group of experiments, large amounts of HBsAg were secreted for a prolonged period, with the following pattern of secretion: after a transient and passive release of HBsAg from the infectious serum for the first 4 days of culturing, active secretion occurred by day 6 or 7. This secretion gradually increased up to day 10 to reach values corresponding to a P/N ratio of 10 and sometimes higher (Fig. 2A). This secretion was generally maintained for up to 3 weeks. In addition, HBeAg production was constantly observed, paralleling the curve of HBsAg secretion. In the second group of experiments, after the transient release of HBsAg, the cells failed to actively secrete viral antigens, and cultures remained negative for HBsAg and HBeAg after 5 days (Fig. 2B). In the third group of experiments, HBsAg secretion either remained constantly expressed after 5 days, although at a very low level (P/N ratio, <10), or was limited to two or three peaks during culturing. HBeAg secretion was very weak or undetectable (data not shown).

HBV DNA was only detected in the media containing the highest amounts of HBsAg and HBeAg in experiments of the first group. However, Southern blot analysis of hepatocyte cellular DNA failed to demonstrate the presence of HBV DNA replicative intermediates (data not shown).

Although we found that infection was efficient with 22 assays and with 10 sera, only three assays fit in the first group, indicating that only limited viral replication occurred in this model. Table 1 also shows that infectivity was subject to variations according to both hepatocyte origin and infectious serum origin.

Effect of DMSO on HBV infection. To determine if viral replication might be stimulated by the addition of DMSO to the culture medium, we infected 3-day-old hepatocyte monolayers, maintained either in pure cultures or in cocultures,

TABLE 1. Variations in infectivity for human hepatocytes experimentally infected with HBV-positive human sera[a]

Group	No. of:		
	Experiments	Donors	Sera
1	3	3	3
2	55	22	17
3	19	14	7

[a] Infection assays were classified in three groups according to the level and the pattern of viral antigen secretion: group 1, HBsAg positive (P/N ratio, >10) and HBeAg positive after 2 weeks; group 2, HBsAg and HBeAg negative after 5 days; group 3, HBsAg positive (P/N ratio, <10) and HBeAg negative after 5 days.

with human infectious serum (chosen because of its high titer of viral DNA) and tested them for viral replication. HBsAg was found to be actively secreted; the secretion rate followed the same pattern as that observed in cocultures in the absence of DMSO. In addition, the secretion levels of HBeAg were considerably higher than those obtained from cultures maintained without DMSO. Both antigens were expressed throughout culturing (data not shown).

To characterize extracellular and intracellular extrachromosomal HBV DNAs, we collected the medium at different times after infection and harvested cells just after infection and on day 11. Cellular DNA was analyzed with the Southern blot procedure for the presence of HBV DNA replicative intermediates. Hybridization with the HBV DNA probe revealed in the undigested cellular DNA of freshly infected

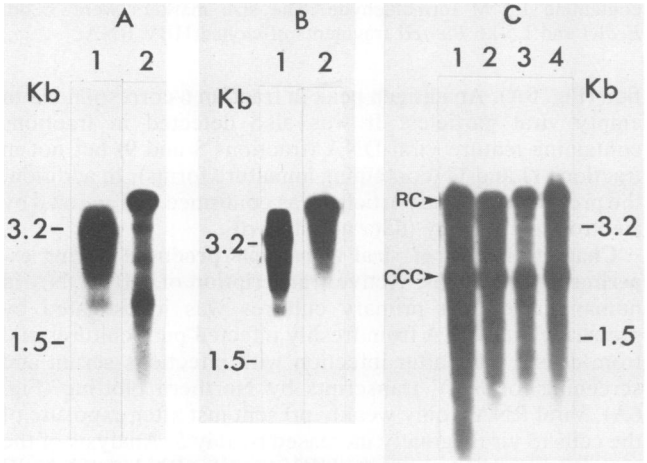

FIG. 3. Southern blot analysis of DNA present in the two virus sources and of intracellular extrachromosomal HBV DNA from infected human hepatocyte primary cultures. (A) Viral DNA extracted after proteinase K treatment from 1 ml of 10-fold-diluted infectious serum (lane 1) and from 1 ml of the $HepG_2$-transfected cell (clone F1 2215) supernatant (lane 2). (B) DNA extracted after proteinase K treatment from the Hirt supernatant of human hepatocyte pure cultures that were maintained in the presence of 1.5% DMSO, infected with either HBV-positive serum (lane 1) or conditioned medium from F1 2215 cells (lane 2), and harvested just after exposure to virus. (C) DNA extracted from pure cultures (lanes 1 and 2) and cocultures (lanes 3 and 4) that were infected with either HBV-positive serum (lanes 1 and 3) or conditioned medium from F1 2215 cells (lanes 2 and 4) and harvested 11 days after infection. One-fourth of the DNA extracted from a 75-cm^2 flask was deposited onto a 1.5% agarose gel. RC and CCC DNA species are indicated. The size markers were cloned HBV DNAs digested with *Eco*RI (3.2 kb) and *Bam*HI (1.5 kb).

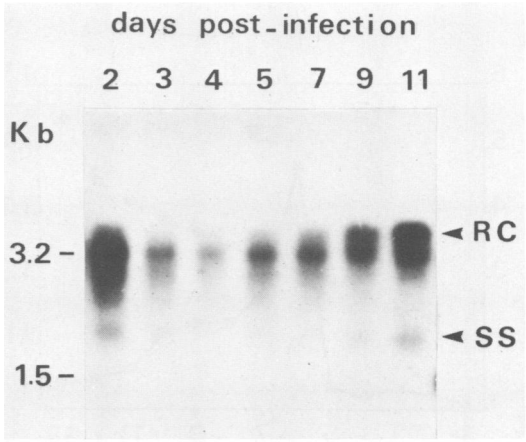

FIG. 4. Southern blot analysis of extracellular HBV DNA separated on a 1.5% agarose gel. Extracellular viruses were isolated by pelleting particles from 4 ml of culture medium harvested at 2, 3, 4, 5, 7, 9, and 11 days after infection. Hepatocytes were infected with human HBV-positive serum and maintained in pure cultures in the presence of 1.5% DMSO. The size markers were cloned HBV DNAs digested with *Eco*RI (3.2 kb) and *Bam*HI (1.5 kb). RC and single-stranded (SS) species are indicated.

cells (Fig. 3B, lane 1) a main band corresponding to a 3.2-kilobase (kb) DNA fragment and a short smear, with no evidence of low-molecular-weight DNA forms. This pattern differed only slightly from that observed with the serum used for infection (Fig. 3A, lane 1); indeed, the serum Dane particles had a short smear ranging from 4 to 2 kb. In contrast, the viral DNA pattern observed in 11-day-old cultures appeared markedly different (Fig. 3C, lanes 1 and 3). A smear extending from the 4-kb DNA position to very-low-molecular-weight DNA positions was observed; two main discrete bands were superimposed on the smear and corresponded to 4- and 2.3-kb DNA fragments, consistent with the relaxed circular (RC) and covalently closed circular (CCC) forms of free HBV DNA (7, 21, 28). In addition, several distinct bands located below the 2-kb position were clearly present, consistent with incomplete HBV DNA minus strands. Identical data were obtained with hepatocytes in pure cultures and in cocultures.

The medium of DMSO-treated cells was also tested for HBV DNA (Fig. 4). The total amount of viral DNA detected by Southern blotting was higher than that found in untreated cultures. Medium collected during the first 2 days after infection contained large amounts of HBV DNA; Southern blot analysis revealed a band located near the 3.2-kb position, a pattern consistent with the partially double-stranded mature form of HBV DNA. The level of HBV DNA in the medium drastically decreased up to day 4 after infection and then increased through day 11. At that time the HBV DNA pattern included completely double-stranded RC HBV DNA and partially double-stranded mature DNA forms. This pattern differed from that observed with the infectious serum, which contained a few completely double-stranded DNA forms.

Infectivity of the viral particles released into the medium by transfected $HepG_2$ cells. To determine if viral particles produced by transfected $HepG_2$ cells were capable of infecting normal human hepatocytes in vitro, we selected the medium from clone F1 2215 for infection, based on the ability of this clone to produce large amounts of viral particles (22), and cultured cell monolayers in the presence of 1.5% DMSO.

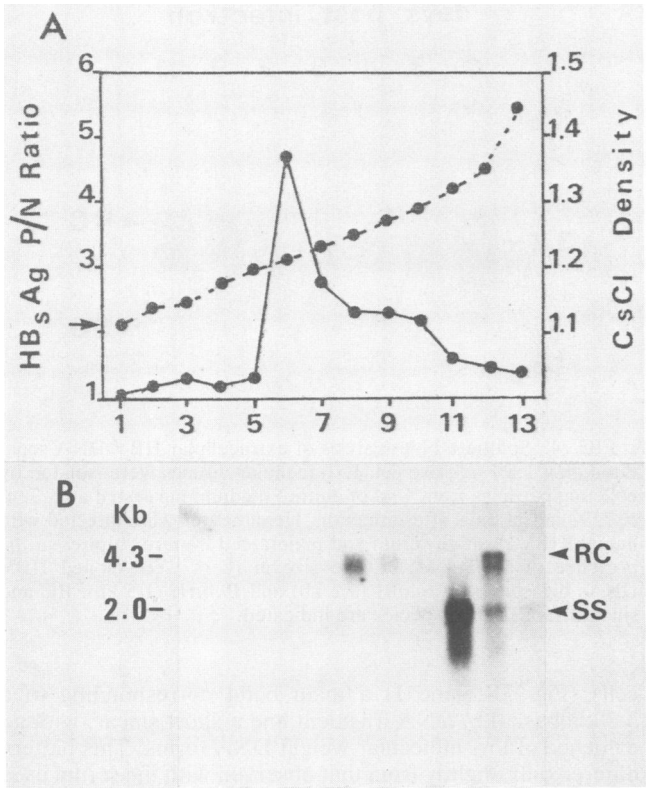

FIG. 5. Analysis of a CsCl gradient of HBV particles in medium from cultures 11 days after infection. CsCl (1.38 g) was added to 4.5 ml of culture medium from hepatocytes infected with the F1 2215 supernatant; the gradient was centrifuged for 60 h at 150,000 × *g*. Thirteen fractions were collected from the top of the centrifuge tube. (A) Analysis of the fractions by radioimmunoassay for the detection of HBsAg (——). Fractions were diluted 10-fold, and the density of each fraction was determined (– – –). (B) Southern blot analysis of the DNA isolated from fractions 3 to 13 and separated on a 1.5% agarose gel. The size markers were *Hin*dIII-digested bacteriophage λ DNAs. RC and single-stranded (SS) species are indicated.

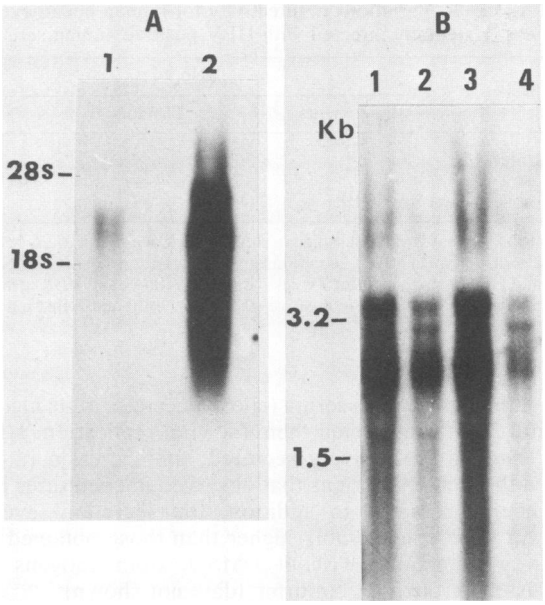

FIG. 6. Kinetic analysis by the Northern blot procedure of the HBV transcripts produced by infected hepatocytes in cultures. Total RNA was extracted as described in Materials and Methods. (A) Viral RNA extracted from cells just after exposure to virus (lane 1) and 5 days later (lane 2). The size markers were 18S and 28S rRNAs. (B) RNA prepared from DMSO-treated pure cultures (lanes 1 and 3) and cocultures (lanes 2 and 4) infected by day 3 either with HBV-positive human serum (lanes 1 and 2) or with the F1 2215 supernatant (lanes 3 and 4). Cells were harvested 11 days after infection. RNA (20 μg) was loaded onto a 1.5% agarose gel containing 1.1 M formaldehyde. The size markers were 3.2-kb *Eco*RI and 1.5-kb *Bam*HI fragments of cloned HBV DNA.

Infected cells secreted high levels of HBsAg and HBeAg in both pure cultures and cocultures. The patterns were similar to those obtained in assays with human infectious serum with an equal titer of viral DNA. However, the HBsAg/HBeAg ratio was slightly diminished (data not shown). The evolution of the intracellular HBV DNA was identical to that observed with serum-infected cultures: a main band corresponding to a 4-kb DNA fragment and a short smear were observed in cells just after exposure to virus, whereas both mature and replicative forms were identified in cells on day 11 (Fig. 3B and C).

Medium from 11-day-old cultures experimentally infected with the $HepG_2$ supernatant was analyzed for particles containing HBV DNA. Particles were separated by cesium chloride density gradient centrifugation. The hybridization pattern obtained indicated that the particles had a buoyant density of 1.23 to 1.26 g/cm^3 (fractions 8 and 9) and 1.32 to 1.35 g/cm^3 (fractions 11 and 12) (Fig. 5B). Particles banding at the density of complete virus (fractions 8 and 9) contained only mature DNA corresponding to partially double-stranded RC forms, whereas particles in fractions 11 and 12 (near a density of 1.34 g/cm^3) contained predominantly single-stranded immature DNA forms.

Fractions of the gradient were assayed for HBsAg secretion (Fig. 5A). An antigen peak in fraction 6 corresponded to empty viral particles. It was also detected in fractions containing mature viral DNA (fractions 8 and 9) but not in fractions 11 and 12 (containing immature forms). In addition, the presence of Dane particles was confirmed in fraction 8 by electron microscopy (data not shown).

Characterization of viral transcripts produced during experimental infections. Active transcription of HBV DNA in human hepatocyte primary cultures was investigated by preparing total RNA from freshly infected pure cultures and from cells 5 days after infection with infectious serum and screening for HBV transcripts by Northern blotting (Fig. 6A). Viral RNAs, only weakly present just after exposure of the cells to virus, greatly increased by day 5. Analysis of the pattern revealed two major HBV-specific RNAs at 2.4 and 3.5 kb by day 5, whereas the 3.5-kb band was not observed early after infection. The 2.4-kb RNA was most likely the messenger for the major envelope protein, and the 3.5-kb RNA may correspond to the pregenome (30). Comparison of the viral transcripts in pure cultures and cocultures infected with either infectious serum or the clone F1 2215 supernatant was done on day 11 (Fig. 6B). In addition to the two major RNA bands, a minor band with an apparent length of 3 kb and a faint low-molecular-weight transcript at 1.7 kb were observed. The pattern of viral transcripts was similar with the two infecting sources under both culture conditions. Comparison of the pure cultures and cocultures revealed smaller amounts of transcripts in the latter.

Individual variations in the ability of human hepatocytes to promote viral replication. Among the early events of the infection process, adsorption of the virus to the cells and

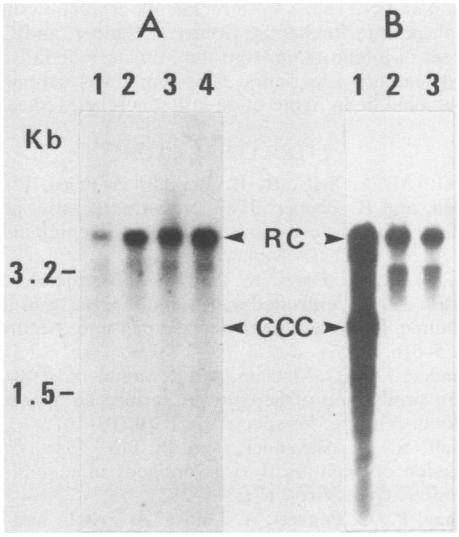

FIG. 7. (A) Southern blot analysis of intracellular HBV DNA from hepatocytes infected with the F1 2215 supernatant and cultured in the presence of 0, 0.5, 1, or 1.5% DMSO (lanes 1 through 4, respectively). Cells were infected on day 3 and collected 5 days later. The size markers were cloned HBV DNAs digested with *Eco*RI (3.2 kb) and *Bam*HI (1.5 kb). (B) Comparative Southern blot analysis of intracellular HBV DNA from hepatocytes prepared from three different donors (lanes 1, 2, and 3) and infected with the F1 2215 supernatant. Cells were cultured in the presence of 1.5% DMSO and harvested 10 days after infection. RC and CCC species are indicated.

penetration of the virus into the cells could play a critical role by boosting infectivity. Since the addition of DMSO to the culture medium increased viral replication, we compared the amounts of inoculating viruses which remained associated with the cells when cultures were maintained in the presence or absence of DMSO (Fig. 7A). Comparative infection assays were done by infecting control and DMSO-treated cells prepared from one individual with the supernatant of transfected $HepG_2$ cells containing infectious particles. The amounts of viral DNA which were associated with the cells significantly increased after the addition of 0.5, 1, or 1.5% DMSO to the culture medium. However, no significant difference could be observed between the different concentrations of DMSO.

Infection assays with hepatocyte preparations from 39 donors revealed differences from one experiment to another. Therefore, we question whether these differences were correlated to variations in the penetration of the virus into the cells or whether they resulted from other events which could play a critical role in the completion of the viral life cycle in the cells. Parenchymal cells prepared from three different donors were cultured with a medium containing 1.5% DMSO and examined for the presence of intracellular replicative intermediates of viral DNA following infection with the culture medium conditioned by the F1 2215 cells. The medium was adjusted to contain equal amounts of viral DNA for each assay. We found that the amounts of viral DNA associated with the cells were high in the three cases (Fig. 7B). However, only cells from one donor had a pattern of replicative intermediates of viral DNA, suggesting individual variations in the ability of human hepatocytes to support replication of the virus present in the cells.

DISCUSSION

This study demonstrates the possibility of obtaining HBV infection of adult human hepatocytes in primary cultures and emphasizes the role of DMSO in this process. Viral replication was revealed by the production of viral antigens, by the appearance of intracellular specific HBV DNA replicative forms and of viral transcripts, and by the production of extracellular mature particles in the culture medium.

The evolution pattern of the intracellular viral DNA indicated that viral particles which were associated with the cells just after infection mostly corresponded to mature forms and that replicative intermediate forms were absent. In contrast, 11 days after infection, large amounts of RC, CCC, and small HBV DNA replicative intermediate forms were identified in the extrachromosomal cellular DNA. Moreover, the respective amount of the CCC DNA was high, probably resulting from abnormal amplification as previously described for cultures of duck HBV-infected duck cells (32). In addition, the amounts of viral RNA, very weak just after infection of the cells, were greatly increased by day 5, strongly suggesting active synthesis of these viral components. These components included the messenger for the major envelope protein and the 3.5-kb RNA pregenome. These results indicate active replication according to the model proposed by Mason et al. (18), Summers and Mason (27), and Will et al. (35); the CCC DNA serves as a template for transcription of the RNA pregenome before synthesis of the DNA minus-strand by reverse transcription.

The rate of daily secretion of viral DNA into the medium increased over time, suggesting active production of virus which corresponded mainly to mature forms and indicating that viral particles are selectively secreted into the medium. Selective secretion of virions from duck HBV-infected primary hepatocytes in duck cells has also been reported by Tuttelman et al. (33). In older cultures, the large number of completely double-stranded virions released into the medium contrasted with the absence of this form in the serum used for infection.

These results differed from those obtained from numerous infection assays performed under the same culture conditions but without DMSO. Indeed, even in the absence of DMSO the survival and functional capacities of hepatocytes increased in cocultures, and HBsAg antigen production was prolonged for at least 3 to 4 weeks. However, only small amounts of HBeAg were secreted, and viral DNA replicative forms were never observed. Similarly, the absence of replicative forms was reported by Shimizu et al. (23), who maintained human hepatocytes under other culture conditions. In contrast, Pekin duck hepatocyte primary cultures rapidly and consistently allowed active viral replication (33). We note that the addition of DMSO to the medium has also been recently found to increase infectivity for duck cells (J. C. Pugh and J. Summers, personal communication). These data demonstrate that viral replication in vitro can be strongly modulated by culture conditions.

We found that viral particles produced by transfected $HepG_2$ cells have the ability to infect normal human hepatocytes maintained in the presence of DMSO. We obtained the same extrachromosomal forms of viral DNA and the same patterns of viral transcripts in the cells as those obtained with the infectious serum, although the two inoculating virions were different. Furthermore, the forms of HBV DNA isolated from the medium of infected cultures possessed a density and electrophoretic mobilities similar to those of the DNA isolated from characteristic serum-derived

viral particles, whereas a great number of immature forms of viral DNA were detected at a density similar to that of immature core particles.

These results strongly emphasize the previous observations with infectious sera: they show that a full viral life cycle occurs in vitro. In addition, they demonstrate that transfected $HepG_2$ cells produce particles that have the ability to infect human cells in tissue cultures. This result correlates well with the recent demonstration that these particles have the capacity to cause hepatitis in chimpanzees (1). Furthermore, these results provide evidence that $HepG_2$ cells have the potential to serve as a source of infectious virus, as efficient as and more reproducible than that from human sera, for analyzing HBV penetration and replication in normal human hepatocyte primary cultures.

We found that the addition of DMSO to the medium of cultured hepatocytes drastically increased the amounts of viral DNA which remained associated with the cells. Since the amounts of viral replicative intermediates were increased in cells maintained in DMSO-supplemented culture medium, it may be questioned whether this compound increases infectivity in favoring penetration of the virus and/or in maintaining at a high level the expression of the liver-specific genes (8, 15), including the regulatory elements which control HBV gene transcription (30). Furthermore, the possibility that DMSO only favors the expression of specific cell surface receptors for attachment of the virus cannot be excluded.

Investigation of infectivity for human hepatocytes with a large number of infection assays demonstrated that infectivity for human hepatocytes was subject to important individual variations. In some experiments these variations could be easily related to the HBV DNA content of the infectious sera; a large amount of HBV was needed for effective infectious experiments in vitro. However, since variations were also observed from one hepatocyte preparation to another following infection with the same infectious serum, at least two other mechanisms could account for these variations: infectious serum may contain determinants that can interact with plasma membrane components of cells from some donors, thereby modifying attachment of the virus to these cells, or infectivity for the cells may vary from one individual to another, independent of the infectious source. Comparative assays of infection performed with the transfected $HepG_2$ cell-conditioned medium confirmed the latter mechanism.

At the present time, we cannot completely exclude a possible involvement of the perfusion procedure in these variations. However, the data strongly suggest that adsorption of the virus is not necessarily followed by viral replication in cultured hepatocytes. It is, therefore, tempting to speculate that a polymorphism could exist within the complex intracellular regulating factors which control virus penetration and/or viral replication in hepatic cells. These individual variations could account, along with those of the immune response, for the highly polymorphic and unpredictable evolution of HBV-related liver diseases in vivo. Long-term primary culturing of human hepatocytes could be a useful tool for investigating these variations and to analyze the complex interactions between the virus and its host cell in humans.

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