

emerged and the 'surrogate' assays were introduced in concert with more-intensive questioning of donors regarding high-risk behavior and by a lessened use of allogeneic blood. Nonetheless, we could show that these combined measures served to decrease hepatitis incidence to 4.5% by 1989 (Fig. 2). Efforts to develop a specific NANB assay continued throughout the 1980s, although the main effort by Chiron was kept well concealed.

During this time, I had developed a panel of sera consisting of duplicate coded samples that had been proved to be infectious in the chimp or non-infectious in humans. By 1989, many different laboratories claimed to have developed a NANB assay and asked to test the panel. None was able to break the code and by 1989, the score was viruses, 20; investigators, zero. At that time, I received a call from George Kuo at Chiron, saying that they too felt they had a NANB assay. I sent George the remnants of the now-dwindling panel and within days received their results followed by several anxious calls asking if I had yet broken the code. When I did, I was excited to find that Chiron had detected all but two of the infectious sera and had properly found all the non-infectious sera to be negative. Further, the two samples that they missed were acute-phase sera, and subsequent samples from these same patients proved to be positive for what Chiron now called the hepatitis C virus. Michael Houghton will describe the events that preceded this discovery.

Using the newly developed assay for antibodies against HCV, we again delved into our repository and were able to rapidly show that 88% of NANB hepatitis cases seroconverted for antibody against HCV, that the development of antibody was in temporal relationship to the course of hepatitis and that in-

fectured patients could be linked to infected donors<sup>18</sup>. Thus, by 1990 it was clear that HCV was the principal agent of NANB hepatitis, and universal donor screening was initiated. We established a new prospective study to measure the effect of such testing and to define the extent of residual hepatitis unrelated to HBV or HCV. The first-generation assay for antibody against HCV resulted in a further 70% decrease in hepatitis incidence to a residual rate of 1.5%, and a more-sensitive second-generation assay, introduced in 1992, nearly eliminated HCV transmission (Fig. 2). Although mathematical modeling indicates that antibody-screened blood might still transmit HCV to 1:100,000 to 1:200,000 recipients, the observed decrease from 33% in 1970 to nearly zero in 1997 stands as a testament to the cumulative effectiveness of a series of donor screening interventions that were evidence-based. Viral nucleic acid testing of donors and improved viral inactivation technologies will soon bring transmission of hepatitis and human immunodeficiency virus from near-zero to absolute zero. I am now looking for another line of work.

#### Acknowledgments

*Throughout almost the entire course of these clinical investigations, my right arm, and sometimes my left as well, has been my dedicated assistant, J.*

*Melpolder. There is no way to adequately acknowledge the substantial contribution she has made in coordinating these studies that have involved thousands of patients. My gratitude is without bounds. I would also like to gratefully acknowledge the manifold contributions of my long-term associate James W. Shih, Ph.D. who so ably supervised the diverse laboratory aspects of these prospective studies.*

## The hepatitis C virus: A new paradigm for the identification and control of infectious disease

### Identification of the hepatitis C virus

The problem of non-A, non-B (NANB) hepatitis emerged in 1975, after serological tests for hepatitis A virus (HAV) and hepatitis B virus (HBV) were developed. It then became evident that most hepatitis cases after transfusion were not due to either HAV or HBV, and that the risk of NANB hepatitis after blood transfusions was as high as 10% or even greater<sup>5</sup>. Later, it also became evident that NANB hepatitis occurred frequently in the form of sporadic, community-acquired infections. A frustrating period of 13 years followed, in which the methods successfully used to identify HAV and HBV all failed to result in the molecular identification of the etiological agent(s) of NANB hepatitis. No NANB-hepatitis-specific antigen, antibody or cell culture system was identified, and this lack of a molecular 'handle' limited progress in identifying the causative agent(s) of NANB hepatitis<sup>19</sup>. However, a chimpanzee model successfully developed by several groups<sup>6,20,21</sup> was exploited to show that one NANB hepatitis agent induced characteristic membranous tubules within the endoplasmic reticulum of infected chimpanzee hepatocytes<sup>22</sup>. Known as the tubule-forming agent, it was later shown to be filterable and to lose infectivity after treatment with organic solvents, consistent with its being a lipid-enveloped virus, possibly a toga-like or flavi-like virus<sup>9,23</sup>. Other data supported the existence of an HBV-like NANB hepatitis agent<sup>19</sup> as well as a chloroform-resistant (non-enveloped) NANB hepatitis virus<sup>23</sup> and possibly other NANB hepatitis agents<sup>22</sup>.

Eventually, what turned out to be the main form of parenterally transmitted NANB hepatitis was identified using a 'blind'

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immunoscreening approach applied to recombinant  $\lambda$ gt11 (ref. 24) cDNA libraries prepared from total RNA and DNA extracted from infectious chimpanzee plasma<sup>25</sup>. Serum from a patient diagnosed with NANB hepatitis was used as a presumed (but unproven) source of NANB-hepatitis-specific antibodies to identify just one viral cDNA clone from a complex cDNA library constituting one million other cDNAs. Formal proof of its etiological origin came from the demonstration that the clone was not derived from the host genome, that it bound a large RNA molecule of around 10,000 nucleotides found only in NANB-hepatitis-infectious materials, and that it was derived from a positive-stranded RNA encoding a protein that induced antibodies only in NANB hepatitis-infected individuals<sup>25,39</sup>. The RNA genome also encoded a large polyprotein of about 3,000 amino acids that had distant primary sequence identity with members of the Flaviviridae family<sup>26</sup>. HCV was therefore identified by direct molecular cloning of its genome in the relative absence of knowledge concerning the nature of the infectious agent and the immune response. This 'blind' method could be of value in the future in unearthing other unknown infectious agents involved in disease. The molecular identification of HCV was the culmination of a team effort<sup>25</sup> spanning 7 years, during which hundreds of millions of bacterial cDNA clones were screened for a putative NANB hepatitis origin using many different approaches. The successful approach involved Qui-Lim Choo in my Laboratory at Chiron and the laboratories of George Kuo (Chiron) and Daniel Bradley (CDC). I accept the award on behalf of these collaborators (Fig. 3).

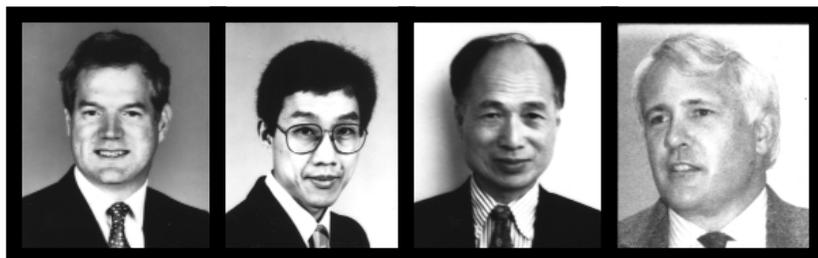


Figure 3 The HCV team (from left to right): M. Houghton, Q-L Choo, G. Kuo and D. Bradley.

have been developed, based on the highly conserved 5' internal ribosome entry site and nucleocapsid gene sequences, which can diagnose new infections before seroconversion to having antibodies against HCV occurs. Implementation of these tests for screening of blood donors will reduce the risk of HCV after transfusion still further (down to approximately one in 300,000 in the US, for example). A test detecting circulating nucleocapsid antigen is also of value in diagnosing infection before seroconversion.

### Properties of HCV

Now known to contain a highly variable RNA genome, HCVs constitute a large genus (the hepacivirus genus) within the Flaviviridae family. Six basic genotypes have been distinguished so far, with more than 100 phylogenetically-distinct subtypes<sup>27</sup>. At any one time, the viral genome exists as a complex quasi-species. The RNA genome contains a conserved 5'-terminal internal ribosome entry site that is responsible for initiating translation of the large polyprotein. The latter is cleaved co- and post-translationally into at least three structural or virion proteins and seven presumed non-structural proteins involved in replication of the virus<sup>28-30</sup> (Fig. 4). The 3' terminus of the RNA genome is composed of a variable region, a polypyrimidine tract and a highly conserved stem-loop secondary structure<sup>29</sup>. Hypervariable regions exist within the large gpE2 glycoprotein domain that may be under immune selection<sup>31</sup>. The virus cannot be grown efficiently in cell culture or purified from infected liver or blood, and thus still has not been characterized morphologically or biochemically.

### Serodiagnosis

The molecular cloning of the HCV genome led to the availability of many recombinant HCV diagnostic antigens. George Kuo purified these and developed numerous experimental EIA tests detecting HCV antibodies. This intensive work allowed the selection of optimal, immunodominant epitopes for inclusion in an evolving series of sensitive and specific blood screening and diagnostic tests for HCV infection<sup>32</sup>. Used to screen blood donors beginning in 1990, these assays have led to the near-disappearance of transfusion-associated hepatitis C. At least 40,000 infections have been prevented each year in the US alone since the implementation of these tests<sup>33</sup>. Such tests have also been of great use in diagnosing hepatitis patients and in their clinical management, and in attributing liver and extra-hepatic diseases to HCV infection. Chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, cryoglobulinemia and porphyria cutanea tarda are all well-established potential clinical sequelae of chronic persistent HCV infection. Other diseases, such as oral lichen planus, Sjögren's-like syndrome and non-Hodgkin lymphoma, have also been linked with HCV infection<sup>28</sup>. Although most HCV-infected individuals have few clinical symptoms and will not progress to a severe disease state, a subset can undergo progressive liver disease in which, often over decades, chronic hepatitis develops into liver cirrhosis and then into hepatocellular carcinoma. Extra-hepatic manifestations can also occur.

Recently, various nucleic acid testing assays

### Education, new therapies and vaccines

Future challenges exist for both developed and developing countries. In the latter, global implementation of blood donor screening for HCV has been recommended recently by the World Health Organization. In many countries in which HCV is endemic, the risks of HCV infection after transfusion are still exceedingly high. Also, the World Health Organization has emphasized education to lower the risks of HCV transmission in both developing and developed countries. The historical use of non-sterile injection devices has been mainly responsible for the huge burden of HCV disease present in many developing countries, as well as cultural practices (such as circumcision) involving the use of non-sterile medical equipment. In developed countries, intravenous drug use involving sharing of needles/syringes is still the main risk factor<sup>34</sup>. Any procedure involving blood transfer (such as tattooing using shared instruments) is not recommended. The Centers for Disease Control also recommends that HCV-infected individuals not share toothbrushes, razors and so on.

The current therapy for HCV consists of a combination of interferon and ribovirin<sup>35</sup>. However, both drugs can produce substantial toxicity, and only a minority of patients responds. In particular, long-term response rates with the most common genotype, type 1, occur in only approximately 30% of patients. Although the imminent introduction of a more stable form of pegylated interferon will improve response rates somewhat, it is apparent that more-effective and less-toxic drugs are required. The HCV genome encodes two proteases involved in processing of the viral polyprotein: a helicase involved in unwinding the RNA strands during replication and translation, and a replicase that copies the positive RNA strand (Fig. 4). The fine structures of all these enzymes have now been resolved by X-ray diffraction methods and are now the subjects of rational drug design<sup>29,30</sup>.

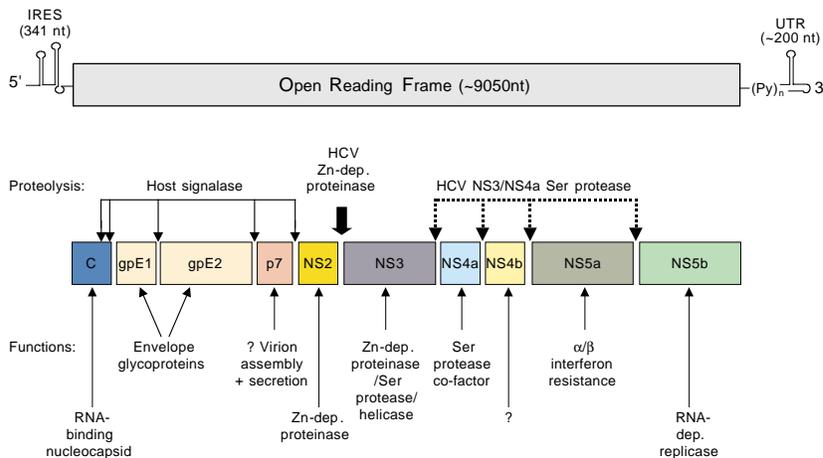


Figure 4 The organization of the hepatitis C positive-stranded RNA genome.

Other therapeutic developments involve ribozyme and antisense strategies involving the conserved 5' internal ribosome entry site and nucleocapsid gene sequences, and nucleoside analog inhibitors of the replicase. A putative receptor for HCV, the CD81 tetraspanin molecule, is also the subject of potential antiviral development<sup>36</sup>. Therefore, we can be optimistic that new, specific drugs against HCV will emerge within the next 5–10 years.

Between 12 and 50% of acute HCV infections spontaneously resolve without progressing to the chronically infected state that is associated with the pathogenic sequelae of infection. Such resolution of acute infection is associated with the induction of broad helper and cytotoxic T-lymphocyte responses to the virus<sup>37</sup>. Thus, appropriate vaccination to prime such immune responses may lower the high chronicity rate associated with HCV infection. Vaccination of chimpanzees with recombinant envelope glycoproteins gpE1 plus gpE2 successfully prevented the development of chronic infection in most animals after challenge with homologous or heterologous subtype 1a virus. In contrast, most control unvaccinated animals develop chronic, persistent infections<sup>37</sup>. These data are encouraging for the development of human vaccines. Human immunoglobulin preparations (containing antibodies against HCV) have been reported to be effective

at reducing the rate of development of chronic infection in the transfusion setting, between sexual partners and in liver transplantation<sup>37</sup>. Response to interferon has also been linked with the endogenous level of intrahepatic HCV-specific cytotoxic T-lymphocyte activity before treatment<sup>38</sup>. If it is confirmed, appropriate vaccination may also be important therapy if such virus-specific cytotoxic T-lymphocyte activity can be boosted during drug therapy.

Finally, although HCV is an RNA virus that does not produce DNA replication intermediates that can integrate into the host genome, it still manages to persist in most cases (usually for life in untreated individuals). Although it may involve the emergence of viral escape mutants to both antibody and T-cell responses, it is very likely that additional mechanisms are in operation to result in such high rates of chronicity. Elucidating these mechanisms represents the most intriguing challenge of future HCV research and, in the process, is likely to open up new strategies for the control of this challenging virus.

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