In vitro assays for drug testing : continuous cell lines

C. Clarysse, J. Fevery, S.-H. Yap

Department of Hepatology, U.Z. Gasthuisberg, Leuven, Belgium.

Hepatitis C virus (HCV) is the major cause of parenterally transmitted and community-acquired non-A, non-B hepatitis, affecting about 3% (0.6-5%) of the population worldwide (1). It frequently leads to chronic hepatitis and cirrhosis and is associated with the development of hepatocellular carcinoma (2). Currently chronic hepatitis C virus infection is treated with interferon-α (IFN-α), preferably in combination with ribavirin. However, from many studies it is clear that a significant number of patients either fail to respond or relapse after cessation of therapy (3). Consequently there is an urgent need for the development of new therapeutics. Such studies, however, are hampered by the lack of an effective in vitro infection and longterm propagation system for HCV.

Several groups have examined the ability to propagate HCV in cell culture following infection with virus-containing inoculum. In vitro replication has been demonstrated in a limited number of cell types including primary human and chimpanzee liver cells (4-6), hepatocyte cell lines (7-15), B lymphocytes (13,16-19), T lymphocytes (9,11,13,18,20-22,24), and in monkey and porcine kidney and hamster lung cell lines (9,12, 15,27). However, in most cases the replication was low with fluctuating titers and with the tendency of the virus to become undetectable or intermittently detectable within days or weeks after infection.

In order to enhance viral infection and replication, various groups have examined several infection and culture conditions. Seipp *et al.* (12) noticed that supplementation with PEG, DMSO and lovastatin during inoculation did not enhance virus replication substantially, but continued stimulation of the low-density lipoprotein (LDL) receptor expression resulted in infections which persisted over 4 months in PK15, Hep G2 and Huh7 cells. Recently Morrica *et al.* (15) described a centrifugation-facilitated inoculation method, known to enhance the recovery of many viruses in tissue cultures. In contrast to the conventional infection procedure yieling only 5% (9), 20% of the cell lines tested now contained HCV RNA 15 days post inoculation. However duration and levels of virus replication were generally modest, suggesting that unknown factors which limit HCV replication in vitro are not overcome by this procedure. Mizutani *et al.* (24) investigated whether viral replication might be affected by conditions of host cell growth. The effect of several antibiotics on HCV replication was tested. Addition of hygromycin B resulted in a prolonged retention of HCV in MT-2C cells compared to the untreated cells. Reduction of the culture temperature from 37°C to 32°C results in an increase of the cell doubling time and led to an HCV infection that persisted during 6 months and 70-100 days in MT-2C and PH5CH cells respectively (14,25)

Several groups using different cell lines, respectively the HPB-Ma, MT-2, PHSCH and Vero cell line, have experienced that some clones were able to support HCV replication more persistently than the parental cells (14,21,23,24,27).

Infectivity of in vitro propagated HCV has been demonstrated by two different methods : by cell-cell transmission and by infection of the cells with culture supernatant from the HCVcarrier cultures. To demonstrate cell-cell transmission of HCV, Shimizu *et al.* (28) produced drug-resistant HPB-Ma cells. During cocultures, the HCV-infected drug-sensitive cells were able to transmit the virus to the drug-resistant cells. Bertolini *et al.* (16) used intracellular HCV from frozen and thawed infected cells for serial passage in CE cells. Infections of cells with the culture supernatant from the HCV-infected HPB-Ma 10-2, MT-2C, HepG2 and Huh7 cells (12,24,25,28) were also successful. Shimizu *et al.* (29) recently infected a chimpanzee with culture supernatant from Daudi cells harvested 58 days after infection. Five to 7 weeks after inoculation, HCV RNA could be detected in its serum, liver and PBMC.

Comparison of sequence analysis of the HCV hypervariable region 1 (HVR1) populations in infected cells with the populations obtained from the inoculum, revealed that despite the complicated quasi-species of the HVRl in the inoculum, limited HVRI sequences became predominant in the cultured cells (8-11,13,18, 25,26). Sugiyama *et al.* (26) examined whether this phenomenon also occurred in other regions of the viral genome. They observed that in more than half of the compared regions, the HCV populations became homogenous. Ikeda *et al.* (13) noticed that when they infected the human T-cell line MT-2 and the human hepatocyte cell line PH5CH with several inocula, different sera had different infectivities in these cell lines.

Correspondence : S.-H. Yap, Department of Hepatology, University Hospital Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium.
Presented at the International Symposium on Viral Hepatitis beyond the Millennium Session of December 10,1999.

These findings suggested that the infective potency of each serum may depend on the type of target cell. To further characterise this cell tropism of HCV, the HCV dynamics in the cultured cells after virus inoculation was examined using the MT-2, PH5CH cell lines and their clones (11,13). They noticed that HCV populations in the infected cells gradually altered with time and converged to two different HCV populations in either cell types. Moreover, Kato *et al.* (11) identified several amino acids positions in the viral core and in the E2 and NS2 proteins which might be involved in the cell tropism of HCV.

Using the previously described infection systems, some preliminary experiments have been performed to examine the influence of interferon (IFN) and antisense oligonucleotides on the HCV replication in vitro. Mizutani *et al.* (23,24) demonstrated that antisense oligonucleotides complementary to the sequences of the core region of the HCV genome could inhibit viral replication in the HCV-infected MT-2C cell line. Using the cell lines HPB Ma 10-2, MT-2C and PH5CH8, Shimizu *et al.* (28), Mizutani *et al.* (24) and Ikeda *et al.* (14) demonstrated an inhibition of viral replication by the addition of IFN-α. Dependent upon the dose and method of administration, a decrease of HCV genomes in the cells or a complete inhibition of viral replication could be achieved.

In a recent study performed in our laboratory, a cell line derived from the human hepatoblastoma cell line Hep G2 was used for HCV infection studies. A new culture method was designed and evaluated in order to prevent unfavourable circumstances coherent with monolayer cultures such as : a) trypsinization which is required for cell passage but can lead to possible temporary loss of permissiveness of the cells for infection, and b) refreshment of the medium, which can result in loss of infectious particles necessary for perpetuation of the infection. Cells were grown in the extracapillary space of a hemodialysis cartridge, in the presence of a HCV positive inoculum, while the culture medium was recirculated through the intracapillary space, supplying the cells with nutrients and oxygen. HCV-RNA could continuously be detected in the cells up to 77 days of culture. Sequence analysis of the HCV hypervariable region 1 (HVR1) revealed that respectively 56% and 75% of the clones obtained from the cells at day 20 and 40 after start of the infection were different from the clones present in the original inoculum. Furthermore, certain nucleotide positions in this region were more susceptible to mutations, leading to an alteration in amino acid sequence. As none of these sequences were present in the clones from the inoculum, it is suggested that new HCV quasispecies have emerged as a result of viral replication in the hepatocytes in vitro. This in vitro HCV propagation system, after improvement of the culture conditions, may therefore be used for drug testing.

References

1. World Health Organization. Hepatitis C : global prevalence. *Weekly Epidemiological Record*, 1997, **72** : 34- 344.
2. SAITO I., MIYAMURA T., OHBAYASHI A. *et al.*, Hepatitis C infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci US A*, 1990, **87** : 65476549
3. REICHARD O., SCHVAREZ R., WEILAND O. Therapy of hepatitis C : alpha interferon and ribavirin. *Hepatology*, 1997, **26** : 108S-llIS.
4. LANFORD R.E., SUREAU C., JACOB J.R., WHITE R., FUERST T.R. Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand- specific RT/PCR. *Virology*, 1994, **202** : 606-614.
5. IACOVACCI S., MANZIN A., BARCA S. *et al.* Molecular characterization and dynamics of hepatitis C virus replication in human fetal hepatocytes infected in vitro. *Hepatology*, 1997, **26** : 1328- 1337.
6. FOURNIER C., SUREAU C., COSTE J. *et al.* In vitro infection of adult normal human hepatocytes in primary culture by hepatitis C virus. *J. Gen. Virol.*, 1998, **79 Part 10** : 2367-2374.
7. TAGAWA M., KATO N., YOKOSUKA O., ISHIKAWA T., OHTO M., OMATA M. Infection of human hepatocyte cell lines with hepatitis C virus in vitro. *J. Gastroenterol. Hepatol.*, 1995, **10** : 523-527.
8. HIJIKATA M., MIZUNO K., RIKIHISA T. *et al.* Selective transmission of hepatitis C virus in vivo and in vitro. *Arch. Virol.*, 1995, **140** : 1623-1628
9. KATO N., NAKAZAWA T., MIZUTANI T., SHIMOTOHNO K. Susceptibility of human Tlymphotropic virus type I infected cell line MT-2 to hepatitis C virus infection. *Biochem. Biophys. Res. Commun.*, 1995, **206** : 863-869.
10. KATO N., IKEDA M., MIZUTANI T. *et al.* Replication of hepatitis C virus in cultured nonneoplastic human hepatocytes. *Jpn. J. Cancer Res.*, 1996, **87** : 787-792.
11. KATO N., IKEDA M., SUGIYAMA K., MIZUTANI T., TANAKA T., SHIMOTOHNO K. Hepatitis C virus population dynamics in human lymphocytes and hepatocytes infected in vitro. *J. Gen. Virol.*, 1998, **79** : 1859-1869.
12. SEIPP S., MUELLER HM., PFAFF E., STREMMEL W., THEILMANN L., GOESER T. Establishment of persistent hepatitis C virus infection and replication in vitro. *J. Gen. Virol.*, 1997, **78** : 2467-2476.
13. IKEDA M., KATO N., MIZUTANI T., SUGIYAMA K., TANAKA K., SHIMOTOHNO K. Analysis of the cell tropism of HCV by using in vitro HCV-infected human lymphocytes and hepatocytes. *J. Hepatol.*, 1997, **27** : 445-454.
14. IKEDA M., SUGIYAMA K., MIZUTANI T. *et al.* Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus. *Virus research*, 1998, **56** : 157-167.
15. MORRICA A., GIORGI M., MAGGI F. *et al.* Susceptibility of human and non-human cell lines to HCV infection as determined by the centrifugation-facilitated method. J. *Virol Methods*, 1999, **77** : 207-215.
16. BERTOLINI L., IACOVACCI S., PONZETTO A., GORINI G., BATTAGLIA M., CARLONI G. The human bonemarrow-derived B- cell line CE, susceptible to hepatitis C virus infection. *Res. Virol.*, 1993, **144** : 281-285.
17. VALLI M.B., BERTOLINI L., IACOVACCI S., PONZETTO A., CARLONI G. Detection of a 5' UTR variation in the HCV genome after a long-term in vitro infection. *Res. Virol.*, 1995, **146** : 285-288.
18. NAKAJIMA N., HIJIKATA M., YOSHIKURA H., SHIMIZU Y.K. Characterization of long-term cultures of hepatitis C virus. *J. Virol.*, 1996, **70** : 3325-3329.
19. IACOVACCI S., BERTOLINI L., MANZIN A. *et al.* Quantitation of hepatitis C virus RNA production in two human bone marrow-derived B-cell lines infected in vitro. *Res. Virol.*, 1997, **148** : 147-151.
20. SHIMIZU Y.K., IWAMOTO A., HIJIKATA M., PURCELL R.H., YOSHIKURA H. Evidence for in vitro replication of hepatitis C virus genome in a human T-cell line. *Proc. Natl. Acad. Sci USA*, 1992, **89** : 5477-5481.
21. SHIMIZU Y.K., PURCELL R.H., YOSHIKURA H. Correlation between the infectivity of hepatitis C virus in vivo and its infectivity in vitro. *Proc. Natl. Acad. Sci US A*, 1993, **90** : 6037-6041
22. NISSEN E., HÖHNE M., SCHREIER E. In vitro replication of hepatitis C virus in a human lymphoid cell line (H9). *J. of Hepat.*, 1994, **20** : 437
23. MIZUTANI T., KATO N., HIROTA M., SUGIYAMA K., MURAKAMI A., SHIMOTOHNO K. Inhibition of hepatitis C virus replication by antisense oligonucleotide in culture cells. *Biochem. Biophys. Res. Commun.*, 1995, **212** : 906-911
24. MIZUTANI T., KATO N., SAITO S., IKEDA M., SUGIYAMA K., SHIMOTOHNO K. Characterization of hepatitis C virus replication in cloned cells obtained from a human T- cell leukemia virus type 1-infected cell line, MT-2. *J. Virol.*, 1996, **70** : 7219-7223.

25. MIZUTANI T., KATO N., IKEDA M., SUGIYAMA K., SHIMOTOHNO K. Long-term human T-cell culture system supporting hepatitis C virus replication. *Biochem. Biophys. Res. Commun.*, 1996, **227** : 822-826.
26. SUGIYAMA K., KATO N., MIZUTANI T., IKEDA M., TANAKA T., SHIMOTOHNO K. Genetic analysis of the hepatitis C virus (HCV) genome from HCV-infected human T cells. *J. Gen. Virol.*, 1997, **78** : 329-336.
27. VALLI M.B., CARLONI G., MANZIN A., NASORRI F., PONZETTO A., CLEMENTI M. Hepatitis C virus infection of a Vero cell clone displaying efficient virus-cell binding. *Res. Virol.*, 1997, **148** : 181-186.
28. SHIMIZU Y.K., YOSHIKURA H. Multicycle infection of hepatitis C virus in cell culture and inhibition by alpha and beta interferons. *J. Virol.*, 1994, **68** : 8406-8408.
29. SHIMIZU Y.K., IGARASHI H., KIYOHARA T. *et al.* Infection of a chimpanzee with hepatitis C virus grown in cell culture. *J. Gen. Virol.*, 1998, **79** : 1383-1386.