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Correlation between the infectivity of hepatitis C virus *in vivo* and its infectivity *in vitro*

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ABSTRACT **A murine retrovirus-infected human T-cell line, HPB-Ma, supported replication of hepatitis C virus (HCV) at least as well as the previously reported Molt4-Ma cells. Cloning of HPB-Ma cells revealed a clonal variation of cellular susceptibility to HCV infection. Using one of the sensitive clones, we tested HCV inocula from different sources for their infectivity titer in cell culture. The *in vitro* titers obtained correlated with the reported infectivity titers of the inocula in chimpanzees. Thus, the system appears to be useful for estimating the *in vivo* infectivity of HCV.**

The molecular cloning of the hepatitis C virus (HCV) genome has made possible a number of new approaches to the delineation of HCV gene function, pathogenesis, and molecular epidemiology (1). Parts of the HCV genes have been expressed by the recombinant DNA technique in various host–vector systems, and immunodiagnostic assays for anti-HCV antibodies have been developed, using the expressed products (2, 3). It is now firmly established that HCV is the major causative agent of transfusion-associated non-A, non-B hepatitis throughout the world (2, 4). HCV contains a positive-strand RNA of approximately 10 kilobases (1, 5, 6). Although the strategy of HCV replication is not yet fully understood, negative-strand viral RNA is believed to be present in infected cells as a replicative intermediate. In fact, the negative strand of HCV RNA has been detected by reverse transcription/polymerase chain reaction (RT/PCR) in infected liver tissue (7–9).

In spite of the progress in molecular biology of HCV, the biological characteristics of this virus remain obscure. This is primarily because biological assays for HCV have been limited to the experimental inoculation of chimpanzees, which are expensive and restricted in number. It is imperative to develop either a less expensive animal model or an *in vitro* system for propagating HCV. We previously reported that a mouse retrovirus-infected human T-cell line, Molt4-Ma, was capable of supporting replication of HCV (7). The level of the viral replication in Molt4-Ma cells, however, was very low and not fully satisfactory for wide application to *in vitro* studies of this virus. Thus, we have continued to examine other cell lines, including HPB-ALL, HPB-Ma, Daudi, HL60, K652, and MT4 for their susceptibility to HCV infection. In this report, we present evidence that a clone of cell line HPB-Ma supports HCV replication more consistently than Molt4-Ma, and we demonstrate a correlation between the infectivity of various HCV inocula *in vivo* and their infectivity *in vitro*.

MATERIALS AND METHODS

Virus. The principal inoculum used in this study was a serum, no. 34, obtained from a chimpanzee (*Pan troglodytes*) infected with the fifth chimpanzee passage of HCV strain F. Inocula used for infectivity titration in HPB-Ma cultures were as follows. Strain H was obtained as plasma collected during the early acute phase of hepatitis C (7 weeks after transfusion) (10). This plasma has been reported to contain $10^{6.5}$ chimpanzee infectious doses (CID) per ml (11). Strain F was obtained as plasma approximately 1 year after onset of chronic transfusion-associated hepatitis C (10). The *in vivo* infectivity titer of plasma F was determined to be $<10^2$ CID/ml: undiluted plasma was infectious in a chimpanzee but 1 ml of the 10^{-2} dilution was not infectious (11). Plasma K was kindly supplied by M. Kohara (Tokyo Metropolitan Institute for Medical Science). One milliliter of a 10^{-4} dilution of this plasma was infectious in one chimpanzee. Plasmas no. 4 and no. 6 were obtained from blood donors who were positive for anti-HCV antibody (donor no. 6 was implicated in the transmission of HCV). They were provided by a research group supported by Grants-in-Aid from the Japanese Ministry of Health and Welfare. One milliliter of a 10^{-5} dilution of plasma no. 6 was infectious in one chimpanzee, but 1 ml of the 10^{-2} dilution of plasma no. 4 was not infectious. The reported *in vivo* infectivity titers (as measured by chimpanzee inoculation) of the inocula and their HCV RNA genome titers (as measured by RT/PCR) are summarized in Table 3, together with other data (see *Results*).

Cell Line. The HPB-Ma cell line was obtained by infecting HPB-ALL human T cells (12) with an amphotropic murine leukemia virus pseudotype of murine sarcoma virus (13). The cells were maintained in RPMI 1640 medium with 8% heat-inactivated fetal calf serum. The HPB-Ma cells were cloned by limiting dilution in 96-well culture plates.

Cycloheximide (CHX) Treatment. In the experiments shown in Figs. 1 and 3*A*, cultures (approximately 5×10^5 cells) were treated with CHX (Sigma) at 50 μg/ml and 37°C for 4 hr before harvest.

Infectivity Titration in Cell Culture. Cloned cells of HPB-Ma (no. 10-2) were suspended at a concentration of 5×10^5 cells per ml. One milliliter of the suspension was mixed with 100 μl of an undiluted sample or a 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, or 10^{-5} dilution of each inoculum. After adsorption at 37°C for 2 hr, the cells were washed twice and suspended in 25 ml of culture medium. The cell suspension was distributed into five Falcon plastic bottles (5 ml each) and cultured at 37°C in a CO_2 incubator without change of medium. One bottle was harvested daily for the detection of intracellular negative-strand HCV RNA.

Detection of HCV Genome. For the detection of HCV genomic RNA, extraction of nucleic acids, RT, and a two-step PCR assay with nested primers were performed as described previously (7). The primers were synthesized, based on the sequence of HCV, and used for the detection of

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Abbreviations: HCV, hepatitis C virus; RT, reverse transcription; CHX, cycloheximide.

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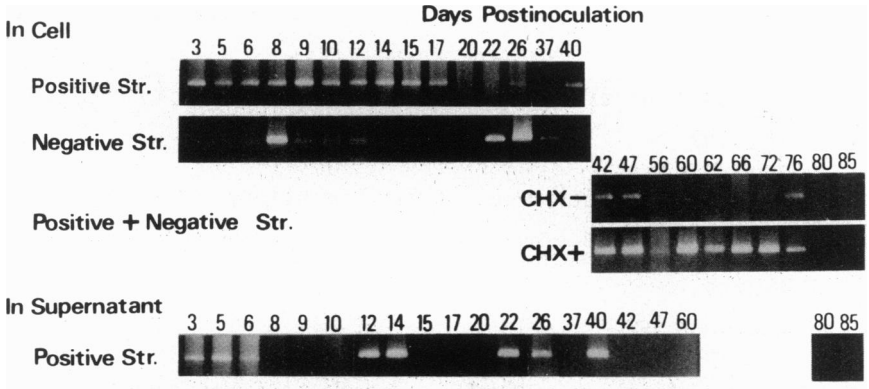

FIG. 1. Detection of viral RNA by RT/PCR in samples from HPB-Ma cultures inoculated with HCV. For the first 40 days of infection, intracellular positive and negative viral RNA strands were detected separately. After day 42, the cells were treated with (+) or without (−) CHX at 50 μg/ml for 4 hr before harvest and RT was carried out in the presence of both sense and antisense primers to detect both viral RNA strands at the same time.

the 5′ noncoding region, known to be highly conserved among HCV isolates (14). Positive- and negative-strand HCV RNAs were detected by performing RT in the presence, respectively, of the antisense primer (5′-GGTGCACGGTCTACGAGACC-3′) or the sense primer (5′-GGCGACACTCCACCATAGAT-3′) to prime cDNA synthesis from the complementary strand. An internal primer pair (5′-TTCACCCAGAAAGCGTCTAG-3′ and 5′-CCCTATCAGGCAGTACCACA-3′) was used for the second round of PCR.

Immunofluorescence. Immunofluorescence was carried out with an anti-HCV core monoclonal antibody produced by immunizing a mouse with a synthetic peptide based on the sequence of the HCV genome. This antibody was kindly supplied by L. Mimms (Abbott) and was used also in our previous study with Molt4-Ma cells (7).

RESULTS

Replication of HCV in HPB-Ma Cells. Because HCV is a positive-strand RNA virus, its replication was evaluated by detection of intracellular negative-strand RNA, which serves as a template for the synthesis of virion RNA.

One-half milliliter of HPB-Ma cell suspension (3×10^6 cells) was mixed with 0.5 ml of undiluted serum no. 34. After adsorption at 37°C for 6 hr, the cells were washed twice, suspended at a concentration of 1×10^5 cells per ml, cultured without medium changes for 6 days, and subcultured at 2- to 4-day intervals thereafter. At intervals, approximately 5×10^5 cells were harvested and assayed by RT/PCR for the presence of HCV RNA. Intracellular positive and negative HCV RNA strands were detected separately for the first 40 days of infection, but after day 42 both strands were assayed at the same time by performing RT in the presence of both sense and antisense primers. Presence of the positive strand in 100 μl of the culture supernatants was also monitored. The results are shown in Fig. 1. HCV RNA was detected for a period spanning 76 days in the cells and 40 days in the supernatants. Intracellular negative-strand RNA first appeared after an incubation period of 6 days and the signal was detectable sporadically thereafter. The positive strand was continuously detected up to 17 days in the cells and up to 6 days in the supernatants. The signals detected shortly after inoculation could have originated from the inoculum. However, the appearance of positive-strand RNA after a negative eclipse period, at 26 and 40 days in the cells and at 12, 14, 22, 26, and 40 days in the supernatants, indicates that HCV replication occurred in cell culture. At least partial viral replication in HPB-Ma cells was confirmed by the results of treatment with CHX, which is known to enhance detection of RNA of other viruses in treated cells. After 42 days of infection, HPB-Ma cells were treated or not treated with

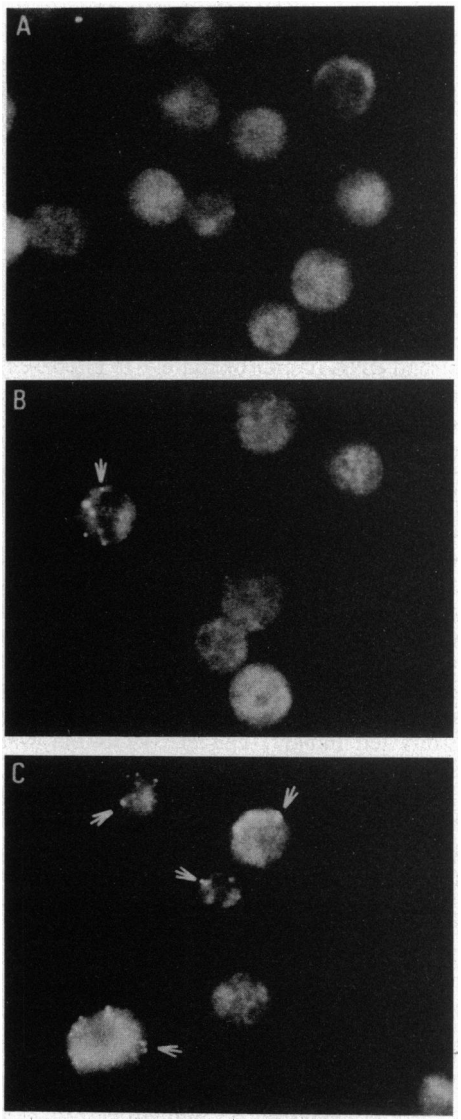

FIG. 2. Indirect immunofluorescent staining of HPB-Ma cells with a mouse monoclonal antibody to HCV core. (*A*) Lack of staining with the antibody in uninoculated HPB-Ma cells. (*B* and *C*) Positive reactions (arrows) in the cells harvested 3 days (*B*) and 7 weeks (*C*) after inoculation with HCV. (×400.)

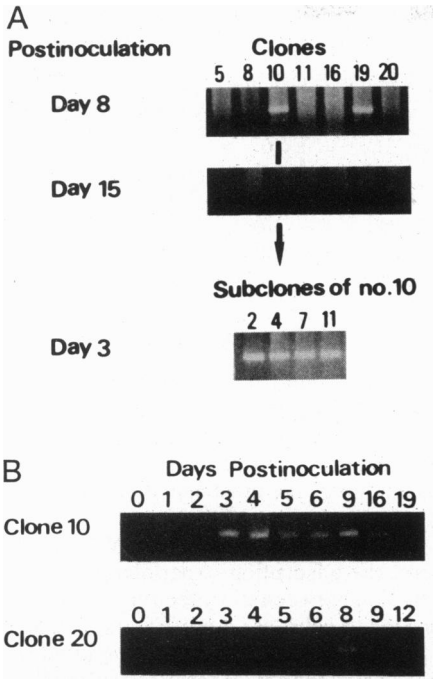

FIG. 3. (*A*) Detection of intracellular negative-strand HCV RNA in HPB-Ma clones and subclones. A cell suspension (1 ml containing 2×10^5 cells) of each clone was mixed with 200 μl of undiluted serum no. 34, incubated at 37°C for 2 hr, washed, and cultured. In the screening of the seven clones (top and middle gels), on days 8 and 15, the cells were treated with CHX at 50 μg/ml and 37°C for 4 hr, harvested, and tested by RT/PCR for the negative-strand HCV RNA. In the screening of the subclones of clone 10, cells were tested in the same manner on day 3 (bottom gel). (*B*) Time of appearance of the negative-strand HCV RNA in clones 10 and 20. A cell suspension (2 ml containing 3×10^6 cells per ml) of clone 10 or 20 was mixed with 200 μl of undiluted serum no. 34, incubated at 37°C for 2 hr, washed, and cultured. Cells were harvested daily without the CHX treatment and tested for negative-strand viral RNA.

CHX at 50 μg/ml and 37°C for 4 hr before harvest. HCV RNA became detectable in treated cells harvested when viral RNA was no longer detectable in untreated cells (on days 62, 66, and 72 in Fig. 1).

When examined by immunofluorescence for HCV core with an anti-HCV core antibody, positive staining was observed in the cytoplasm of cells harvested on day 3 (Fig. 2*B*), and the observed positive cells increased in number at week 7 (Fig. 2*C*).

Clonal Variability. Since there may be clonal variation in susceptibility to HCV infection among HPB-Ma cells, we cloned cells by limiting dilution, obtained seven clones, and tested them for their susceptibility to HCV infection. A cell suspension (1 ml containing 2×10^5 cells) of each clone was inoculated with 0.2 ml of undiluted serum no. 34, washed twice after adsorption at 37°C for 2 hr, suspended in 5 ml of medium, and cultured for 8 days without medium change. On day 8, after addition of an equal volume of medium (5 ml), each culture was divided into two bottles and one of them was harvested after treatment with CHX at 50 μg/ml and 37°C for 4 hr. The other bottle was reincubated and harvested on day 15 after treatment with CHX. Both harvests were assayed by RT/PCR for the presence of intracellular negative-strand viral RNA. As shown in Fig. 3*A*, clones 10 and 19 were positive on day 8 and clone 20 was positive on day 15. The remaining four clones were negative on both days. The observed difference in HCV growth kinetics between clones 10 and 20 was confirmed in daily harvests of the cells; appearance of negative-strand viral RNA was first detected on day 3 in clone 10, but it was delayed until day 8 in clone 20 (Fig. 3*B*). Clone 10 was subsequently subcloned and all of the subclones tested were sensitive to HCV (Fig. 3*A*, bottom gel), indicating that the susceptibility was a genetically stable trait. Clone 10-2 was employed in the experiments described below.

Adsorption of HCV. It has recently been reported (8) that some serum samples contain virions with negative-strand HCV RNA. To estimate the probability of detecting negative strands derived from the inocula in HPB-Ma cells, we examined the rate of adsorption to HPB-Ma cells of presumed virions with positive or negative RNA strands. One-half milliliter of the cell suspension (3×10^5 cells per ml) was mixed with 100 μl of an undiluted sample or a 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, or 10^{-5} dilution of plasma H, no. 6, K, no. 34, no. 4, or F. After adsorption at 37°C for 2 hr, the cells were washed twice with 20 ml of the medium and suspended in 1 ml. The cells were then pelleted by centrifugation and their RNAs were extracted. The whole extract was dissolved in 20 μl of H_2O; 10 μl was used for detection of positive-strand HCV RNA and the remaining 10 μl, for detection of negative-strand RNA. At the same time, 100-μl samples of each dilution of each inoculum were tested by RT/PCR for each strand in the same manner. The results are shown in Table 1. The amount of the positive strand adsorbed was 1/10 of that in the inoculum for H and K, 1/10–1/100 for no. 6, 1/1000 for no. 34 and no. 4, and <1/1000 for F. Thus, adsorption of virions in plasmas with low *in vivo* infectivity was less efficient than that in plasmas with high *in vivo* infectivity, regardless of the titer of RNA genomes as measured by

Table 1. Adsorption of HCV to HPB-Ma clone 10-2 cells

		Detection of HCV RNA by RT/PCR at indicated dilution (log_{10})											
		Positive strand						Negative strand					
Inoculum	Sample	−0	−1	−2	−3	−4	−5	−0	−1	−2	−3	−4	−5
H	Input	NT	+, +	+, +	+, +	+, −	−, −	NT	−, −	−, −	−, −	−, −	−, −
	Adsorbed	+	+, +	+, +	+, −	−, −	−, −	−, −	−, −	−, −	−, −	−, −	−, −
6	Input	NT	+	+, +	+, +	−, +	−	NT	+	−, −	−, −	−, −	NT
	Adsorbed	+, +	+, +	−, +	+, −	NT	NT	−, −	−, −	−, −	−, −	NT	NT
K	Input	NT	+	+	+	−	NT	NT	+	+	−	−	NT
	Adsorbed	+	+	+	−	−	NT	−	−	−	−	NT	NT
34	Input	+	+	+	+	+	−	+	+	+	−	−	−
	Adsorbed	+	+	−	−	−	NT	−	−	−	−	−	NT
4	Input	NT	+	+, +	+, +	−, −	−, −	NT	−	−, −	−, −	−, −	−, −
	Adsorbed	+, +	−, −	−, −	−, −	−, −	NT	−, −	−, −	−, −	−, −	−, −	NT
F	Input	NT	+, +	−, +	+, +	−, −	−	NT	−, −	−, −	−, −	−, −	−
	Adsorbed	−, −	−, −	−, −	−, −	−, −	NT	−, −	−, −	−, −	−, −	−, −	NT

The + and − indicate positive or negative for HCV RNA detected by RT/PCR; NT, not tested. Presentation of two results indicates duplicate experiments.

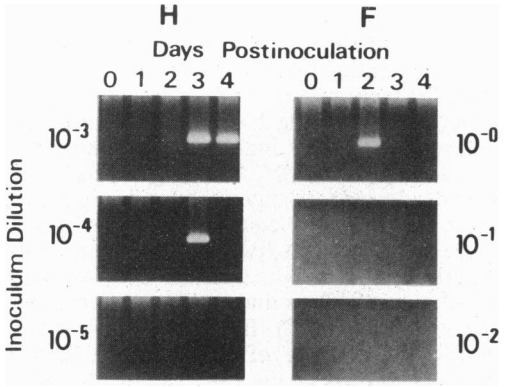

FIG. 4. Infectivity titration of strains H and F in HPB-Ma clone 10-2 cultures. The cells (5×10^5) were inoculated with 100 μl of a 10^{-3}, 10^{-4}, or 10^{-5} dilution of strain H or with 100 μl of an undiluted sample, or a 10^{-1} or 10^{-2} dilution of strain F, harvested daily for 5 days, and tested by RT/PCR for negative-strand HCV RNA.

RT/PCR. On the other hand, the adsorption of negative-strand RNA was not detected in any of the inocula. This suggests that negative-strand RNA is packaged differently than positive-strand RNA and indicates that the probability of detecting carry-over negative strands in the cells is negligible.

Relationship Between *in Vivo* and *in Vitro* Infectivity. The well-characterized inocula of HCV, strains H and F with high and low *in vivo* infectivity, respectively, were serially diluted in 10-fold increments and used for infectivity titration in HPB-Ma clone 10-2 cultures. The cells were inoculated with 100 μl of a 10^{-3}, 10^{-4}, or 10^{-5} dilution of strain H or 100 μl of a 10^{0}, 10^{-1}, or 10^{-2} dilution of strain F as described in *Materials and Methods*. The cells were harvested daily for 5 days after inoculation and assayed by RT/PCR for intracellular negative-strand HCV RNA. As shown in Fig. 4, in the cells inoculated with dilutions of plasma H, a signal for the negative-strand viral RNA was detected on days 3 and 4 in cells inoculated with a dilution of 10^{-3} and in addition on day 3 in cells inoculated with a dilution of 10^{-4}. In the cells inoculated with the dilutions of plasma F, the signal appeared on day 2 only in cells inoculated with undiluted plasma. Thus, the infectivity titer in cell culture was estimated to be 10^5/ml for strain H and 10^1/ml for strain F, demonstrating a correlation between the titers measured in cell cultures and those in chimpanzees (see Table 3). The *in vitro* infectivity titers of other inocula were also measured. The *in vivo* infectivity titer of these samples had been determined previously in chimpanzees. As the apparent time of appearance of the negative strand varied, the signal revealed at any time during the 5-day observation was judged as positive. Table 2 shows the results. The *in vitro* infectivity titers of inocula no. 6, K, and no. 34 were estimated to be $10^{2.5}$/ml and that of no. 4 was $<10^1$/ml (Table 2).

Table 2. Infectivity titration in cell culture

Inoculum	Detection of negative-strand HCV RNA by RT/PCR at indicated dilution ($\log_{10}$)					
	−0	−1	−2	−3	−4	−5
H	NT	+, +	+, +	+, +	+, −	−, −
6	+, +	−, +	+, −	−, −	−, −	NT
K	+, +	+, +	−, −	−, −	−, −	NT
34	+, +	+, +	−, −	−, −	−, −	NT
4	−, −	−, −	−, −	−, −	−, −	NT
F	+, −	−, −	−, −	−, −	−, −	NT

Detection of the negative-strand HCV RNA by RT/PCR at any time during 5 day-observation is tabulated. Symbols are as in Table 1.

Table 3. Titers (per ml) of inocula by different tests

Inoculum	Chimp *in vivo*	Genome (RT/PCR)	Adsorption (positive strand)		HPB-Ma *in vitro*
			Input	Adsorbed	
H	$10^{6.5}$	10^7	10^5	10^4	10^5
6	$\geq 10^5$	10^5	10^5	$10^{3.5}$	$10^{2.5}$
K	$\geq 10^4$	10^5	10^4	10^3	$10^{2.5}$
34	NT	10^5	10^5	10^2	$10^{2.5}$
4	$<10^2$	10^4	$10^{4.5}$	$10^{1.5}$	$<10^1$
F	$<10^2$	10^5	10^4	$<10^1$	10^1

DISCUSSION

In this study we found a new cell line, HPB-Ma, to be superior to Molt4-Ma for replication of HCV. The HCV genome persisted as long as 76 days, which is roughly 3 times longer than the duration we reported for Molt-Ma cells (7). In addition, selection of the most sensitive clone ensured more consistent infection. Using HPB-Ma clone 10-2, we observed a correlation between infectivity of HCV *in vivo* and *in vitro*. Furthermore, the adsorption experiments revealed an additional correlation between the infectivity of HCV inocula and their adsorption to cells, as summarized in Table 3. Thus, the system is useful for predicting the *in vivo* infectivity of HCV and for future development of assays for virus neutralization. The question of whether HCV replicates to completion in HPB-Ma cells remains to be answered. At present, there is no conclusive evidence, although persistence of HCV RNA in the cells for more than 10 weeks and appearance of the positive-strand RNA in the medium after an incubation period favor complete replication of the virus. To determine whether this system is abortive or productive for infectious HCV particles, additional studies must be performed. But, the system, as currently constituted, does provide a useful estimate of the infectivity of HCV strains.

Presence of HCV negative-strand RNA in plasmas or sera (ref. 8 and this report) made the data in which detection of the negative strand was used as a criterion of the HCV replication difficult to interpret. Although detection of negative-strand RNA is not due to an inadvertent PCR amplification from positive-strand RNA template (7), one could not exclude the possibility that the detected negative strand was derived from the inoculum. However, the data from the adsorption experiments clarified this ambiguity. As shown in Table 1, we could not detect adsorption of the negative strand, strongly suggesting that detected negative-strand RNA in cells was not derived from the inoculum.

It was also revealed that the rate of adsorption to cells of virions in plasma with low *in vivo* infectivity (F and no. 4) was less than that of plasma with high *in vivo* infectivity (H, no. 6, and K), regardless of the titer of RNA genome as measured by RT/PCR. On the basis of our previous study (15), in which plasmas F and no. 4 were found to contain noninfectious virions (probably bound to antibodies) at a higher ratio than plasmas H and no. 6, it is possible that the less efficient adsorption of virions in plasmas F and no. 4 to cells is related to anti-HCV antibodies bound to the virion surface.

In some instances in which the negative-strand RNA was detected in cells, the positive strand was not detectable (e.g., days 22 and 37 in Fig. 1) and there was an eclipse period before the HCV genome could be detected again (day 20 in Fig. 1). In the previous experiments using rotavirus and poliovirus as double- and single-stranded RNA controls, respectively, detection of the double-stranded RNA was about 1/1000 as sensitive as that of the single-stranded RNA (7). Thus, it is possible that we might not have efficiently detected the double-stranded form of intracellular HCV RNA under the conditions we used for the present study.

The ratio of the negative and positive HCV RNA strands in infected livers was reported to be usually 1:10-1:100 but on occasion could be 1:1 (7, 8). In HPB-Ma cells, synthesis of positive-strand RNA did not appear significantly greater than that of negative-strand RNA. This situation is quite unusual for a positive-strand virus. Since the negative-strand RNA may function as an antisense RNA to suppress expression of virus genes (16), synthesis of the negative-strand RNA of HCV at a higher than normal ratio in cells may be related to low titer of virus *in vivo* and *in vitro*.

CHX has been shown to cause an increase in virus-specific RNA in treated cells (17, 18) and is believed to cause stabilization of viral RNA. Treatment with CHX increased the detection of the intracellular HCV RNA in this study. With a rare sample positive for both positive and negative RNA strands, we found CHX preferentially increased the detection of the negative strand. CHX, however, did not always enhance the detection of HCV RNA (data not shown). The enhancement may depend upon some unknown factors such as the stage of the virus replication, cell physiology, etc.

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