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An Assessment of the Use of Chimpanzees in Hepatitis C Research Past, Present and Future: 2. Alternative Replacement Methods

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Summary — The use of chimpanzees in hepatitis C virus (HCV) research was examined in the report associated with this paper (*1: Validity of the Chimpanzee Model*), in which it was concluded that claims of past necessity of chimpanzee use were exaggerated, and that claims of current and future indispensability were unjustifiable. Furthermore, given the serious scientific and ethical issues surrounding chimpanzee experimentation, it was proposed that it must now be considered redundant — particularly in light of the demonstrable contribution of alternative methods to past and current scientific progress, and the future promise that these methods hold. This paper builds on this evidence, by examining the development of alternative approaches to the investigation of HCV, and by reviewing examples of how these methods have contributed, and are continuing to contribute substantially, to progress in this field. It augments the argument against chimpanzee use by demonstrating the comprehensive nature of these methods and the valuable data they deliver. The entire life-cycle of HCV can now be investigated in a human (and much more relevant) context, without recourse to chimpanzee use. This also includes the testing of new therapies and vaccines. Consequently, there is no sound argument against the changes in public policy that propose a move away from chimpanzee use in US laboratories.

Key words: *chimpanzee, hepatitis C, hepatitis C virus, hepatocellular carcinoma,* Pan troglodytes.

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Introduction

Hepatitis C affects hundreds of millions of people worldwide, including an estimated four million in the USA and five million in Europe (1–3). Serious consequences include liver cirrhosis and hepatocellular carcinoma (HCC), and around 5% of infected people eventually die as a result (4–6). It is therefore a significant healthcare burden, which is set to increase dramatically as the number of infected people rises (2). Research to understand the virus and the disease has been a priority since its discovery in 1989 (7), and this research has involved extensive use of chimpanzees.

However, invasive research involving the use of captive chimpanzees has been banned, or at least severely restricted, across much of the world (8). The continued use of chimpanzees in invasive research in the USA is therefore highly controversial, and there exist compelling arguments against it — for example, the extent of suffering of those chimpanzees involved, and concern over the lack of human relevance of the data produced (8–17). The latter — i.e. the scientific validity of chimpanzee experimentation with respect to human medicine — has been extensively investigated with respect to hepatitis C in the associated paper, *1: Validity of the Chimpanzee Model* (18). This previous paper, which examined, in detail, claims concerning the past contributions of chimpanzee experiments to hepatitis C research, as well as the need for chimpanzee use in current and future investigations, concluded that these claims were exaggerated and unjustifiable, respectively. Major scientific, ethical, economic and practical caveats of the chimpanzee model in hepatitis C virus (HCV) research were highlighted, and the contributions of other, non-chimpanzee methods of research were evaluated. On balance, it was concluded that extensive chimpanzee use in the study of the virus and the disease has made relatively negligible contributions to the body of knowledge and to tangible progress, as compared to non-chimpanzee methods, and that the chimpanzee model must be considered to be scientifically redundant, given the array of alternative methods of inquiry now available.

While Paper 1 makes this argument in its own right by evaluating the degree to which chimpanzee data are predictive of and relevant to human HCV infection, and by illustrating the extent of the contribution of other methods, this complementary paper makes an important further contribution, by reviewing the breadth and com-

prehensive nature of non-chimpanzee methods, and illustrating how *in vitro* approaches, supported by clinical, epidemiological, *ex vivo* and *in silico* methods, can provide all the necessary information about HCV and its pathology to enable the development and testing of HCV therapies.

In Vitro Systems to Study HCV

In the search for a more complete understanding of the HCV life-cycle, in order to facilitate the discovery of new therapeutics, one thing above all else has been cited as a serious impediment and rate-limiting factor, namely, the lack of a reliable and robust cell culture system (e.g. 1, 19–28). This is not surprising, as cell and viral culture are acknowledged as having been pivotal in the development of vaccines against other viruses, such as the polio and measles viruses (29, 30). This is because cell culture approaches, among other things:

— permit the investigation of the entire viral life-cycle, from host-cell attachment and entry, through replication, to the assembly and release of progeny virions;

— facilitate reverse-genetics analyses to elucidate detailed information about viral genomics and proteomics, including interactions with host factors;

— permit the study of the humoral immune response and neutralising antibodies to aid vaccine development; and

— greatly assist in the identification of therapeutic targets and in the screening and testing of anti-viral compounds (1, 27, 31).

In view of their importance, many attempts have been made to establish cell culture systems for HCV. As relatively long ago as 2001, reviews cited dozens of systems that had been assessed and that had succeeded to varying degrees (25). More recently, many good reviews have outlined developments in this field and have illustrated the major breakthroughs these represent and the insights and progress they should deliver (1, 27, 32–34).

Infection of cultured cells with HCV

The simplest approach involves the straightforward infection of primary human cell cultures with HCV-infected sera. This has been successfully achieved with primary fetal (28, 35, 36) and non-fetal (37, 38) human hepatocytes and primary human peripheral blood mononucleocytes (PBMCs; 39). While some investigators reported that the efficiencies of these systems could be low and/or hard to control, others described relatively long-term, reliable, efficient and reproducible systems that supported HCV infection and replication. These successes were reflected in opinions that this approach constitutes a valuable model for the study of HCV replication, the identification of relevant viral and cellular factors associated with successful infection, the evaluation of antiviral agents, the further analysis of serum infectivity, and the cloning of novel HCV genomes from patients (28, 37, 38).

At the same time, various immortalised cell lines were exhibiting support of HCV infection and replication, such as the human T-lymphocyte and B-lymphocyte cell lines MOLT-4Ma, HPB-Ma, MT-2, and Daudi (25, 40, 41). Some of these cell lines were able to support long-term (≥ 12-month) infections (42, 43), and demonstrated a good correlation of their measured infectivity titres with the infectivity titres measured in chimpanzees (42). The immortalised human hepatoma cell lines, HepG2, Huh-7 and PH5CH (25, 44, 45), also supported HCV infection and replication, and efforts to increase the efficiency of these and the lymphocyte-based systems led to the culture of primary hepatocytes and PBMCs from persistently-infected patients (46, 47) that produced higher viral titres. Research involving infected cell lines such as these has led to some important discoveries, and progress was being realised in improving cell culture conditions and performance. For instance, this approach revealed that the low-density lipoprotein receptor (LDLR) is involved in the process of HCV entry into the host cell (48), and a human B-cell line, SB, was shown to continuously, reliably and reproducibly produce infectious HCV virions, which were subsequently able to infect a number of human cell lines, including hepatocytes *in vitro*, potentially facilitating the study of the entire viral life-cycle (49). However, by the time these discoveries were made, these culture systems were generally not deemed robust enough to support reliable in-depth studies of the HCV life-cycle and the development of anti-viral agents. This, combined with the difficulty of sourcing and maintaining primary cells, and the desire to create a system that enabled infection with defined HCV genomes to facilitate reverse genetics studies, meant that attempts at viral culture had progressed from using virus-containing sera or infected cells to the use of HCV molecular clones.

Infectious molecular clones of HCV

As one of the more salient examples put forward by advocates of chimpanzee research of the importance of chimpanzees as a research tool, a thorough account of the development and use of

infectious molecular clones in HCV research is given in the associated paper (18). In summary, HCV clones are produced *in vitro* by using HCV genomic RNA from infectious serum samples as a template. These clones then serve as templates for the production of RNA molecules with precise 3′ termini, representing HCV genomic RNAs (50), which can be used *in vitro* or *in vivo* to initiate the viral life-cycle (32). To overcome the initially unsuccessful attempts which utilised screened clones to infect chimpanzees (51, 52), lessons were learned from previous work with other related viruses (e.g. 53), in which *consensus* sequences of the specific viral isolates being used were determined, in order to overcome mutation effects (54–56). While it has been asserted that the development of infectious clones was dependent on chimpanzee use (22, 24), there existed several contemporaneous *in vitro* approaches for which investigators had claimed validity for the study of the HCV life-cycle, including replication, persistence and pathogenicity, and to test anti-viral agents (57, 58). It had been claimed that problems existed with regard to the measurement of HCV replication in these *in vitro* systems (59), but a number of sensitive and reliable methods were actually available, to specifically detect and quantify HCV replicative activity. For example:

— the reverse transcription-polymerase chain reaction (RT-PCR) and/or ribonuclease protection assay of negative strand RNA intermediates only produced during viral replication;

— the incorporation of radioactive nucleosides, such as [^{3}H] uridine, into *de novo* RNA molecules;

— the *in situ* RT-PCR of HCV RNA present in long-term cultured cells;

— the infection of freshly cultured non-infected cells with culture medium from transfected cell lines, indicating the production of infectious HCV;

— the immunostaining of viral proteins; and

— the visualisation of virus or virus-like particles via electron microscopy (57, 58).

Furthermore, recent examinations of 14 HCV clones and their properties *in vitro* and *in vivo* (54, 59) list parallel references for the *in vitro* and *in vivo* studies of each HCV clone. Finally, it may be true that the existence of an established *in vivo* system which used chimpanzees for determining the clone infectivity of other viruses, led to the continuation of chimpanzee use out of convenience, rather than necessity (52).

While chimpanzees continued to be used to investigate the characteristics of HCV infectious clones, many *in vitro* approaches were highly informative. For example, with regard to the HCV proteins, *in vitro* studies furthered our understanding of sub-cellular localisation and assembly (60–63), function (60, 64), effects on host-cell growth and gene expression (65, 66), and effects on IFN-induced intracellular signalling (67). Such approaches had also elucidated the anti-viral effects and modes of action of interferon (IFN) and ribavirin (68, 69), the role of CD26 in HCV infection (70), the determinants of membrane association of the viral polymerase (71), and various properties and characteristics of the HCV NS3-NS4A complex (72).

Subgenomic and genomic replicon systems

Replicons (autonomously replicating HCV-derived RNAs) perhaps represented the first highly reliable, reproducible and robust *in vitro* method to investigate HCV replication. Their development was considered a “great leap forward” (1), a “significant advance” (31), a “milestone” (27), and a “highlight” and a “major breakthrough” (50), which greatly facilitated research into many aspects of the HCV life-cycle. The importance of replicons is reflected in their citation, alongside full recombinant HCV culture, in discussions of the crucial contributions *in vitro* approaches have made to HCV research (6, 27). These contributions include, for example, the elucidation of the roles of specific HCV genome segments and proteins in replication, the determination of intracellular HCV protein localisation, the characterisation of virus–host interactions, and the identification of anti-viral agents (20, 32–34, 73–75). Many more examples, too numerous to discuss here, are summarised in a recent very detailed and comprehensive review, including, for example, the importance of the HCV untranslated regions (UTRs) and other *cis*-acting elements in viral replication, the elucidation of roles, functions and criticality of HCV viral proteins and protein domains, the involvement of various host proteins with the endoplasmic reticulum, the association of the HCV core protein with host lipids, and the screening and testing of viral inhibitors (1).

Subgenomic replicons comprise truncated viral minigenomes containing, for instance, the HCV 5′ and 3′ UTRs plus the viral polymerase gene, but also direct the synthesis of a marker gene (such as the neomycin resistance gene). This permits the selection of stable cell lines containing highly replication-competent HCV RNAs (1, 50). Their development, which notably was contemporary to, and independent of, experiments involving infectious molecular clones in chimpanzees, was based on prior experiments involving the related *Flaviviridae* Kunjin virus (76), the bovine viral diarrhoea virus (BVDV) (77), and poliovirus (78), in which transcripts from subgenomic molecular clones were shown to be capable of autonomous intracellular replication *in vitro*.

The first subgenomic replicon for HCV was based on a consensus genotype 1b sequence derived from a chronically infected patient, and exhibited efficient replication when transfected into Huh7 human hepatoma cell lines (26). Subsequent *in vitro* analyses revealed the evolution of various adaptive mutations in the subgenomes, which further increased their replicative capacities by several orders of magnitude, even up to 10,000-fold (33, 79, 80). Knowledge of these adaptive mutations permitted the development of extremely efficient *in vitro* replicon systems, which rendered the inclusion of selectable marker genes redundant. The consequent exclusion of heterologous RNA from replicons, among other things, led to a further increase in replication efficiency (81), facilitated the study of unmodified HCV replicons in a more 'native' environment, assisted the development of full-length replicon systems (82) — including those involving other HCV genotypes (e.g. 1a [81] and 2a [83]), and opened the door to the *in vitro* investigation of full viral assembly (1). Furthermore, it was discovered that the cell line itself — even different passages of the same cell line — could affect replication efficiency by up to 100 times (84), and that 'curing' cell lines of HCV RNA via IFN treatment could lead to subsequent higher permissiveness (85). The identification, isolation and use of highly permissive cell lines have also facilitated the use of the replicon system by increasing efficiency. Examples of these cell lines include the Huh7.5 (85), Huh7.5.1 (33) and Huh7-Lunet (86) lines.

Whilst replicon systems were proving to be extremely valuable for research into most aspects of the HCV life-cycle, they were proving unsuitable for the investigation of viral assembly and release. Although full-length replicons were able to produce HCV viral proteins, many did not produce detectable progeny virions — presumably due to adaptive cell culture mutations favouring replicon replication at the expense of viral assembly and release (1). However, some systems did reliably produce infectious progeny virions (87), despite this being at a low level (1). Fortunately, alternative and highly productive *in vitro* approaches to this type of study have been developed. The use of HCV virus-like particles (VLPs) and HCV pseudoparticles (HCVpp) has been productive, and *trans*-encapsidation of HCV subgenomic replicons has been achieved via the baculovirus-directed expression of HCV structural proteins (88), the generation of a packaging cell line (89), and the use of helper viruses (90).

HCV virus-like particles (VLPs) and HCV pseudoparticles (HCVpp)

There are a number of detailed and extensive recent reviews of the application of these methods to HCV research, and of the data they can provide with regard to HCV binding, membrane fusion and entry, antibody-mediated neutralisation, screening of anti-HCV agents and vaccine development, and other aspects of the HCV life-cycle (1, 27, 33). Briefly, the techniques range from the simple expression of the HCV envelope glycoproteins (E1 and E2) in cell culture, through the expression of HCV structural proteins by using recombinant baculovirus, to the creation of HCVpp in which the HCV envelope proteins are incorporated into other enveloped viruses, in place of their own envelope proteins.

Experiments involving the expression of HCV envelope proteins in cell lines implicated the CD81 cell-surface protein and scavenger receptor class B type I protein (SR-BI) as mediators, for example, of HCV attachment and entry (91–93). However, more-advanced investigations with *bona fide* VLPs were more productive, permitting detailed *in vitro* studies of virion assembly, genome encapsidation, virus–host interactions and vaccine research.

VLPs were first reported following the expression of HCV structural proteins by using the eukaryotic baculovirus expression system, whereby these proteins self-assembled into enveloped VLPs with highly similar biophysical properties to virions isolated from HCV-infected humans (94). These VLPs have been subsequently used in a number of ways. For example:

— to elucidate humoral immunity in acute and chronic hepatitis C patients, including responders and non-responders to IFN therapy (95);

— to determine structural differences in HCV envelope proteins, depending on their context, and consequent effects on CD81 binding (96, 97);

— to study early events in host-cell entry, including the role of lipoproteins (98, 99) and low-density lipoprotein (LDL) receptors (100);

— to demonstrate the role of cell-surface heparan sulphate proteoglycans (HSPGs) in HCV envelope–target cell interaction (101), and to map the viral and cellular determinants of this interaction (102);

— to establish the crucial involvement of the claudin-1 (CLDN-1) protein late in the viral entry process (103);

— to study HCV uptake, antigen processing and presentation in human dendritic cells (104); and

— to analyse the genome-wide host-cell response following HCV binding of human liver and hepatoma cells (105).

Infectious HCVpp contributed to the study of early events in HCV infection further, as, unlike

VLPs, HCVpp are infectious and permit the functional investigation of putative receptors involved in cell entry (106). HCVpp are formed via the transfection of human cell lines with: a) expression vectors encoding the HCV envelope proteins (E1 and E2); b) a 'viral genome' that can be encapsidated, which contains a reporter gene such as the green fluorescent protein; and c) a 'packaging construct' encoding core proteins from another enveloped 'carrier' virus, such as murine leukaemia virus (MLV; 106) or human immunodeficiency virus (HIV; 107). Upon expression within the cell of the transfected constructs, assembly of the hybrid virion occurs, which comprises an encapsidated RNA 'genome' containing the reporter gene, with the HCV envelope proteins at the virion surface. These HCVpp are secreted into the culture medium and can be subsequently used in assays for infectivity, since they are easily quantifiable via the measurement of reporter gene expression (106–109).

HCVpp systems are robust and reliable, and have therefore facilitated many important discoveries in HCV research, including the elucidation of HCV tropism, the identification of HCV envelope glycoproteins and host-cell receptors/co-receptors, and the determination of their involvement in the infection process. Also, HCVpp systems have permitted measurement of the extent of neutralisation by patient sera and monoclonal antibodies, leading to the mapping of envelope protein neutralisation epitopes, which is highly relevant to vaccine studies (for extensive and comprehensive reviews, see 1, 33, 110–112). For example, HCVpp experiments involving a variety of HCV envelope proteins of different genotypes have:

— demonstrated the effects on HCV infectivity of lipoproteins such as high-density lipoproteins (HDL) and low-density lipoproteins (LDL) (113–119);

— permitted the analysis of cross-neutralisation, as well as genotype-specific neutralisation (106, 119–125); and

— elucidated the roles of CD81 (92, 107, 114, 126–129), SR-BI (107, 114, 116, 118), clathrin (107, 114, 130–132), claudin-1 (103), C-type lectins (133, 134), and pH (107, 114) in viral attachment and entry.

Overall, the HCVpp system was clearly technically uncomplicated, versatile, highly productive and an extremely important facet of HCV research. However, HCVpp involve chimaeric virions and can only be used to investigate the early steps of the infective process. Therefore, a more native, physiologically relevant and comprehensive 'full life-cycle' system was still required.

Full life-cycle infectious HCV cellular clones (HCVcc)

The above information underscores the progress made in the development of *in vitro* techniques to study the life-cycle of HCV and identify antiviral therapies (33). A suite of approaches involving HCV-infected primary and immortalised cell lines, subgenomic and full-length replicons, and HCV VLPs and pseudoparticles, facilitated the investigation of many aspects of the virus and the disease — for example, virus attachment and entry into host cells, its replication and the assembly of progeny virions, host defences against HCV infection, host–virus interactions, immune escape, and the screening of anti-viral agents. Each method has some limitations. For instance, primary cell culture can be demanding, and replicons harbour adaptive mutations. Also, VLPs are not secreted by host cells, and HCVpp are hybrids with non-HCV cores, and they are not associated with lipoproteins (33). A full life-cycle system was therefore necessary, in which all aspects of HCV infection, including reverse genetics studies, could be investigated more easily and more reliably, all in the context of the complete viral life-cycle.

The major breakthrough in the ultimate realisation of HCVcc was made in 2005, when the development of the first *in vitro* system supporting the infection, replication and production of infectious HCV virions was announced (135). This system utilised the genotype 2a clone, JFH1, derived from an HCV isolate obtained from a Japanese patient with fulminant hepatitis (136), which was transcribed *in vitro* and the RNA transfected into Huh7 cells. This was based on earlier experiments involving a JFH1 subgenomic replicon, which, unlike all similar replicons, did not require adaptive mutations in order to be highly replicative in a number of different cell lines (83, 137, 138). The presence of adaptive mutations in replicons had been widely assumed to favour replication at the expense of the formation of viral progeny (74, 139). It was therefore hypothesised that these JFH1 replicons might be more conducive to the formation of new virus particles — hence the successful experiments of Wakita *et al.* (135).

Subsequent investigations greatly improved this culture system. The transfection of other cell lines, such as Huh7.5 and Huh7.5.1, with JFH1 transcripts resulted in increased replication and viral titres, in systems that could be initiated at low multiplicities of infection, serially passaged, and which reflected the clinically-observed effects of IFN treatment (140, 141). Furthermore, the construction of JFH1 chimaeras with other HCV isolates, such as J6, Con1 and H77S, has also improved efficiency (140, 142, 143), and has succeeded both for intragenotypic chimaeras as well as intergenotypic chimaeras — the latter being crucial for research

into other HCV genotypes and the development of anti-viral therapies against them (144–146). The serial passage of Huh7.5 cells infected with chimaeric J6/JFH1 HCV has given rise to culture variants with adaptive mutations, which demonstrate enhanced infectivity, and which could be useful for the study of HCV entry (147). Dedicated systems for HCV genotype 1a (strain H77 [87]) and 4a (148) also exist, and are showing promise. In addition, alternative systems involving the transfection of DNA plasmid clones instead of RNA transcripts are also in the course of development for HCV genotype 1a, 1b and 2a strains. In these systems, intracellular ribozyme-directed cleaving of transcripts produces functional viral RNA molecules that give rise to HCV particles for each strain (149).

While these HCVcc systems are relatively recent developments, they have already been used to provide useful data on the life-cycle of the virus. They have corroborated with and expanded upon HCV receptor experiments, by illustrating, for example, that HDL and the CD81 and SR-BI proteins are required for HCV infection (150–154), and that viral entry is mediated by clathrin vesicles (130). HCVcc have also demonstrated the close association of apolipoprotein C1 (ApoC1) with HCV. ApoC1 associates with HCV intracellularly via its C-terminus during viral morphogenesis, and plays a crucial role in HCV infectivity (155). HCVcc have also shown, via small interfering RNA (siRNA) analysis of 140 cellular-membrane trafficking genes, that HCV replication utilises host gene products involved in cytoskeletal regulation and endocytic trafficking, and also a critical lipid kinase PI4K-IIIalpha (156).

Summary and outlook of *in vitro* systems

Current consensus endorses and validates the assertions that cell culture systems were imperative for the progress of HCV research. Given the realisation and continued development of the *in vitro* methods summarised here, the prospects for further significant advances in the understanding of HCV, its life-cycle, the ensuing pathology, and prophylactic and therapeutic therapies, are substantial and unprecedented (1). The full life-cycle of HCV is now easily accessible to reliable and reproducible *in vitro* study. While the use of cellular clones represents the current vanguard, other more-restricted, yet more-focused, systems are still widely used and are delivering important data. Allied technologies, such as genomics and proteomics, will augment these methods further, providing enhanced detail of virus–host interactions and the molecular basis of resistance and progression to chronicity, thus illuminating putative targets of new anti-virals and vaccines. There is still scope for improvement in HCVcc systems, however. Higher viral titres and a broader range of host cells are desirable, while extension of HCVcc to genotypes other than 2a is crucial. It has recently been established that primary and passaged cultures of human fetal hepatocytes (HFH) support the replication of unmodified HCV (i.e. devoid of adaptive mutations) for up to two months (28), either following the transfection of consensus transcripts of HCV strain H77 (genotype 1a; 51) or infection with patient sera containing HCV of various genotypes, including 1a, 1b, 2a, 2b and 3. Progeny viruses were also released into the culture supernatants and proved to be infectious. This system is extremely stable and reproducible, as HFH cells maintain their phenotype for months and express LDL receptor and CD81, which play important roles in the HCV life-cycle.

In Vitro & Clinical Findings in HCV Research

Recap

A great many examples of crucial *in vitro* and clinical findings concerning HCV infection have already been discussed above. Infected primary cell lines implicated the LDL receptor in HCV entry. Subgenomic and full-length replicon systems have elucidated:

— the subcellular localisation of HCV proteins and their assembly into VLPs;

— the functions of wild-type and mutant HCV proteins;

— the effects of HCV proteins on host-cell growth and gene expression;

— the functional roles of HCV UTR stem-loops;

— the anti-viral effects and modes of action of IFN and ribavirin;

— the role of CD26 in HCV infection;

— the effects of HCV proteins on IFN-induced intracellular signalling;

— the determinants of membrane association of the viral polymerase;

— various properties and characteristics of the HCV NS3-NS4A complex;

— the roles of specific HCV proteins and genome segments, including UTRs and other *cis*-acting elements in replication;

— virus–host interactions;

— the identification and testing of anti-viral agents; and

— the association of the HCV core protein with host lipids.

HCV VLPs and HCVpp have greatly informed our understanding of:

— HCV attachment, membrane fusion and entry;

— virion assembly, genome encapsidation and virus–host interactions;

— the involvement of CD81, SR-BI, LDLRs, HSPGs and CLDN-1 in the viral life-cycle;

— the genome-wide host-cell response following HCV binding to human liver and hepatoma cells;

— antigen processing and presentation in human dendritic cells; and

— antibody-mediated neutralisation and other aspects of humoral immunity in acute and chronic hepatitis C patients.

HCV VLPs and HCVpp have also facilitated the screening of anti-HCV agents and potential vaccine development, including the mapping of envelope protein neutralisation epitopes, which was crucial for vaccine studies.

HCVcc have augmented and corroborated much previous work with VLPs and HCVpp, and have further informed our understanding of the roles of clathrin vesicles, ApoC1, and many host genes in the HCV life-cycle. Human studies were vital from the inception of HCV research, demonstrating that 'non-A, non-B hepatitis' (NANBH) was the salient complication of transfusion therapy, defining NANBH's natural history, identifying surrogate markers of the disease, such as alanine aminotransferase, and, as a result of these studies, lowering the incidence of transfusion-associated NANBH, even prior to the definitive identification of HCV.

Studies of people with hepatitis C have been, and continue to be, extremely valuable and informative (reviewed by Seeff [157]). Several clinical studies, some of which utilised human liver biopsies, and some of which were prospective (thus enabling the study of early infection in asymptomatic HCV-positive people), have elucidated the roles of cellular and humoral immunity during acute HCV infection and viral clearance, and following recovery. These studies revealed the importance of HLA alleles, the role of IFNs, and the genetic mechanisms and miRNAs associated with infection, progression to fibrosis, viral clearance and the outcomes of IFN therapy, in both responders and non-responders.

The power and potential of 'alternatives' — further examples

Analysis of HCV structure and physical properties

Electron microscopy and immunoelectron microscopy of patient sera are helping to inform our understanding of the structure and morphogenesis of the virus (158–163), and molecular analyses of human sera have revealed the association of HCV virions with lipoproteins and immunoglobulins (164–170). Although some subsequent infectivity studies have involved the use of chimpanzees (171, 172), there are many other studies that have described the association of lipoproteins and apolipoproteins with HCV (and the consequences of this association), that did not use chimpanzees. These were based instead, for example, on HCVpp studies (116, 118, 119) and physiochemical, immunological and electron microscopical studies of human sera (165, 169, 173–175). HCVcc systems have also been useful, by revealing, for example, the determinants of interactions between HCV NS5A and HCV core proteins, and their subcellular localisations (176).

Pathogenesis/viral clearance and persistence

Prospective clinical studies permit the study of early events in infection and at disease onset, including the recognition of spontaneous resolution, and allow the pairing of 'matched controls' for comparative studies. Both prospective and retrospective studies have generated important data on disease progression, such as rates of fibrosis, cirrhosis, HCC, and death. So-called retrospective-prospective studies take advantage of serendipitous discoveries of distant, well-characterised, point-source outbreaks of hepatitis C, where data from all infected persons are recoverable and the subjects can be followed prospectively. Some of these studies have allowed follow-up periods of up to 35 years (177), and have revealed that infected children and young women had the highest rates of spontaneous resolution, along with the lowest rates of development of cirrhosis and HCC, and of death from liver disease. These studies also revealed that individuals who are immune suppressed, and those who are infected but who have normal aminotransferase levels, progress at very different rates (157). Human studies have also shown that:

— there might be no correlation of HCV-specific T-cell responses with viral clearance, in contrast to previous reports (178);

— strong $CD8^+$ T-cell responses are not sufficient to prevent progression to chronicity (179–181);

— mutations in HLA class I-restricted epitopes targeted by $CD8^+$ cells occur early in HCV infection and are associated with persistence (182);

— particular HLA class I alleles were associated with viral clearance or persistence in a cohort of women accidentally infected with HCV (183); and

— an increase in the diversity of the viral population (evolution of quasispecies) is associated with progression to chronicity via immune evasion (184).

Human and *in vitro* studies have revealed that inadequate cellular immunity might be a factor in HCV persistence and the development of chronic hepatitis C (185, 186), and that viral employment of 'decoy' antigens (187) or inhibition of host IFN responses could be involved in this process (188, 189).

Studies of neutralising antibodies, immune escape and the roles of immune responses in HCV infection and clearance have been, and continue to be, greatly aided by clinical and *in vitro* approaches. Some HCV-infected patients with primary antibody deficiencies have accelerated rates of disease progression (190). Observations that HCV infection might not induce protective immunity were made in a study of thalassaemic children (191), while studies with hypogammaglobulinaemic humans indicated that antibodies were not required for HCV clearance (192) — a finding corroborated in a prospective study of prisoners, some of whom spontaneously cleared HCV, but never developed anti-HCV antibodies (178, 193, 194).

HCVcc systems have revealed the role of cholesterol and sphingolipid in HCV infection and virion maturation (176), the molecular mechanisms by which the HCV core protein triggers hepatitis-associated neoangiogenesis (195), and the mechanism of the role of HCV NS2 protein in viral assembly (196). Magnetic absorption of HCV virions (HCVcc) and/or HCVpp to nanoparticles enables a synchronous infection to be induced via the natural entry route of HCV. This is important for both the accurate determination of the kinetics of viral entry and subsequent viral and cellular events (including the early stages of infection), and the improvement of efficiency and infectivity (197). Microarray studies have also been used in tandem with siRNA analyses and with HCVcc systems, in order to identify genes whose expression is altered by HCV, which are important to pathogenesis, and which contribute to hepatocellular damage. Arginase I, for example, is elevated following HCV infection, and stimulates hepatocellular growth (198). Serial analysis of gene expression (SAGE) techniques have also been applied to the study of gene expression profiles in human hepatitis C and HCC livers. These techniques have identified many different genes and genetic pathways involved in HCV infection and pathogenesis (199).

Various *in vitro* methods, allied with replicon and HCVcc systems, have permitted the study of the HCV NS2/3 and NS2 proteins, previously shown by HCVcc approaches to be necessary for HCV virion assembly and viral infectivity (200, 201). Via mutagenesis and ensuing alterations in NS2/3 degradation, the importance of NS2 regulation for the HCV viral life-cycle, and the identification of specific regions and residues of NS2 for infectious virion assembly, were demonstrated (202, 203). High-throughput (HT) mutational analyses, making use of HCVcc, have also been applied to the entire HCV genome. This has permitted the profiling of HCV *cis*-elements and protein domains, confirming previously identified functional regions of the HCV genome (204). *In vitro* approaches have also revealed a number of different mechanisms by which viral proteins mediate IFN resistance (205). The tagging of HCV non-enveloped capsid-like particles with a fluorescent protein has permitted observation of the intracellular trafficking, in hepatocytes and various human immune cells, of naked capsids via live microscopy (206). This should increase our understanding of their biological significance and their role in pathogenesis (206). Cell culture, patient-based research, and a variety of *in vitro* data, have all highlighted a role for the HCV Alternative Reading Frame Protein (ARFP; also known as F [frameshift] and core+1) in the HCV life-cycle, and implicated it in HCV-related advanced liver disease and HCC (207).

Genome-wide association studies (GWASs) are accelerating, thanks to improved genotyping and single-nucleotide polymorphism (SNP)-discovery technologies. By scanning maps of genetic markers to identify differences in allele frequency between hepatitis C patients and appropriate controls, genomic regions affecting HCV infection and hepatitis C pathology can be identified, adding important new data to our knowledge of HCV pathophysiology and pharmacology (208). For example, a GWAS involving several hundred patients scanned more than 12,000 genes to discover greater than 1,600 SNPs associated with advanced HCV-related fibrosis (209). Of these, two SNPs were considered to be significantly associated with advanced fibrosis, and another SNP with decreased risk of advanced fibrosis. These markers should be important in predicting fibrosis risk in HCV patients. Furthermore, 361 of the discovered SNPs were selected for 'signature building', leading to a predictive signature for cirrhosis in Caucasian patients (208), and a seven-gene signature to differentiate high risk and low risk of cirrhosis (210).

Proteomic technologies have had a major impact in advancing virology in general, and are believed to be a key approach to elucidating the pathogenesis of many viruses, including HCV (reviewed by Liu *et al.* [211]). For example, MALDI-TOF mass spectrometry and liquid chromatography–tandem mass spectrometry (LC-MS/MS) have been used to analyse hepatoma cell lines to identify proteins involved in the pathogenesis of HCV that contribute to its carcinogenic properties, and (in conjunction with the yeast two-hybrid system) to identify host receptors and other virus protein–host protein interactions (211).

Host factors and response

Prospective and retrospective clinical studies have also identified numerous host factors that influence disease progression. These include:

— age (4, 177, 212–214), gender (4, 213, 215–217) and race (218, 219);

— genetic polymorphisms in HLA (220, 221) and profibrogenic cytokine (222, 223) genes;

— metabolic factors, in patients with steatosis (224, 225), diabetes (226, 227) and obesity (228, 229); and

— co-infection/co-morbidity associated with HIV (230–234), hepatitis B (235, 236), schistosomiasis (237, 238) and haemochromatosis (239), as well as smoking (240, 241) and high alcohol intake (242–244).

Microarray studies of human liver core needle biopsies have been "extensively utilised for global transcriptional profiling of the host response to HCV infection from a variety of disease states including fibrosis, cirrhosis and hepatocellular carcinoma" (245–251; as reviewed by Walters & Katze [6]). RNAi screening, by using siRNAs, permits the analysis of specific 'knockdowns' of host genes, in order to determine their effects on (and the role of those genes in) viral infection (252, 253). By coupling an siRNA-based cell-array screening system with HT fluorescence microscopy, an automated method of analysing images of single cells has been developed that can identify cells with altered HCV infections (254, 255). This permits the quantification of viral replication and the identification of genes involved in replication and viral entry. *In vitro* methods have provided considerable data on the importance of microRNAs (miRNAs) in the liver during HCV infection. For example, they have been shown to regulate the expression of HCV transcripts, acting as important cofactors or inhibitors, assembling into RNA-induced silencing complexes. They are affected by IFNs, and so act as targets for anti-viral therapies, and they differ between non-responders and early-responders to IFN therapy (256). A functional screen of a small hairpin RNA (shRNA) library, in concert with siRNA knockdown experiments, identified cellular factors involved in regulating the replication of HCV subgenomic replicons. The host-cell lipid kinase PI4KIII-alpha (produced by the *PI4KA* gene) was shown to be essential for the replication of all HCV genotypes tested (1a, 1b and 2a; 257). The results of these studies are augmented and corroborated by other *in vitro* and clinical analyses, which establish the genes/proteins as genuine therapeutic targets (e.g. 258–261). These include, for example, real-time quantitative RT-PCR, a technique that has permitted very accurate and quantitative gene expression analysis in hepatitis C patients, leading to "new insights into the role of gene networks and regulatory pathways" in hepatitis C and the development of fibrosis and HCC (246, 247). This technique has been used to establish that DNA methylation of specific genes is an important event preceding cirrhosis and HCC (208, 262–267). It has also facilitated the investigation of differential miRNA expression in liver tissue from uninfected and HCV-infected patients, which revealed that pathways relating to immune response, antigen presentation, cell-cycle, proteasome, and lipid metabolism were each activated by HCV (268).

Replicon systems have demonstrated that:

— oxidative stress induces an anti-HCV status via the activation of the MEK–ERK1/2 signalling pathway, in response to various anti-HCV nutrients and agents (269–274);

— proanthocyanidin from blueberry leaves inhibits HCV RNA expression, possibly mediated via heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 (275);

— the 17-allylaminogeldanamycin (17-AAG) inhibitor of heat-shock protein 90 (Hsp90) suppresses HCV replication in a dose-dependent manner (276);

— unsymmetrical dialkyl-hydroxynaphthalenoyl-benzothiadiazines 2 and 3 inhibit HCV replication (277); and

— honeysuckle extracts (pheophytin a from *Lonicera hypoglauca* Miq.) have potent anti-HCV properties via inhibition of NS3, that are synergistic with INF-alpha-2a (278).

HCVcc systems have revealed that:

— the cytokine oncostatin M (OSM; a member of the IL-6 family) has an anti-HCV activity that is synergistic with IFN-alpha (279); and

— the HCV genome encodes single-stranded RNA (ssRNA) ligands of Toll-like receptor 7 (TLR7) that significantly activate innate immunity and induce IFN-alpha production (280).

Other *in vitro* investigations have elucidated the mechanism of the anti-HCV action of cyclosporin, and illustrated the essential role of the host cyclophilin A (CyPA; 281) and the host SYNCRIP (synaptotagmin-binding, cytoplasmic RNA-interacting protein; 282) in HCV replication.

Development and improvement of therapies and vaccines: Identification of therapeutic targets

Rational drug-design, with regard to 'Specifically Targeted Anti-viral Therapy for hepatitis C' (STAT-C), has been made possible via the crystallisation and determination of high-resolution

three-dimensional (3-D) structures of key viral enzymes, often complexed with their substrates, cofactors and/or inhibitors, the activities of which are tested by specific *in vitro* assays (summarised by Locarnini & Bartholomeusz [283]). The yeast two-hybrid system, with HCV core protein used as 'bait' to screen a human liver cDNA expression library, has been used to identify cellular proteins that interact with the HCV core protein (284).

Gondeau *et al*. comprehensively reviewed cellular models in current use for the screening and development of anti-HCV agents, highlighting their utility and productivity. The review listed HCVpp, HCVcc, subgenomic and productive replicons, and primary and immortalised hepatocyte cultures as being used to test more than 55 different anti-viral agents (285). It is asserted that the use of these models will "allow the prediction of the pharmacokinetics of the new chemical entities as well as possible drug interactions and liver toxicity."

In vitro assays have established that cyclophilin B (CyPB), a normal cellular protein, boosts HCV replication by increasing the activity of the HCV polymerase via improvement of its RNA-binding capacity (286). This has important consequences for the design and development of anti-HCV drugs, as a number of HCV inhibitors, such as cyclosporin and related derivatives, function by sequestering cyclophilins (286–289). HT mutational analyses with HCVcc have identified regions of the HCV genome as being suitable candidates for therapeutic and vaccine targets (204).

A new and superior method of screening and identifying innate immunostimulatory molecules, based on primary leukocyte cultures co-cultured with HCV replicon-expressing cells, has been recently announced, which should aid the development of HCV therapeutics (290). A replicon-based HT assay has been developed that permits the screening of inhibitors of HCV genotypes 1a and 1b, and the determination of inhibitor cytotoxicity, within a single well (291). A medium-throughput assay based on flow cytometry, which is able to screen for entry inhibitors that impede the interaction between HCV E2 envelope glycoprotein and host-cell CD81 receptors, is also available (292). A 3-D hollow-fibre culture system, incorporating an immortalised primary human hepatocyte (HuSE/2) cell line, has been created. This system permits the study of blood-borne HCV from patients, as well as the long-term proliferation of the virus and production of infectious virions (293). The developers state that it: "reproduces strain-dependent events reflecting viral dynamics and virus–cell interactions at the early phase of blood-borne HCV infection, …and allows the development of new anti-HCV strategies specific to various HCV strains."

A recently established reporter cell-line, which utilises secreted alkaline phosphatase (SEAP) as the reporter molecule, enables much more rapid and sensitive assays of HCV infectivity and replication to be performed, as compared to previous time-consuming and labour-intensive methods (140). It also facilitates the investigation of virus entry and the HT screening of entry inhibitors and other anti-viral agents (294). This method is advantageous, because it allows cell integrity to be maintained, by enabling reporter molecule measurement in the culture medium. It can also be used with any inter-genotypic or intra-genotypic derivative of HCV JFH1, and potentially with other future cell culture-infectious viral isolates. The screening of human siRNA libraries and miRNA expression profiling have identified hundreds of genes that support HCV replication, providing new targets for anti-viral therapy and enabling the *in vitro* testing of the effects on HCV replication of inhibitors of those genes (295–298). In addition, expression profiling has led to the discovery of specific miRNAs that are differentially expressed in HCC/HCV-induced fibrosis/cirrhosis, as well as their target genes (299–303).

The existence of antibodies that cross-neutralise different genotypes of HCV, was demonstrated by using the HCVpp system (119, 120, 122) — a finding that should aid the design of vaccine strategies, and which is permitting the determination of correlates of protection (123).

Development and improvement of therapies and vaccines: Assessment of treatment, vaccine efficacy and toxicity

Clinical research is making progress toward establishing human biomarkers of HCV drug and vaccine efficacy and toxicity, which will lead to safer, quicker and more-effective clinical trials. For example, serum biomarkers of liver fibrosis have already been discovered (304). SNP analysis of human biological samples has characterised particular SNPs that indicate the likelihoods of successful response to IFN therapy, spontaneous viral clearance and progression to fibrosis (305, 306). Microarray profiling of human tissue has provided gene expression signatures for IFN treatment in responders and non-responders, and identified mechanisms of failed response (307–309).

A study performed with cell lines derived from HCV patients, illustrated that expression levels of, and polymorphisms in, the SOCS3 gene, represent important biomarkers for the *a priori* prediction of response to HCV anti-viral therapy (310). Comparative studies of Caucasian and North American Aboriginal populations revealed that ethnicity might influence responses to IFN-alpha (via IL-10 production; 311, 312), which could explain the enhanced propensity of the latter group to clear HCV infection (313). Another new

cell culture method has been developed to model relapse after the end of IFN therapy, which involves the use of IFN-alpha-resistant, genome-length HCV RNA-harbouring cells (314). This model has been used to assess alternative anti-HCV agents and has shown that those with a different mode of action to that of IFN-alpha can prevent relapse.

A fully-human modular immune *in vitro* construct ('MIMIC') has been developed, which should serve as a reliable and human-specific *in vitro* test system for proposed HCV vaccines. By using white blood cells from volunteer donors, it involves "optimised PBMC culture systems via recombination of distinct leukocyte components of the immune system, T-cells, B-cells, and dendritic cells (DCs), at ratios similar to those found at sites of *in vivo* lymphocyte activation" (315). Autologous DCs pulsed with test vaccines are introduced to the cultures, and the humoral and cell-mediated immune responses induced by the vaccine candidate can be studied. A 'peripheral tissue equivalent' (PTE) module serves to model events at the vaccination site and/or point of microbial attack, providing data concerning the innate immune response in terms of cytokine production/inflammation and maturation of antigen-presenting cells; similarly, a 'lymphoid tissue equivalent' (LTE) module models adaptive immunity/lymph node events, assessing B-cell and T-cell activities and antibody production. Advantages of the MIMIC system include its capacity to test adjuvants, vaccine components and complete vaccines, and its use to assess the quality of established vaccines. Also, each well of the 96-well plate provided, represents the immune system of a specific individual human being — thus reflecting human biological and immunological diversity across the whole plate. The stated goals of this system are to obviate preclinical, animal-based vaccine tests and to identify optimal human vaccine formulations. Given the performance of this system to date, there is robust evidence that its use will reduce the risk of adverse events in clinical trials, elucidate why some vaccines work in certain populations of people but not in others, and address safety and immunogenicity issues. Furthermore, this method is applicable, not just to vaccines, but also to the immunotoxicological evaluation of other drugs and biologicals.

Mathematical models

Mathematical models have helped to further our understanding of HCV dynamics and clinical trial results in humans (reviewed by Shudo *et al.* [316]). Based on data acquired from people undergoing IFN-alpha treatment, the mechanism of action and efficacy of IFN-alpha were elucidated, along with the dynamics of HCV production and clearance (317). Other mathematical models have:

— revealed that both rapid and slow biological processes occur following HCV infection, the knowledge of which has impacted the use of anti-virals in HCV patients (318);

— permitted the physiologically-based pharmacokinetic modelling of putative new drugs, in order to predict their properties in the human body (e.g. 316, 319, 320);

— provided insights into the mode of action of ribavirin (321); and

— generated valuable data concerning the different responses of diverse patient populations to anti-viral therapy (245).

Discussion and Conclusions

This paper, together with the associated paper (18), sought to critically evaluate claims of necessity for the use of chimpanzees in hepatitis C research. This is timely and of high importance, due to the controversial nature of invasive chimpanzee research (which is now practised only in the USA), and the reintroduction of the *Great Ape Protection Act* (GAPA) bill in the US House of Representatives that seeks to end it. This bill has significant consequences, not just for the 1,000 chimpanzees in US laboratories, but also for many millions of people relying on science to provide treatments and cures for hepatitis C and other diseases.

Paper 1 (18) examined the scientific validity of the chimpanzee model. By directly assessing the claims of advocates of chimpanzee HCV research that it made crucial contributions to past progress — claims that have been subject to little critical analysis to date — the chimpanzee model's proposed critical role in future investigations can begin to be gauged. The conclusions of the review were that these assertions were exaggerated and unjustifiable. While chimpanzees have been involved in many areas of HCV research, the contribution of chimpanzee data to the advancement of knowledge and to tangible progress was negligible, and was eclipsed by that of other methods of inquiry. When considered alongside acknowledged and serious scientific caveats of the chimpanzee model of hepatitis C, as well as ethical, economic and practical caveats of chimpanzee use in general, it was concluded that the chimpanzee model in HCV research must now be considered redundant.

This complementary review augments the findings of the first paper, by illustrating how non-chimpanzee methods have contributed to the development of important *in vitro* methods for

studying the HCV life-cycle. While full life-cycle infectious cellular clones represent the long awaited and most comprehensive *in vitro* system for many aspects of HCV study, all the *in vitro* methods employed, including HCV-infected cultured primary and immortalised cells, infectious molecular clones, subgenomic and genomic replicons, and virus-like particles and pseudoparticles, have added greatly to the body of knowledge on the hepatitis C virus and hepatitis C pathology, as well as enhancing progress toward new treatments. Furthermore, this review describes how full life-cycle infectious clones (HCVcc), which were urgently called for by the research community for decades, can provide all the necessary data to facilitate the development and testing of HCV therapies, when supported by clinical, epidemiological, *ex vivo* and *in silico* methods.

It is now possible to investigate the complete HCV life-cycle, from host-cell attachment to release of progeny, immune responses to infection, the roles of host factors, identification of therapeutic targets, testing of new therapies and vaccines, and so on, in a human, and therefore completely relevant, context. While it must be acknowledged that all methods of investigation have inherent imperfections and caveats, including the use of human cultured cells *per se,* and the biological differences between the use of HCV *in vitro* in human cells and the *in vivo* situation in humans, the evidence suggests that the use of human cells *in vitro* is much more relevant to human biology than the use, albeit *in vivo*, of another animal entirely (i.e. the chimpanzee) as a 'model' organism. Notably, while chimpanzee use is declining markedly (in 2008, the NIH funded 220 human hepatitis trials, as compared to just 35 chimpanzee projects, the latter having decreased by 50% over the past 30 years), the use of the various human-cell culture systems in the study of HCV is continuing to produce large amounts of important data, particularly when used alongside other methods.

In summary, there is a very strong argument against any scientific requirement for the use of chimpanzees in hepatitis C research, and an equally strong argument in support of the concentration of research effort in human-specific clinical and *in vitro* technologies. Prohibiting chimpanzee use would accelerate progress by releasing extra funds for more-productive and scientifically-superior alternatives, benefiting the hundreds of millions of human beings infected with, or at risk of being infected by, HCV, as well as for the approximate 1,000 chimpanzees in US laboratories.

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