Viral Hepatitis

Viral Hepatitis

Diagnosis, Therapy, and Prevention

EDITED BY

Steven Specter

College of Medicine
University of South Florida, Tampa

Springer Science+Business Media, LLC

Originally published by Humana Press Inc. in 1999
Softcover reprint of the hardcover 1st edition 1999

This publication is printed on acid-free paper. ♾
ANSI Z39.48-1984 (American Standards Institute) Permanence of Paper for Printed Library Materials.

Cover illustration:

Cover design by Patricia F. Cleary.

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel: 973-256-1699; Fax: 973-256-8341; E-mail: humana@humanapr.com, or visit our Website at www.humanapress.com

10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging-in-Publication Data
Viral hepatitis: diagnosis, therapy, and prevention/edited by Steven Specter.
p. cm.
Includes index.
ISBN 978-1-61737-033-5 ISBN 978-1-59259-702-4 (eBook)
DOI 10.1007/978-1-59259-702-4
1. Viral hepatitis. I. Specter, Steven.
[DNLM: 1. Hepatitis, Viral, Human--diagnosis. 2. Hepatitis, Viral, Human--therapy. WC 536V81342 1999]
RC848.H43V5733 1999
616.3'623--dc21
DNLM/DLC
for Library of Congress 98-36841
CIP

Preface

Hepatitis continues to be a major health threat in both developed and developing nations. This occurs despite the recognition over the past few decades of the viral agents responsible for this disease, and despite development of methods to detect these viruses and of vaccines to prevent the spread of some of them. Nevertheless, the number of viruses that are known to cause hepatitis continues to increase. This has been both helpful in developing strategies to combat viral hepatitis and disconcerting as we realize that the problem is more complex than was realized.

The artificial grouping of these totally unrelated viruses, based on the disease they cause, requires careful examination of the biology, pathogenesis, and epidemiology of these agents. *Viral Hepatitis: Diagnosis, Therapy, and Prevention* examines the five well-recognized hepatitis viruses, A through E, and examines some newer agents (so called non–A-E hepatitis viruses or GB viruses), whose description has emerged in the past few years. These agents can all be transmitted by human blood products, which makes them potentially important pathogens. The book's chapters will examine the biological nature of each of the viruses, the pathogenesis of the disease(s) they cause, both acute and chronic, information on how, and how readily, they are transmitted, and the clinical signs and symptoms of the diseases. This will provide an in depth understanding of the current state of knowledge of these agents.

The hepatitis viruses are tested for during the diagnosis of disease. The specific types of diagnosis and the most practical way to approach this will be evaluated.

The prevention of and therapy for hepatitis virus infections are a major public health goal. Significant progress has been made in this regard through the 1980s and 1990s. There are now vaccines for hepatitis B virus (and consequently hepatitis D virus) and for hepatitis A virus. In addition, interferon and some antiviral drugs are now being used for therapy. These are all discussed in chapters devoted to each of the topics noted.

Viral Hepatitis: Diagnosis, Therapy, and Prevention concludes with a look at where the study of viral hepatitis is headed and what impact new developments in this field might have on world health.

Steven Specter

Contents

Contributors

MAURO BENDINELLI • *Dipartmento di Biomedicina Sperimentale, Infettiva e Pubblica, Universita di Pisa, Pisa, Italy*
RICHARD L. HODINKA • *Clinical Virology Laboratory, Children's Hospital of Philadelphia, Philadelphia, PA*
THOMAS P. LEARY • *Abbott Laboratories, North Chicago, IL*
JOHN A. LEWIS • *Merck Research Laboratories, West Point, PA*
FABRIZIO MAGGI • *Dipartmento di Biomedicina Sperimentale, Infettiva e Pubblica, Universita di Pisa, Pisa, Italy*
DILIP MOONKA • *Division of Gastroenterology, Hospital of the University of Pennsylvania, Philadelphia, PA*
ISA K. MUSHAHWAR • *Abbott Laboratories, North Chicago, IL*
CHRISTOPHER O'BRIEN • *Division of Gastroenterology, Hospital of the University of Pennsylvania, Philadelphia, PA*
MAURO PISTELLO • *Dipartmento di Biomedicina Sperimentale, Infettiva e Pubblica, Universita di Pisa, Pisa, Italy*
ZHI-REN LIU • *Department of Animal and Dairy Science, Auburn University, Auburn, AL*
ANTONIA SMEDILE • *Servizio Sanitario Nazionale, Regione Piemonte; Divisione di Gastroenterologia, Azienda Ospedaliera, S. Giovanni Battista di Torino, Ospedale Molinette, Torino, Italy*
CHRISTOPHER W. J. SMITH • *Department of Biochemistry, University of Cambridge, United Kingdom*
STEVEN SPECTER • *Department of Medical Microbiology and Immunology, University of South Florida College of Medicine, Tampa, FL*
JACK T. STAPLETON • *Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, IA*
ALBERT W. TAM • *GeneLabs Technologies, Redwood City, CA*
MARIA LINDA VATTERONI • *Dipartmento di Biomedicina, Sperimentale, Infettiva e Pubblica, Universita di Pisa, Pisa, Italy*
GIORGIO VERME • *Servizio Sanitario Nazionale, Regione Piemonte; Divisione di Gastroenterologia, Azienda Ospedaliera, S. Giovanni Battista di Torino, Ospedale Molinette, Torino, Italy*
GIRISH N. VYAS • *Departments of Laboratory Medicine and Pathology, University of California, San Francisco,CA*
PATRICE O. YARBOUGH • *GeneLabs Technologies, Inc., Redwood City, CA*
T.S. BENEDICT YEN • *Department of Laboratory Medicine and Pathology, University of San Francisco, San Francisco, CA*

Introduction

Steven Specter

Hepatitis as an entity has been recognized for thousands of years. The description of jaundice, i.e., hepatitis, dates to ancient Chinese writings, and the first European reference dates to the third century A.D. Recognition that hepatitis has an infectious etiology has been in the modern literature since early in this century *(1)*, and the terms hepatitis A and B were first used in 1947 *(2)*. However, proof that viruses are responsible for this disease was first published in 1968 with the description of hepatitis B virus (HBV) particles in *Nature (3)*.

Shortly before this, Blumberg and colleagues had reported that a unique protein isolated from Australian aborigines, which was referred to as Australia antigen, was associated with serum hepatitis *(4)*. Interestingly, rather than being the result of serologic testing for hepatitis, these studies resulted from Blumberg's interests in anthropology. While looking for a distinguishing protein in aborigines, Blumberg discovered the Australia antigen. Thereafter, when one of the technical staff developed hepatitis and subsequently seroconverted to positive for the antigen, the association with hepatitis was made. Prior to this time, there clearly had been evidence that disease entities that included hepatitis were caused by viruses but not the two forms of classically described hepatitis, serum and infectious hepatitis. While before this discovery, viruses were believed to cause hepatitis, this was the first solid evidence of a viral etiology for classic hepatitis.

The discovery of a second hepatitis virus, hepatitis A virus (HAV) was confirmed in 1973 *(5)*. At that time, it was believed that the major causes of acute hepatitis were identified, with HAV being determined to be responsible for infectious or short-incubation hepatitis and HBV the agent of serum or long incubation hepatitis. However, as virus identification and screening of blood became commonplace, it became clear that other agents were involved, and the third form of hepatitis became known as non-A, non-B or NANB hepatitis.

Over the past two decades, we have added hepatitis D virus (HDV), hepatitis C virus (HCV), and hepatitis E virus (HEV). HDV, which was

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

initially designated the delta agent, was identified as a unique agent in 1983 *(6)*. HCV was initially described in 1989 using molecular biology techniques *(7)* and only visualized in the 1990s. It is interesting to note here that this was the first time that discovery of an infectious entity resulted solely from molecular biological techniques. HEV was first visualized in 1983 *(8)*. In the past few years, additional agents have been described that have been referred to both as the GB agents and hepatitis G virus (HGV). There are three GBV agents described and referred to as GBV-A, GBV-B, and GBV-C. Through homology studies, it appears that GBV-C and HGV are variants of the same virus. Their role as clinical entities is still not very clear (chapter 7; ref. *9*). A more comprehensive history of viral hepatitis has been presented by Zuckerman in the initial chapter of a text on hepatitis *(10)*, with the exception of events in more recent years.

The viruses that comprise the group of "hepatitis viruses" are completely unrelated taxonomically and are grouped solely by the fact that the primary disease that they cause is inflammation of the liver. These viruses cause a continuum of disease that ranges widely in severity, ability to cause chronic infection and disease, epidemiology, diagnosis, prevention, and treatment. This is, of course, related to the diverse biology of the etiologic agents. Each virus and the properties indicated above are described in the chapters that follow. In addition to the hepatitis viruses, A–G, there are many other viruses that include hepatitis in the constellation of symptoms that they may induce. This includes several human herpesviruses, rubella, yellow fever, dengue, and other hemorrhagic fever viruses *(11)*. The current volume is limited to the examination of the hepatitis viruses only, discussing their biology, pathogenesis, epidemiology, clinical descriptions, diagnosis, prevention, and therapy. By examining all aspects of these viruses and their disease, we attempt to present the reader with a comprehensive overview of the clinical entity viral hepatitis caused by the classic hepatitis viruses. In this regard, a table that should serve as a quick reference is provided that summarizes some of the properties dealt with in this text (*see* Table 1).

The list of viruses presented is not necessarily complete. The reader may have noticed that there are no hepatitis F viruses included. A putative hepatitis F virus was described associated with a human stool sample and was transmissible in primates *(12)*; however, this awaits confirmation. The GB agents, which are blood borne, as described by Mushahwar and colleagues at Abbott Laboratories (*see* chapter 7), include potentially three separate viruses. Additionally, there may be as yet undetected agents capable of causing hepatitis that will be recognized as we eliminate the known viruses from the blood supply. Thus, the list of "hepatitis viruses" may yet grow.

Table 1
Comparison of Hepatitis Viruses, Their Properties, and Diseases[a]

Virus	HAV	HBV	HCV	HDV	HEV	GBV–C/HGV
Nucleic acid	ssRNA(+)	dsDNA/ssDNA	ssRNA(+)	ssRNA	ssRNA(+)	ssRNA
Family	Picornavirus	Hepadnavirus	Flavivirus	Viroid?	Calicivirus–like	Flavivirus
Particle size (nm)	27	42 (Dane)	55–65	30–35	27–34	30–60?
Transmission	fecal-oral	Parenteral/ sexual	Parenteral/ sexual	Parenteral/ sexual	Fecal-oral	Parenteral & fecal–oral
Incubation (d)	15–50 d	45–160 d	14–180 d	42–180 d	15–64 d	?
Onset	Abrupt	Insidious	Insidious	Abrupt	Abrupt	?
Severity	Mild	Occasionally severe	Usually subclinical	Co-infect. with HBV occas. severe; superinfect. with HBV often severe	Mild except in pregnancy	Uncertain; some assoc. with fulminant hepatitis
Chronic	No	Yes	Yes	Yes	No	Yes
Other disease	None	HCC, cirrhosis	HCC, cirrhosis	Cirrhosis, fulminant hepatitis	None	?
Mortality	<0.5%	1–2%	0.5–1%	High	1–2%-normals ~20%-pregnancy	?
Lab diagnosis	Serology, no isolation for all viruses, molecular probes for HCV. GBV-C					
Vaccine	Yes	Yes	No	Yes-HBV vaccine	No	No
Passive Ab	ISG	HBIG	ISG–uncertain	No	No	No
Therapy	None	Chronic-IFNα	Chronic-IFNα	None	None	None

[a]HCC = hepatocellular carcinoma; Ab = antibody; ISG = immune serum globulin; HBIG = hepatitis B immune globulin; IFNα = interferon alpha.

Laboratory diagnosis for hepatitis viruses has been an evolving field since its introduction in the early 1970s with testing for "Australia antigen." Chapter 8 details the current state of testing for these viruses to determine the etiology of established hepatitis infection. Hepatitis testing has become an important tool for tracking the prognosis of chronic hepatitis and therapy for this condition.

Therapeutics for hepatitis have been developed only recently with the introduction of interferon alpha for treatment of chronic hepatitis. Chapter 9 details the accumulated information on development of antiviral compounds for treatment of hepatitis, including studies with newer antiviral drugs. Antiviral treatment for hepatitis is finally showing some promise, and the next few years should provide us with effective weapons for dealing with some or all of these hepatitis viruses.

The development of vaccines for both HAV and HBV have been major accomplishments, which occurred during the past few decades. In the case of HBV, the first vaccines were used in the 1970s, whereas HAV vaccine was not licensed until 1995. These have been described in separate chapters (10 and 11) in this text. In both cases, vaccination does not currently use live vaccines. For HBV, vaccination is currently performed using a recombinant HBV surface antigen (HBsAg), the major surface protein of the virus that can also be found in the blood of infected patients as independent particles. For HAV, the vaccine is intact inactivated virus. A major benefit of the HBV vaccine is that it also is protective against HDV, since HDV is a defective virus dependent on HBsAg for maturation. Vaccine development for other hepatitis viruses has pretty much been limited to HCV. To date, studies have been unsuccessful in the development of an HCV vaccine candidate.

The status of hepatitis as an entity has changed dramatically as a result of advances in scientific knowledge. The viruses responsible for the vast majority of infections have been identified. As a result of this identification, we have developed diagnostic tests, vaccines, and therapeutics. Screening of blood for donation for HBV initially lead to a decrease in transfusion-associated hepatitis. This led to the emergence of HCV as the leading cause of transfusion-associated hepatitis. The addition of HCV screening has allowed for interventions that have greatly reduced the transmission of HBV and HCV in the blood supply in developed nations. Additionally, screening for cytomegalovirus has further reduced blood-borne hepatitis. We cannot become complacent regarding our successes with these viruses. Hepatitis caused by the hepatitis viruses A–G is still a major problem worldwide. Acute and chronic hepatitis still result in thousands of deaths annually, and sequelae such as cirrhosis and hepatocellular (HCC) are a significant cause of mortality. Further interruption of modes of transmission through epide-

miology efforts and education can greatly reduce the incidence of these infections, especially in developing nations. This can further be enhanced by continued development of our understanding of the molecular biology of these viruses, their pathogenesis, better diagnostic techniques, as well as methods of prophylaxis and therapy.

References

1. Cockayne, E. A. (1912) Catarrhal jaundice, sporadic and epidemic, and its relation to acute yellow atrophy of the liver. *Quart. J. Med.* **6,** 1–28.
2. Havens, W. P., Jr (1947) The etiology of infectious hepatitis. *J. Am. Med. Assoc.* **134,** 653–655.
3. Bayer, M. E., Blumberg, B. S., and Werner, B. (1968) Particles associated with Australia antigen in the sera of patients with leukemia, Down's syndrome and hepatitis. *Nature* **218,** 1057–1059.
4. Blumberg, B. S., Gerstley, B. J. S., Hungerford, D. A., London, W. T., and Sutnick, A. I. (1967) A serum antigen (Australia antigen) in Down's syndrome and hepatitis. *Ann. Int. Med.* **66,** 924–931.
5. Feinstone, S. M., Kapikian, A. Z., and Purcell, R. H. (1973) Hepatitis A: detection by immune electron microscopy of a viruslike antigen associated with acute illness. *Science* **182,** 1026–1028.
6. Rizzetto, M., Bonino, F., Verme, G., Purcell, R. H., and Gerin, J. L. (1987) Nomenclature: a proposal to designate the delta agent as hepatitis D virus, in *The Hepatitis Delta Virus and Its Infection* (Rizzetto, M., Gerin, J. L., and Purcell, R. H., eds.), Alan R. Liss, New York, pp. xx–xxi.
7. Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244,** 359–362.
8. Balayan, M. S., Andjaparidze, A. G., Savinskaya, S. S., Ketiladze, E. S., Braginsky, D. M., Savinov, A. P., and Poleschuk, V. F. (1983) Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. *Intervirology* **20,** 23–31.
9. Versalovic, J. (1997) Hepatitis G virus. *Clin. Micro. Newslett.* **19,** 161–164.
10. Zuckerman, A. J. (1983) Introduction. A history of viral hepatitis from antiquity to the present, in *Viral Hepatitis: Laboratory and Clinical Science* (Deinhardt, F. and Deinhardt, J. eds.), Marcel Dekker, New York, pp. 3–32.
11. White, D. O. and Fenner, F. J. (1986) *Medical Virology*, 3rd ed., Academic, Orlando, FL, pp. 627–629.
12. Bowden, D. D., Moaven, L. D., and Locarini, S. A. (1996) New hepatitis viruses: are there enough letters in the alphabet? *Med. J. Australia* **164,** 87–89.

2

Hepatitis A Virus

Biology, Pathogenesis, Epidemiology, Clinical Description, and Diagnosis

Jack T. Stapleton

1. Introduction

Type A viral hepatitis, or "infectious" hepatitis, is almost certainly an ancient disease, with descriptions of contagious jaundice described as long ago as the fifth century B.C. (reviewed in ref. *188*). Although infectious causes of hepatitis other than hepatitis A virus (HAV) may have been responsible for some of these outbreaks, the description of many outbreaks of epidemic jaundice appear to have resulted from HAV infection. Recognition of infectious hepatitis as a distinct clinical entity did not begin to evolve until the first part of the twentieth century *(106,188)*. Infectious hepatitis was a major problem for military forces during World War II, prompting studies that demonstrated the existence of at least two distinct transmissible hepatitis agents, one responsible for short-incubation, "infectious" jaundice (subsequently renamed type A hepatitis) and the other causing long-incubation, "homologous serum" jaundice (type B hepatitis) *(62,98,99,113,114)*. These two agents were distinguished by differences in incubation periods, apparent modes of transmission, and lack of cross-protection in human challenge studies *(113)*. Several decades later, with the advent of specific tests for hepatitis A and B viruses, it became apparent that most cases of post-transfusion hepatitis could be attributed to neither of these viruses *(2)*. This led to the realization that there were other distinct types of viral hepatitis, termed "non-A, non-B" hepatitis. The most common etiologic agent of this entity was identified in 1989, and it has since been named hepatitis C *(16)*.

Hepatitis A virus was first identified in feces from humans in 1973 *(39)*, and in vitro replication of the virus in cell culture was first reported in

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

1979 *(127)*. These relatively recent developments have allowed a new level of investigation into the virology, pathogenesis, and epidemiology of hepatitis A, and have led to the development of inactivated and live-attenuated HAV vaccines *(7,35,41,80,101,150,181)*. The virus differs in many fundamental ways from hepatitis B virus (HBV), hepatitis C virus (HCV) and other viral causes of hepatitis. HAV is a small, nonenveloped, RNA-containing picornavirus *(1,22,25,108,112)*, whereas HBV is a larger, enveloped, DNA-containing hepadnavirus. HCV is an enveloped, RNA-containing virus that is related distantly to the flaviviruses *(16)*. Unlike HBV and HCV, persistent infection with HAV has not been clearly documented in humans, and HAV does not cause chronic hepatitis. Nonetheless, HAV is responsible for substantial human illness and among the hepatitis viruses has unparalleled potential for epidemic dissemination *(63,79,115,141)*.

2. Biology and Pathogenesis

2.1. Genetic Organization

The complete nucleotide sequence of the genomic RNA is known for several HAV strains *(19,22,75,106,112)*. The virus genome consists of single-stranded, positive-sense RNA, which is polyadenylated at the 3' end and covalently linked to a small protein (VPg) at the 5' end *(22,112,178)*. The RNA is approx 7.5 kb in length, sediments at 33S, and full-length complementary DNA and in vitro transcribed RNA is infectious *(21)*. Analysis of the nucleotide sequence reveals a long, continuous open reading frame beginning 735 bases from the 5' end. When this open reading frame is expressed in eukaryotic expression systems, the polyprotein undergoes cotranslational, protease-mediated cleavage into nonstructural polypeptides and structural proteins which assemble into virus particles (Fig. 1A) *(133,159,160,182)*.

2.2. Viral Proteins

The polyprotein produced by translation of the open reading frame undergoes a highly regulated cascade of proteolytic processing events to produce mature viral proteins in other picornaviral systems, and presumably this also occurs in HAV (reviewed in refs. *85,119*). These processing events can be divided into three categories. Primary cleavage separates the virus capsid precursor (P1) from the nascent chain, while secondary cleavages process the structural and nonstructural precursor proteins into individual viral proteins. Finally, cleavage of 1AB (VP0) to 1A (VP4) and 1B (VP2) probably occurs during virion assembly, but not all copies of VP0 found in virions are cleaved *(4,5,9,65,135)* (nomenclature adopted from ref. *138*).

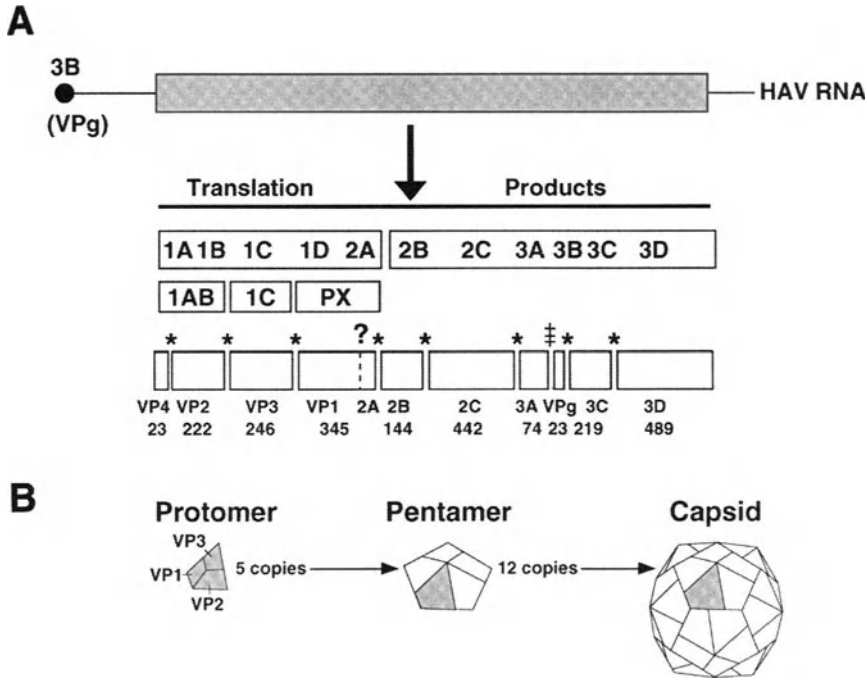


Fig. 1. **(A)** Hepatitis A virus RNA bound by 3B protein (VPg) is depicted on the top line, with its translation product and proteolytic processing end products noted below. The asterisk represents cleavage sites that have been identified by amino terminal amino acid sequencing. The 3A-3B (VPg) cleavage site has been indirectly identified *(178)*, and the exact carboxyl terminus of 1D (VP1) has not been conclusively identified. **(B)** One copy of each structural protein form the protomer (depicted as 1B, 1C, and 1D; however, 1AB and PX may substitute for 1B and 1D, respectively). Five protomers assemble into the pentamer, and 12 pentamers form the viral capsid. Thus, each capsid contains 60 copies of each structural protein.

Initial processing of the HAV polyprotein was postulated to occur between 1D (VP1) and 2A *(22)*; however, Anderson and Ross *(4)* described a novel HAV particle associated 40 kDa protein recognized by anti-1D serum that they called "PX." They speculated that PX may represent 1D2A and suggested that primary processing in HAV occurs between 2A and 2B as in aphtho- and cardioviruses. This protein (PX), along with one or more immunoreactive proteins with a molecular mass intermediate between 1D and PX, have subsequently been detected in HAV-infected cells by others *(8,15,173)*. With the exception of the 1A-1B cleavage, HAV 3C protease has been shown

to be responsible for all identified cleavage events *(67,142)* (Fig. 1A). How PX is processed into 1D remains unknown, and the original prediction for the location of the carboxy terminus of 1D *(22)* appears to be incorrect *(33)*, as the 1D protein found in HAV-infected cell cultures migrates approx 4 kDa faster than originally predicted.

The HAV nonstructural proteins have been characterized by evaluation of both cell-culture-derived HAV and recombinant proteins expressed in vitro. In other picornaviruses, the 2A region is remarkably variable in size and function *(119)*. In HAV, the region originally called 2A is actually part of 1D (VP1) and is present on protomers and pentamers *(4,159,183)* (Fig. 1A). In recombinant systems, empty capsids also contain 1D-2A (PX), although it is not clear if PX is present in nonrecombinant HAV empty capsids *(4,9)*. The 2A protein is predicted to be 45-amino-acids long and may be cleaved in several sites between the carboxy terminus of 1D and the start of 2B (Stapleton et al., unpublished data).

The function of the HAV 2B protein is unknown, although it has been implicated in morphogenesis *(66)*. 2B is approx 26 kDa *(53,142)*, and mutations in this protein are associated with adaptation to growth in cell culture *(36,37,54,75)*. HAV 2C protein appears to be larger than originally predicted (442 amino acids) *(67,102,143)* and has amino acid homologies with other picornaviral 2C proteins *(22)*. In poliovirus, 2C has helicase activities and is thought to be involved in RNA strand separation during replication *(125)*.

HAV 3A and 3B proteins are believed to be necessary for anchoring 2C protein to the endoplasmic reticulum and thus are required for RNA replication *(125)*. The small (23 amino acid) 3B protein also serves as the VPg protein, which is covalently attached via a phosphodiester bond to the 5' terminal uridylyl residue of the RNA *(69,178)*. In other picornaviral systems, 3B appears to serve as a primer for viral RNA synthesis, and removal of VPg appears to direct viral RNA to ribosomes early in the replication cycle *(137)*.

The HAV 3C region encodes a 219 amino acid serine-like, cysteine proteinase *(6,22,29,77,100)*. The active site nucleophile is located at position 172, and it is the only protease thus far identified in HAV polyprotein processing *(142,143)*. It autocatalytically processes itself from the polyprotein, and is active both in trans and in *cis (67)*. The structure of HAV 3C was the first picornaviral protease structure solved *(6)*.

HAV 3D encodes an RNA-dependent RNA polymerase *(22)*. This protein has the most homology with poliovirus of any of the HAV proteins (29%), and it contains an 18-amino-acid domain thought essential for GTP binding *(22,179)*.

2.3. Morphogenesis

Virus particles (HAV virions) have been identified in liver tissue, bile, feces, serum, and occasionally saliva of naturally or experimentally infected humans and nonhuman primates *(18,49,57,83,87,91,96,128,167)*. The virion is a spherical, nonenveloped particle with icosahedral symmetry and an approximate diameter of 27 nm *(39,182)*. Infectious particles band isopycnically in cesium chloride at 1.32 g/mL to 1.34 g/mL have a sedimentation coefficient of 156S and contain multiple copies of at least three structural polypeptides (VP1–VP3) that range in size from 33–27 kDa *(25,94)*. A fourth virion polypeptide (VP4), consisting of either 17 or 23 amino acids, is predicted by the sequence of the viral RNA but has not been identified directly in virions *(4,9,135)*. This protein is formed by the cleavage of VP0 (1AB) into VP2 (1B) and VP4 (1A) (Fig. 1A). Five copies of the three structural proteins interact to form a precursor particle termed the "pentamer," and 12 pentamers assemble into empty capsid particles, which become infectious when viral RNA is packaged (Fig. 1B). Thus, each empty capsid or infectious hepatitis virion contains 60 copies of the three major structural proteins. Empty capsids and infectious virus particles appear to be identical in their antigenic and immunogenic properties *(94,146,159)*.

2.4. Classification

HAV was originally classified within the picornavirus family as a member of the enterovirus genus (enterovirus 72) on the basis of its primary mode of transmission (fecal-oral) and its biophysical and biochemical characteristics (acid stability, sedimentation velocity, buoyant density, size, and organization of the RNA genome and structural proteins) *(56,69)*. These characteristics are shared with several members of this genus, including poliovirus, coxsackievirus, and echovirus. However, a comparison between HAV nucleotide and amino acid sequences and other picornaviruses reveals much less homology than is shared among other enteroviruses (reviewed in ref. *11*). HAV has only one serotype, is generally not cytopathic in cell culture, and does not react with an enteroviral-specific monoclonal antibody (reviewed in ref. *11*). HAV also has extraordinary resistance to environmental stresses, such as heating and drying, which makes it unique among the picornaviruses *(105,120,147,149)*. In addition, the secondary structure of virion RNA within the 5' noncoding region and several other features of the organization of the genome that have become clear within the past few years, distinguish HAV from the enteroviruses (reviewed in refs. *11,169*). Consequently, HAV was recently reclassified as the type species of a new genus, hepatovirus, within the family Picornaviridae *(110)*.

2.5. *Growth in Cell Culture*

A high level of resistance to unfavorable environmental conditions is probably a major factor in the maintenance and spread of HAV within populations. Infectivity is reduced only fivefold after 16 wk incubation at 4°C and only 100-fold when HAV is held at room temperature over a 4-wk period *(105,120,147,149)*. However, inactivation of HAV may be accomplished by chlorination or sufficient heat *(120,121)*. HAV suspended in a buffered saline solution becomes noninfectious within 4 min at 70°C and virtually instantaneously at 85°C *(120)*. When HAV is suspended in milk and pasteurized, 0.1% of infectivity remains after 30 min at 62.8°C and 1% of infectivity is present after 15 s at 71.6°C *(120)*. Nonetheless, a recent study demonstrated that HAV suspended in a commercial factor VIII stabilizer and heat treated at 60°C for 10 h underwent less than a 5 $\log_{10}$ reduction in virus infectivity *(64,89)*.

HAV has been propagated in several types of primate cells. Infection of cell cultures is generally characterized by viral persistence with no measurable inhibition of host cell macromolecular synthesis *(20,27,40,127)*. Therefore, HAV is usually not cytopathic in cell culture, although cytopathic variants have been selected for in vitro *(3,26)*. Replication of HAV generally has been detected by immunologic methods, including immunofluorescent antibody staining of infected cell cultures, solid-phase immunoassay, and an indirect plaque assay based on autoradiographic visualization of foci of viral replication *(42,46,92,103)*. cDNA-RNA hybridization and RT-PCR methods are sensitive means of detecting the virus in culture and feces *(74,76,145,170,171)*. Only moderate titers of virus (10^7–10^8 infectious U/mL) are usually obtained in infected cell cultures (*27*, reviewed in ref. *158*). These relatively low yields of virus continue to pose a major problem for vaccine development. Adaptation to growth in cell culture has been shown to result in attenuation of some strains of the virus *(19,20,28,37,54,168)*. Isolation of wild-type virus from clinical specimens is often unsuccessful, and requires weeks to months and occasionally several blind passages. Consequently, culture is not a useful diagnostic tool.

Immunologic methods, including radioimmunoassay, virus cross-neutralization techniques, and sophisticated monoclonal antibody analysis have failed to discern significant antigenic differences between human HAV strains recovered from widely separated regions of the world *(90,153,154)*. However, strain differences may be demonstrated by comparison of nucleotide sequences obtained from PCR–amplified cDNA *(131)*. Studies carried out with neutralizing monoclonal antibodies and experimentally produced HAV mutants that resist neutralization by these antibodies have demon-

strated an immunodominant neutralization antigenic site on the surface of the virus *(157)*. In addition, there appears to be at least one other functionally independent neutralization antigenic site on virions *(124)*. The immunodominant antigenic site is highly conserved, even among human HAV strains demonstrating substantial genetic divergence *(90,93,124,157)*. Significant antigenic variation has been demonstrated recently among several simian HAV strains recovered from naturally infected nonhuman primates, but it is likely that simian and human HAV strains elicit cross-protective immunity *(90,154)*. Although data are scant, these simian strains do not appear capable of causing disease in humans.

Amino acid residues within both the VP3 and VP1 capsid proteins are involved in the structure of the immunodominant antigenic site *(111,123,124)*, and disruption of virus by detergents largely destroys antigenicity *(159,182)*. Thus, tertiary structure or conformational arrangements between different surface polypeptides are critical to antigenicity *(159,182)*. Consequently, recombinant HAV structural proteins expressed individually (VP1, VP2, VP3) have not elicited high levels of neutralizing antibody *(48,78,118,126,135,184)*, limiting the feasibility of a recombinant HAV vaccine. When the entire HAV polyprotein is expressed in eukaryotic systems (baculovirus—refs. *107,133,160*, and vaccinia virus—refs. *159,182*), processing of the polyprotein proceeds accurately, resulting in pentameric (14S) and empty capsid (70S) particles, which are immunogenic in mice *(159)*.

2.6. HAV Receptor

Radiolabeled HAV is able to bind many cell lines of mammalian origin and requires calcium for attachment *(32,155,187)*. The binding involves a specific receptor, since binding is saturable, and can be blocked by unlabeled HAV but not unlabeled poliovirus *(155)*. Neutralization-escape mutants bind cells approximately as well as neutralization-sensitive HAV strains, suggesting that the immunodominant, neutralization antigenic site on HAV is not directly involved in attachment to cells *(155)*. Recently, Kaplan and colleagues identified a mucin-like protein on monkey kidney cells that appears to specifically bind to HAV *(81)*. Following transfection of this protein into nonsusceptible murine Ltk-cells, HAV attachment and productive infection was demonstrated *(81)*.

2.7. Infection and Host Immune Response

HAV is predominantly transmitted by the fecal-oral route. Following exposure to HAV, there is an incubation period of 15–50 d (average 28), the duration of which may be inversely related to the titer of the HAV inoculum (reviewed in refs. *11,49,57,88,136*). The virus traverses the intestine and

reaches the liver by an undetermined mechanism. Replication in the gastrointestinal tract is suspected *(18)*; however, the data to support this hypothesis are not entirely conclusive. Once HAV reaches the liver, it replicates in hepatocytes. Virus is released from hepatocytes into the bloodstream (viremia) at the same time it is present in the bile and shed in the feces. Fecal shedding and viremia are maximal just prior to or shortly after the onset of liver function abnormalities and terminate at the time that humoral immunity to HAV is detected *(49,57)* (Fig. 2). HAV infection is limited to man and several nonhuman primate species (reviewed in ref. *88*).

By the time that clinical symptoms of hepatitis develop, serum antibody to HAV has almost invariably risen to detectable levels. The initial antibody response consists of IgM, IgG, and IgA antibodies, with 7S (IgA or IgG) antibodies demonstrable as early as 2 d after the onset of illness (reviewed in refs. *11,49,57,88,95,136,158,169*) (Fig. 2A). The HAV antibodies detected by commercial immunoassays recognize only particulate forms of the viral proteins (pentamers, empty capsids, and infectious virus) and do not recognize isolated structural proteins or nonstructural proteins *(159,182)*. IgM anti-HAV typically disappears within 3 mo of symptoms; however, sensitive immunoassays may occasionally detect IgM for up to 1 yr following acute hepatitis *(151,162)*. Viral isolation and detection of viral antigen or RNA is complicated, expensive, and not standardized; consequently detection of virus is not useful for diagnosis. Fortunately, IgM anti-HAV is easily detectable by immunoassay. Therefore, detection of IgM anti-HAV is the gold standard of specific diagnosis of HAV. IgG anti-HAV is the major class of antibody found in convalescence, and current evidence indicates that IgG is the primary defense against reinfection (reviewed in ref. *183*).

Currently available diagnostic immunoassays cannot distinguish between persons who acquired their anti-HAV by natural infection or by immunization with formalin-inactivated vaccine. Both a radioimmunoprecipitation method and an ELISA method for detecting antibody that binds HAV nonstructural proteins have been successfully employed following infection of chimpanzees and following natural infection of humans *(132,161)*. Since the inactivated vaccine preparation contains only infectious virus particles and empty capsids (structural proteins), antibody to nonstructural proteins will not be elicited by vaccination *(148)*. Consequently, the ELISA and radioimmunoprecipitation assays may serve to distinguish between natural infection and immunization *(132,161)*. It has not yet been determined if antibody to nonstructural proteins is elicited by present versions of live-attenuated vaccines.

It is clear that antibody to HAV is critical and sufficient by itself to prevent reinfection *(183)*; however, several lines of evidence indicate a

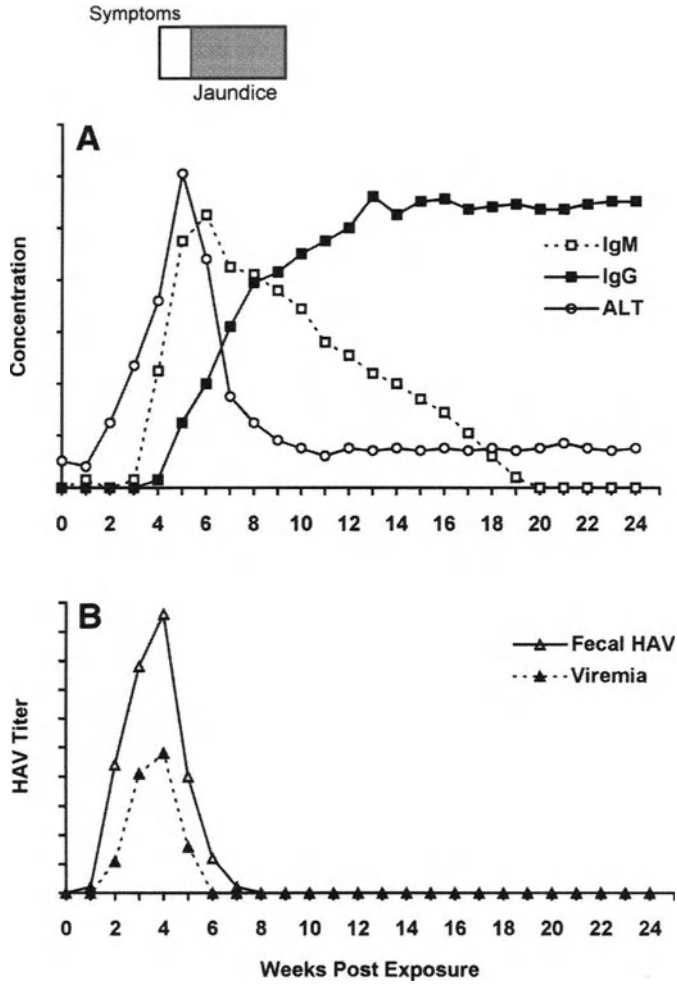


Fig. 2. **(A)** Model of clinical and serologic events occurring during hepatitis A virus (HAV) infection. Pre-icteric symptoms develop prior to jaundice. **(B)** Exposure to HAV at time 0 is followed by viremia and fecal shedding between 1 and 3 wk.

potential role for cellular immunity in the clearance of HAV from hepatocytes and in HAV pathogenesis *(43,174,175)*. HAV persistently infects cells in cell culture and does not cause a direct cytopathic effect *(57)*, and peak viral shedding in feces and peak viremia in experimental HAV infection occurs before the development of symptoms *(57,91)*. Anti-HAV is almost always present at the time of symptoms, and in the unusual cases where it is

not, interferon gamma is detectable prior to seroconversion *(186)*. All of these data indirectly suggest that cell-mediated immune destruction of HAV-infected hepatocytes leads to hepatocyte destruction (hepatitis).

Among the potential mediators of cell-mediated immunity, it appears that antibody-dependent cellular cytotoxicity does not occur in HAV infection *(47)*. Conflicting reports about the presence *(86)* or absence *(45)* of natural killer cell responses to HAV-infected cells exist. Cell-mediated cytotoxicity has been demonstrated to be important in the immune response to hepatitis A infection *(47)*. Virus-specific cytotoxic T lymphocytes (CTL) develop in the course of HAV infection *(174,175)*. Clonal analysis of the T lymphocytes in the liver during acute HAV infection identified antigen-specific CD8+ T lymphocytes from liver biopsies obtained during acute HAV infection, implicating these cells as responsible for the destruction of infected hepatocytes *(43)*. Nearly 50% of liver-derived T cell clones displayed HAV specific cytotoxicity compared with <1% of peripheral blood lymphocyte clones, further supporting the role of CTL in the pathogenesis of acute hepatitis A. Cytolytic T-cell determinants have been demonstrated on both HAV nonstructural proteins *(82)* and on HAV structural proteins *(184)*. Since the formalin-inactivated vaccine contains the structural proteins present in these antigenic regions, vaccination could potentially elicit these CTL responses.

3. HAV Epidemiology and Clinical Course

3.1. Epidemiology

Hepatitis A virus accounts for an estimated 143,000 cases of acute hepatitis in the United States each year, resulting in approx 80 deaths and an estimated $200 million in medical care and lost productivity *(59)*. Worldwide, the incidence of HAV infection exceeds 1.4 million cases with a cost of $1.5–3 billion annually *(59)*. Because HAV is transmitted by the fecal oral route, cases in developed countries usually are identified in individuals who have had close contact with an index case or who have traveled to an underdeveloped area where the virus is endemic *(59,144)*.

Preschool day-care centers are important foci of HAV transmission *(60,61,122,130)*. In a study conducted within the United States, 11% of all reported cases of hepatitis A had contact with children attending a preschool day-care center *(60)*. In a second study, approximately one third of all type A or type-unspecified hepatitis cases reported in one city could be related to day-care centers *(61)*. Factors favoring day-care-center-associated transmission of HAV include enrollment of nontoilet-trained children and large enrollment within a center. The infection is usually asymptomatic in day-

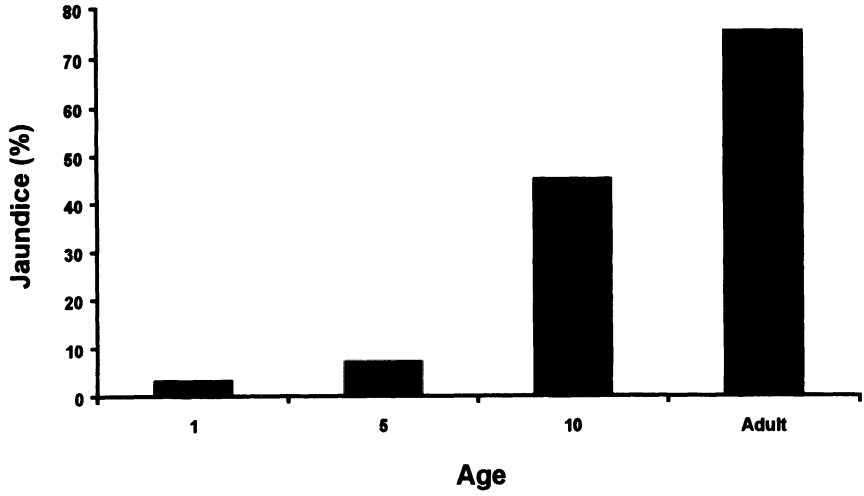


Fig. 3. Severity of illness correlates with degree of jaundice. There is an inverse relationship between jaundice and the age of hepatitis A virus infection.

care attendees under the age of 2 yr, which tends to mask the source of the outbreak (Fig. 3). However, such asymptomatically infected children may be primarily responsible for transmission *(130,139)*. In contrast, the majority of infections in parents, older siblings, and day-care staff are accompanied by jaundice or other clinical evidence of hepatitis. The aggressive use of immune serum globulin (ISG) for prophylaxis in children and employees of day-care centers following the identification of an outbreak of hepatitis was shown to substantially reduce the transmission of HAV *(60,61)*. Furthermore, such aggressive intervention apparently lowered rates of hepatitis A in the general population *(61)*. Thus, many cases of hepatitis A in the community may represent tertiary transmission from day-care-center-related cases.

Although spectacular epidemics of hepatitis A have occasionally been traced to a food handler or another common source, these outbreaks are relatively unusual and account for only a small proportion of cases of hepatitis A *(44)*. Secondary cases in common-source outbreaks usually occur one incubation period after the index case but are generally restricted to household contacts *(44,59)*. A history of raw shellfish consumption may be present in some patients with acute hepatitis A *(59)*. Bivalve shellfish appear to concentrate HAV from sewage-polluted water; the shellfish do not serve as a host for viral propagation *(59)*. Prolonged survival of the virus in living shellfish has been demonstrated *(38,185)*; thus shellfish must be thoroughly

cooked to inactivate HAV. Non-bivalve shellfish (shrimp, lobster) do not impart the same risk. Sexual transmission of HAV has been described, and an increased frequency of hepatitis A has been observed in homosexual men attending a sexually transmitted disease clinic *(23,84)*.

In recent years, several outbreaks of hepatitis A have been recognized among parenteral drug abusers in the United States and Western Europe *(12,70,116,180)*. It is not known whether these outbreaks represent transmission related to poor personal hygiene or needle-borne transmission of virus present in blood, but the latter is likely to contribute substantially to spread of the infection among these individuals *(12)*. Transmission of HAV by blood transfusion is well documented but occurs rarely *(69)*. The donor must be in the viremic, prodromal phase of infection at the time of blood donation. Surveys of multiple transfused children, posttransfusion hepatitis patients, and hemodialysis patients and staff members do not show a greater than expected frequency of anti-HAV antibody, arguing against transfusion as a major source of transmission (reviewed in ref. *69*). However, evidence that emerged during 1992 suggests that treatment with highly purified, solvent-detergent-inactivated factor VIII concentrates may transmit the infection relatively frequently *(89,117)*. Nosocomial transmission of HAV is rare but is well documented *(30,34)*.

The clinical course of hepatitis A can range from a mild, anicteric illness to severe, prolonged, icteric hepatitis (reviewed in refs. *49,57,165*). Inapparent or anicteric hepatitis occurs in over 90% of infected children under the age of 5, compared with as many as 80% of adults (Fig. 3). In devoping countries, HAV is endemic, and virtually all individuals are infected early in life. However, as developing regions of the world improve their overall level of sanitation, the acquisition of HAV occurs at an older age, when symptomatic infection is more likely (Fig. 4). This creates a paradoxical situation, where the overall rate of HAV infection decreases at the same time that the incidence of clinically apparent disease is increasing.

Several patterns of age-specific prevalence of HAV infection have been identified in different parts of the world (Fig. 4). In developing countries, virtually all individuals are infected with HAV early in childhood and remain anti-HAV antibody positive throughout life *(177)* (Fig. 4, pattern A1). The percent of people who are antibody positive may decline slightly in older adults because of the level of antibody falling below the limits of detection *(163)*. Because of the age-specific incidence of jaundice (Fig. 3), clinical hepatitis A is unusual in these settings, and outbreaks do not occur. In endemic countries with a lower incidence of infection (Mediterranean area), the vast majority of individuals ultimately are infected with HAV (Fig. 4, pattern A2). The rate of saturation is slower than in pattern A1, and thus

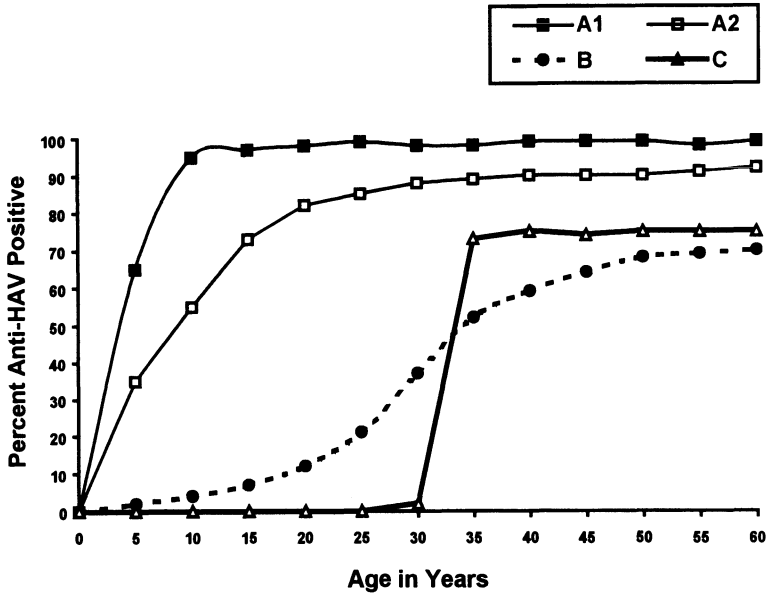


Fig. 4. Age-specific patterns of hepatitis A virus antibody in various populations (*see* text).

more individuals at risk of clinical disease are susceptible to infection until adulthood *(140,177)*.

In highly developed countries with steady improvement of sanitation, over a period of several decades (United States, Western Europe), there have been both a reduction in the overall rate of infection and a delay in the age of acquisition of HAV infection *(59,144)* (Fig. 4, pattern B). Seroepidemiologic studies carried out within the United States indicate that the risk of prior infection is related to age and socioeconomic status *(144)*. In studies carried out over a decade ago, 10–20% of Americans had antibody by age 20 and 50% by age 50 *(163,164)*. Studies in Europe, Australia, and Thailand, where the prevalence of anti-HAV is similarly age-related, indicate that the age-related nature of this seroprevalence curve reflects a cohort effect, with a declining overall incidence of infection with HAV over a prolonged period of time *(58,72)*. Thus, the higher prevalence of antibody among older persons is caused by a greater endemicity of HAV during the early years of life.

A third type of epidemiologic pattern has been identified in closed communities, when HAV is introduced into the community *(104,152)*. Since most of the population is susceptible, there is a high rate of infection, and the virus is unable to maintain itself. Consequently, HAV infection disappears

until it is reintroduced into the community. When this happens, the outbreak is confined to children born after the original outbreak and adults who did not reside in the community during the prior outbreak (Fig. 4, pattern C). The finding of this pattern provides evidence that chronic infections with persistent or intermittent virus shedding do not occur *(104,152)*. While these generalizable patterns are helpful in understanding HAV epidemiology, it is important to remember that all three patterns may exist in large countries, particularly within different groups of the population.

3.2. Clinical Patterns

The incubation period of hepatitis A (the time between exposure to HAV and the onset of clinical symptoms or biochemical evidence of liver disease) may range from 15 to 50 d with a mean of 28 d *(57)* and appears to be inversely related to the titer of the inoculum of virus *(57,68)*. A short prodomal or preicteric phase, varying from several days to more than a week, often precedes the onset of jaundice. This phase may be characterized by fever, fatigue, malaise, myalgia, anorexia, nausea and vomiting. Right-upper-quadrant pain may occur secondary to hepatomegaly. Splenomegaly is present in 10–15% of patients. Diarrhea is uncommon and may be caused by an enteric bacterial pathogen acquired from the same fecal-contaminated source as the HAV *(45,57,165)*. The icteric phase of HAV infection may begin with the appearance of dark, golden brown urine due to bilirubinemia, followed one to several days later by pale stools and yellowish discoloration of the mucous membranes, conjunctivae, sclera, and skin. Palmar erythema and spider angiomata may be observed *(57,68,165)*. The duration of hepatitis A is variable, but clinical and biochemical indicators of hepatitis usually resolve within 4–8 wk of the onset of illness *(45)*. Unfortunately, based on clinical findings, hepatitis A cannot be distinguished from the other forms of viral hepatitis.

Hepatitis A does not cause chronic liver disease, although several complications can accompany HAV infection. A cholestastic form of hepatitis characterized by fever, pruritis, prolonged jaundice, and weight loss has been described, and symptoms may last for more than 6 mo *(52)*. Skin rashes including urticaria *(31)*, cryoglobulinemia *(71)*, meningoencephalitis *(10)*, Guillain-Barre syndrome, renal failure *(13)*, hematologic *(55)*, and cardiovascular complications *(51)* have all been reported to accompany HAV infection. Hepatitis A may relapse, and up to 10% of hospitalized patients in Argentina suffered a second episode *(166)*. Increasingly, this biphasic course of hepatitis A is recognized *(14,17,24,50,73,106,151,176)*. Fulminant hepatitis A is uncommon, but is estimated to account for 5–20% of all cases of fulminant viral hepatitis *(97)*.

Fulminant hepatitis is characterized by increasing severity of jaundice, deterioration in liver function, drowsiness, fetor hepaticus, and eventually encephalopathy and coma *(129)*. Hepatitis A has an overall fatality rate of approx 0.15 deaths per 1000 cases *(57)*; however, the mortality is age related. For example, a recent study reported 381 deaths out of 115,000 cases, for a case fatality rate of 3.3 per 1000 *(59)*. In this outbreak, more than 70% of the deaths occurred in adults over the age of 49, and the calculated case fatality rate within this age group was 27 per 1000, or 2.7% *(59)*.

Chemistry laboratory findings in hepatitis A are similar to those found in other types of viral hepatitis and unfortunately cannot be used to distinguish one form of hepatitis from another. The levels of serum aspartate (AST) and alanine aminotransferase (ALT) rise abruptly, with peak levels ranging from less than 300 to more than 3000 IU/L. In most clinical cases, the AST and ALT values are greater than 500 IU/L *(45,57)*. Serum aminotransferase levels do not correlate well with the degree of liver cell damage. Serum total bilirubin levels typically peak between 5 and 15 mg/100 mL, equally divided between the conjugated and unconjugated fractions *(57)*. Liver enzymes typically normalize within 4–6 mo, and are invariably normal by 12 mo. Thrombocytopenia, mild leukopenia and anemia, aplastic anemia, hypoalbuminemia and prolonged prothrombin and partial thromboplastin times have all been seen in association with hepatitis A but are rare *(55)*. Thus, serology is necessary to confirm a diagnosis of HAV infection.

3.3. Prevention and Management

Three forms of prevention for hepatitis A are available. First, hygienic measures including the sanitary disposal of human waste and the maintenance of adequate standards for the purity of drinking water, are critical for preventing hepatitis A. Infections from contaminated drinking water or contaminated shellfish may be prevented by chemical or heat inactivation of the virus *(57,74)*. Second, passive immunization with ISG can be used to protect individuals after known exposure to HAV or in those traveling to high-risk regions of the world *(183)*. Passive prophylaxis with ISG within the first 2 wk following exposure to HAV may reduce icteric illness by more than 80% but does not necessarily prevent infection *(183)*. The fact that pooled human IgG is effective indicates that antibody to HAV is sufficient for protection against hepatitis A, and cellular immunity against the virus is not required for protection *(154)*.

A growing concern is that commercial lots of ISG contain variable concentrations of antibody to HAV *(156,183)*. In developed countries of the world, the proportion of blood donors who are immune to HAV has

decreased to the point where ISG effectiveness may decline *(153)*. For this reason, some countries (e.g., Switzerland) import batches of ISG from countries with a higher seroprevalence for use as hepatitis A prophylaxis. Finally, active immunization against HAV is now possible and will be discussed in a subsequent chapter.

Patients with hepatitis A generally do not require hospital admission, unless complications arise such as fulminant hepatitis; coagulopathy; encephalopathy; intractable nausea, vomiting, or abdominal pain; or when there is uncertainty concerning the diagnosis *(57,165)*. Patients requiring hospitalization traditionally have been placed in isolation using both enteric and blood precautions. In general, the viremic stage of HAV is not extensive enough to pose a significant problem of disease transmission through direct contact with blood. Only strict enteric precautions, therefore, are generally necessary for hepatitis A. The situations where nosocomial transmission is most likely involves neonatal or infant HAV infection, where the duration of viremia and fecal shedding may be prolonged *(134)*. This is particularly true if there is close contact between hospital staff and the infected individual or the individual has open wounds such as burns *(30)*.

The probability of transmission of HAV within the hospital is low, but precautions must be taken when staff members come in direct contact with an infected patient's feces. The extent of these precautions should include wearing gloves when handling bedpans or fecal material or when using instruments that come into contact with the intestinal tract. Strict handwashing procedures should be observed, and gowns should be worn if contact with feces is anticipated. A private room is not needed unless the patient is fecally incontinent.

Currently, the management of HAV infection is supportive and symptomatic care only. There is no indication that bed rest or limiting physical activity affects the outcome of HAV infection. Dietary restrictions (including modest amounts of alcohol use) similarly do not appear to influence outcome *(172)*; however, recommendation of abstinence from alcohol is conventional since alcohol use has been associated with relapse of jaundice *(109)*. Antibody levels in immune globulin recipients are several orders of magnitude below those found in patients with acute hepatitis A *(156,183)*, thus the administration of ISG is neither rational nor justified.

In extreme cases of fulminant hepatitis A, liver transplantation has been successfully performed. However, since 60% of patients with fulminant hepatitis A (including patients with grade 4 hepatic encephalopathy) recover without transplantation, selecting patients for transplantation is difficult. It is important to remember that except for the rare case of fulminant hepatitis A, patients with HAV illness will resolve their infection without sequelae.

References

1. Aach, R. D., Szmuness, W., Mosely, J. W., Hollinger, F. B., Kahn, R. A., Stevens, C. E., and Edwards, V. M. (1981) Serum alanine aminotransferase of donors in relation to the risk of non-A, non-B hepatitis in recipients: the transfusion-transmitted viruses study. *N. Engl. J. Med.* **304,** 989–994.
2. Alter, H. J., Purcell, R. H., Feinstone, S. M., Holland, P. V., and Morrow, A. G. (1978) Non-A, non-B hepatitis: a review and interim report of an ongoing study, in *Viral Hepatitis: A Contemporary Assessment of Etiology, Epidemiology, Pathogenesis, and Prevention* (Vyas, G. N., Cohen, S. H., and Schmid, R., eds.), Franklin Inst. Press, Philadelphia, PA, pp. 359–369.
3. Anderson, D. A. (1987) Cytopathology, plaque assay, and heat inactivation of hepatitis A virus strain HM-175. *J. Med. Virol.* **22,** 35–44.
4. Anderson, D. A. and Ross, B. C. (1990) Morphogenesis of hepatitis A virus: isolation and characterization of subviral particles. *J. Virol.* **64,** 5284–5289.
5. Arnold, E., Luo, M., Vriend, G., Rossmann, M. G., Palmenberg, A. C., Parks, G. D., Nicklin, M. J. H., and Wimmer, E. (1987) Implications of the picornavirus capsid structure for polyprotein processing. *Proc. Natl. Acad. Sci. USA* **84,** 21–25.
6. Bergmann, E. M., Mosmann, S. C., Chernaia, M. M., Malcolm, B. A., and James, M. N. (1997) The refined crystal structure of the 3C gene product from hepatitis A virus: specific proteinase activity and RNA recognition. *J. Virol.* **71,** 2436–2448.
7. Binn, L. N., Bancroft, W. H., Lemon, S. M., Marchwicki, R. H., LeDuc, J. W., Trahan, C. J., Staley, E. C., and Keenan, C. M. (1986) Preparation of a prototype hepatitis A virus vaccine from infected cell culture. *J. Infect. Dis.* **153,** 749–756.
8. Bishop, N. E. and Anderson, D. A. (1991) Morphogenesis of Hepatitis A virus. Europic 91. *Seventh Meeting European Study Group Picornavirus*, G10.
9. Borovec, S. V. and Anderson, D. A. (1993) Synthesis and assembly of hepatitis A virus-specific proteins in BS-C-1 cells. *J. Virol.* **67,** 3095–3102.
10. Bromberg, K., Newhall, D. N., and Peter, G. (1982) Hepatitis A and meningoencephalitis. *JAMA* **247,** 815.
11. Cederna, J. B. and Stapleton, J. T. (1994) Hepatitis A, in *Manual of Clinical Microbiology* (Yolken, R. H., ed.), *Am. Soc. Microbiol.* Washington.
12. Centers for Disease Control (1988) Hepatitis A among drug abusers. *MMWR* **37,** 297–305.
13. Chio, F., Jr. and Bakir, A. A. (1992) Acute renal failure in hepatitis A. *Int. J. Artif. Organs* **15,** 413–416.
14. Chiriaco, P., Guadalupi, C., Armigliato, M., Bortolotti, F., and Realdi, G. (1986) Polyphasic course of hepatitis type A in children. *J. Infect. Dis.* **153,** 378–379.
15. Cho, M. W. and Ehrenfeld, E. (1991) Rapid completion of the replication cycle of Hepatitis A virus subsequent to reversal of guanidine inhibition. *Virology* **180,** 770–780.
16. Choo, Q. L., Kuo, G., Weiner, A., Overby, L., Bradley, D. W., and Houghton, M. (1989) Isolation of a cDNA derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244,** 359–362.
17. Cobden, I. and James, O. F. W. (1986) A biphasic illness associated with acute hepatitis A virus infection. *J. Hepatol.* **2,** 19–23.
18. Cohen, J. I., Feinstone, S., and Purcell, R. H. (1989) Hepatitis A virus infection in a chimpanzee: duration of viremia and detection of virus in saliva and throat swabs. *J. Infect. Dis.* **160,** 887–890.

19. Cohen, J. I., Rosenblum, B., Ticehurst, J. R., Daemer, R. J., Feinstone, S., and Purcell, R. H. (1987) Complete nucleotide sequence of an attenuated hepatitis A virus: comparison with wild-type virus. *Proc. Natl. Acad. Sci. USA* **84,** 2497–2501.
20. Cohen, J. I., Rosenblum, R., Daemer, R. J., Ticehurst, J. R., Feinstone, S. M., and Purcell, R. H. (1988) Attenuation of hepatitis A virus: molecular analysis, in *Viral Hepatitis and Liver Disease* (Zuckerman, A. J., ed.), Alan R. Liss, New York, pp. 43–47.
21. Cohen, J. I., Ticehurst, J. R., Feinstone, S. M., Rosenblum, B., and Purcell, R. H. (1987) Hepatitis A virus cDNA and its RNA transcripts are infectious in cell culture. *J. Virol.* **61,** 3035–3039.
22. Cohen, J. I., Ticehurst, J. R., Purcell, R. H., Buckler-White, A., and Baroudy, B. M. (1987) Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. *J. Virol.* **61,** 50–59.
23. Corey, L. and Holmes, K. K. (1980) Sexual transmission of hepatitis A in homosexual men. *N. Engl. J. Med.* **302,** 435–446.
24. Cornu, C., Lamy, M. E., Geubel, A., and Galanti, L. (1984) Persistence of immunoglobulin M antibody to hepatitis A virus and relapse of hepatitis A infection. *Eur. J. Clin. Microbiol.* **3,** 45–46.
25. Coulepis, A. G., Locarnini, S. A., Westaway, E. G., Tannock, G. A., and Gust, I. D. (1982) Biophysical and biochemical characterization of hepatitis A virus. *Intervirology* **18,** 107–127.
26. Cromeans, T., Sobsey, M. D., and Fields, H. A. (1987) Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. *J. Med. Virol.* **22,** 45–56.
27. Daemer, R. J., Feinstone, S. M., Gust, I. D., and Purcell, R. H. (1981) Propagation of human hepatitis A virus in African green monkey kidney cell culture: primary isolation and serial passage. *Infect. Immun.* **32,** 388–393.
28. Day, S. P., Murphy, P., Brown, E. A., and Lemon, S. M. (1992) Mutations within the 5' nontranslated region of hepatitis A virus RNA which enhance replication in BS-C-1 cells. *J. Virol.* **66,** 6533–6540.
29. Dinakarpandian, D., Shenoy, B., Pusztai-Carey, M., Malcolm, B. A., and Carey, P. R. (1997) Active site properties of the 3C proteinase from hepatitis A virus (a hybrid cysteine/serine protease) probed by Raman spectroscopy. *Biochemistry* **36,** 4943–4948.
30. Doebbeling, B. N., Li, N., and Wenzel, R. P. (1993) An outbreak of hepatitis A among health care workers: risk factors for transmission. *Amer. J. Public Health* **83,** 1679–1684.
31. Dollberg, S., Berkun, Y., and Gross-Kieselstein, E. (1991) Urticaria in patients with hepatitis A virus infection. *Pediatr. Infect. Dis. J.* **10,** 702–703.
32. Dotzauer, A., Feinstone, S. M., and Kaplan, G. (1994) Susceptibility of nonprimate cell lines to HAV infection. *J. Virol.* **68,** 6064–6068.
33. Dotzauer, A., Vallbracht, A., and Keil, G. M. (1995) The proposed gene for VP1 of hepatitis A virus encodes for a larger protein than that observed in HAV-infected cells and virions. *Virology* **213,** 671–675.
34. Edgar, W. M. and Campbell, A. D. (1985) Nosocomial infection with hepatitis A. *J. Infect.* **10,** 43–47.
35. Ellerbeck, E., Lewis, J., Midthun, K., Gershman, K., Nalin, D., McCaughtry, H., Provost, P., and Caladra, G. (1990) Safety and immunogenicity of an inactivated

hepatitis A virus vaccine, *1990 Intern. Symposium Viral Hepatitis Liver Dis. Abstr.* **43,** 50.

36. Emerson, S. U., Huang, Y. K., and Purcell, R. H. (1993) 2B and 2C mutations are essential but mutations throughout the genome of HAV contribute to adaptation to cell culture. *Virology* **194,** 475–480.
37. Emerson, S. U., Huang, Y. K., McRill, C., Lewis, M., Shapiro, M., London, W. T., and Purcell, R. H. (1992) Molecular basis of virulence and growth of hepatitis A virus in cell culture. *Vaccine* **10(Suppl. 1),** S36–S39.
38. Enriquez, R., Frösner, G. G., Hochstein-Mintzel, V., Riedemann, S., and Reinhardt, G. (1992) Accumulation and persistence of hepatitis A virus in mussels. *J. Med. Virol.* **37,** 174–179.
39. Feinstone, S. M., Kapikian, A. Z., and Purcell, R. H. (1973) Hepatitis A: detection by immune electron microscopy of a virus like antigen associated with acute illness. *Science* **182,** 1026–1028.
40. Flehmig, B. (1981) Hepatitis A virus in cell culture. II. Growth characteristics of hepatitis A virus in Frhk-4/R cells. *Med. Microbiol. Immunol.* **170,** 73–81.
41. Flehmig, B., Heinricy, U., and Pfisterer, M. (1989) Immunogenicity of a killed hepatitis A vaccine in seronegative volunteers. *Lancet* **1,** 1039–1041.
42. Flehmig, B., Ranke, M., Berthold, T., and Gerth, H. J. (1979) A solid phase radioimmunoassay for detection of IgM antibodies to hepatitis A virus. *J. Infect. Dis.* **140,** 169–175.
43. Fleischer, B., Fleischer, S., Maier, K., Wiedmann, K. H., and Vallbracht, A. (1990) Clonal analysis of infiltrating T lymphocytes in liver tissue in viral hepatitis A. *Immunology* **69,** 14–19.
44. Francis, D. P., Hadler, S. C., Prendergast, T. J., Peterson, E., Ginsberg, M. M., Lookabaugh, C., Holmes, J. R., and Maynard, J. E. (1984) Occurrence of hepatitis A, B., and non-A/non-B in the United States. CDC Sentinel County Hepatitis Study 1. *Amer. J. Med.* **76,** 69–74.
45. Friedman, L. S. and Dienstag, J. L. (1984) The disease and its pathogenesis, in *Hepatitis A* (Gerety, R. J., ed.), Academic, London, pp. 55–79.
46. Frosner, G. G., Overby, L. R., Flehmig, B., Gerth, H. J., Haase, H., Decker, R. H., Ling, C. M., Zuckerman, A. J., and Frosner, H. R. (1977) Seroepidemiological investigation of patients and family contacts in an epidemic of hepatitis A. *J. Med. Virol.* **1,** 163–173.
47. Gabriel, P., Vallbracht, A., and Flehmig, B. (1986) Lack of complement-dependent cytolytic antibodies in hepatitis A virus infection. *J. Med. Virol.* **20,** 23–31.
48. Gauss-Muller, V., Zhou, M. Q., von der Helm, K., and Deinhardt, F. (1990) Recombinant proteins VP1 and VP3 of hepatitis A virus prime for neutralizing response. *J. Med. Virol.* **31,** 277–283.
49. Gerety, R. J. (1984) *Hepatitis A*. Academic, London.
50. Glikson, M., Galun, E., Oren, R., Tur-Kaspa, R., and Shouval, D. (1992) Relapsing hepatitis A. Review of 14 cases and literature survey. *Medicine* (Baltimore) **71,** 14–23.
51. Gordon, S. C., Patel, A. S., Veneri, R. J., Keskey, K. A., and Korotkin, S. M. (1989) Case report: acute type a hepatitis presenting with hypotension, bradycardia, and sinus arrest. *J. Med. Virol.* **28,** 219–222.
52. Gordon, S. C., Reddy, K. R., Schiff, L., and Schiff, E. R. (1984) Prolonged intrahepatic cholestasis secondary to acute hepatitis A. *Ann. Int. Med.* **101,** 635–637.

53. Gosert, R., Cassinotti, P., Siegl, G., and Weitz, M. (1996) Identificationn of hepatitis A virus non-structural protein 2B and its release by the major virus protease 3C. *J. Gen. Virol.* **77,** 247–255.
54. Graff, J., Normann, A., Feinstone, S. M., and Flehmig, B. (1994) Nucleotide sequence of wild-type hepatitis A virus GBM in comparison with two cell culture-adapted variants. *J. Virol.* **68,** 548–554.
55. Gundersen, S. G., Bjoerneklett, A., and Bruun, J. N. (1989) Severe erythroblastopenia and hemolytic anemia during a hepatitis A infection. *Scand. J. Infect. Dis.* **21,** 225–228.
56. Gust, I. D., Coulepis, A. G., Feinstone, S. M., Locarnini, S. A., Moritsugu, Y., Najera, R., and Siegl, G. (1983) Taxonomic classification of hepatitis A virus. *Intervirology* **20,** 1–7.
57. Gust, I. D. and Feinstone, S. M. (1988) Hepatitis A. CRC Boca Raton, Florida.
58. Gust, I. D., Lehmann, N. I., and Lucas, C. R. (1978) Studies on the epidemiology of hepatitis A in Melbourne, in *Viral Hepatitis: A Contemporary Assessment of Etiology, Epidemiology, Pathogenesis, and Prevention* (Vyas, G. N., Cohen, S. H., and Schmid, R., eds.), Franklin Inst. Press, Philadelphia, PA, pp. 105–112.
59. Hadler, S. C. (1991) *Viral Hepatitis and Liver Disease.* Williams & Wilkins, Baltimore, MD, pp. 14–20.
60. Hadler, S. C. and McFarland, L. (1986) Hepatitis in day care centers: epidemiology and prevention. *Rev. Infect. Dis.* **8,** 548–557.
61. Hadler, S. C., Webster, H. M., Erben, J. J., Swanson, J. E., and Maynard, J. E. (1980) Hepatitis A in day-care centers. A community-wide assessment. *N. Engl. J. Med.* **302,** 1222–1227.
62. Hall, W. T., Bradley, D. W., Madden, D. L., Zimmerman, D. H., and Brandt, D. E. L. (1977) Comparison of sensitivity of radioimmunoassay and immune electron microscopy for detecting hepatitis A antigen in fecal extracts. *Proc. Soc. Exp. Biol. Med.* **155,** 193–198.
63. Halliday, M. L., Kang, L. Y., Zhou, T. K., Hu, M. D., Pan, Q. C., Fu, T. Y., Huang, Y. S., and Hu, S. L. (1991) An epidemic of hepatitis A attributable to the ingestion of raw clams in Shanghai, China. *J. Infect. Dis.* **164,** 852–859.
64. Hamman, J., Zou, J., and Horowitz, B. (1994) Removal and inactivation of hepatitis A virus (HAV) during processing of factor VIII concentrates. *Vox Sanguinis* **67,** s**1,** 72–77.
65. Harber, J. J., Bradley, J., Anderson, C. W., and Wimmer, E. (1991) Catalysis of poliovirus VP0 maturation cleavage is not mediated by serine 10 of VP2. *J. Virol.* **65,** 326–334.
66. Harmon, S. A., Emerson, S. U., Huang, Y. K., Summers, D. F., and Ehrenfeld, E. (1995) Hepatitis A viruses with deletions in the 2A gene are infectious in cultured cells and marmosets. *J. Virol.* **69,** 5576–5581.
67. Harmon, S. A., Updike, W., Jia, X.-Y., Summers, D. F., and Ehrenfeld, E. (1992) Polyprotein Processing in cis and trans by hepatitis A virus 3C protease cloned and expressed in escherichia coli. *J. Virol.* **66,** 5242–5247.
68. Hollinger, F. B. and Glombicki, A. P. (1990) Hepatitis A virus, in *Principles and Practices of Infectious Diseases* (Mandell, G. L., Douglas, R. G., Jr., and Bennett, J. E., eds.), Churchill Livingstone, New York, pp. 1383–1399.
69. Hollinger, F. B. and Ticehurst, J. (1990) Hepatitis A virus, in *Virology* (Fields, B. N. and Knipe, D. M., eds.), Raven, New York, pp. 631–667.

70. Holter, E. and Siebke, J. (1988) Hepatitis A in young Norwegian drug addicts and prison inmates. *Infection* **16,** 91–94.
71. Inman, R. D., Hodge, M., Johnston, M. E. A., Wright, J., and Heathcote, J. (1986) Arthritis, vasculitis, and cryoglobulinemia associated with relapsing hepatitis A virus infection. *Ann. Int. Med.* **105,** 700–703.
72. Innis, B. L., Snitbhan, R., and Hoke, C. H. (1991) The declining transmission of hepatitis A in Thailand. *J. Infect. Dis.* **163,** 989–995.
73. Jacobson, I. M., Nath, B. J., and Dienstag, J. L. (1985) Relapsing viral hepatitis type A. *J. Med. Virol.* **16,** 163–169.
74. Jansen, R. W., Newbold, J. E., and Lemon, S. M. (1985) Combined immunoaffinity cDNA-RNA hybridization assay for detection of hepatitis A virus in clinical specimens. *J. Clin. Microbiol.* **22,** 984–989.
75. Jansen, R. W., Newbold, J. E., and Lemon, S. M. (1988) Complete nucleotide sequence of a cell culture-adapted variant of hepatitis A virus: comparison with wild-type virus with restricted capacity for in vitro replication. *Virology* **163,** 299–307.
76. Jansen, R. W., Siegl, G., and Lemon, S. M. (1990) Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. *Proc. Natl. Acad. Sci. USA* **87,** 2867–2871.
77. Jewell, D. A., Swietnicki, W., Dunn, B. M., and Malcolm, B. A. (1992) Hepatitis A virus 3C proteinase substrate specificity. *Biochemistry* **31,** 7862–7869.
78. Johnston, J. M., Harmon, S. A., Binn, L. N., Richards, O. C., Ehrenfeld, E., and Summers, D. F. (1988) Antigenic and immunogenic properties of a hepatitis A virus capsid protein expressed in Escherichia coli. *J. Infect. Dis.* **157,** 1203–1211.
79. Joseph, P. R., Millar, J. D., and Henderson, D. A. (1965) An outbreak of hepatitis traced to food contamination. *N. Engl. J. Med.* **273,** 188–194.
80. Kallinowski, B., Gmelin, K., Kommerell, B., Bock, H. L., Clemens, R., Scheiermann, N., Wohland, B., Hofmann, F., and Theilmann, L. (1992) Immunogenicity, reactogenicity and consistency of a new, inactivated hepatitis A vaccine—a randomized multicentre study with three consecutive vaccine lots. *Vaccine* **10,** 500–501.
81. Kaplan, G., Totsuka, A., Thompson, P., Akatsuka, T., Moritsugu, Y., and Feinstone, S. M. (1996) Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus. *EMBO J.* **15,** 4286–4296.
82. Karayiannis, P., O'Rourke, S., Waters, J., Watts, R., and Thomas, H. C. (1994) Studies of cytotoxic T lymphocyte activity in Tamarins with acute hepatitis A virus infection, in *Viral Hepatitis and Liver Disease* (Nishioka, K., Suzuki, H., Mishiro, S., and Oda, T., eds.), Springer-Verlag, Tokyo, pp. 155–157.
83. Karron, R. A., Daemer, R. J., Ticehurst, J. R., D'Hondt, E., Popper, H., Mihalik, K., Philips, J., Feinstone, S., and Purcell, R. H. (1988) Studies of prototype live hepatitis A virus vaccine in primate models. *J. Infect. Dis.* **157,** 338–345.
84. Katz, M. H., Hsu, L., Wong, E., Liska, S., and Anderson, L. (1997) Seroprevalence of and risk factors for hepatitis A infection among young homosexual and bisexual men. *J. Infect. Dis.* **175,** 1225–1229.
85. Krausslich, H.-G. and Wimmer, E. (1988) Viral proteinases. *Ann. Rev. Biochem.* **57,** 701–754.
86. Kurane, I., Binn, L. N., Bancroft, W. H., and Ennis, F. A. (1985) Human lymphocyte responses to hepatitis A virus-infected cells: interferon production and lysis of infected cells. *J. Immunol.* **135,** 2140–2144.

87. LeDuc, J. W., Lemon, S. M., Keenan, C. M., Graham, R. R., Marchwicki, R. H., and Binn, L. N. (1983) Experimental infection of the new world owl monkey (Aotus trivirgatus) with hepatitis A virus. *Infect. Immun.* **40,** 766–772.
88. Lemon, S. M. (1985) Type A viral hepatitis. New developments in an old disease. *N. Engl. J. Med.* **313,** 1059–1067.
89. Lemon, S. M. (1995) Hepatitis A virus and blood products: virus validation studies. *Blood Coagulation and Fibrinolysis* **6,** s20-s22.
90. Lemon, S. M. and Binn, L. N. (1983) Antigenic relatedness of two strains of hepatitis A virus determined by cross-neutralization. *Infect. Immun.* **42,** 418–420.
91. Lemon, S. M., Binn, L. N., Marchwicki, R., Murphy, P. C., Ping, L. H., Jansen, R. W., Asher, L. V. S., Stapleton, J. T., Taylor, D. W., and LeDuc, J. W. (1990), in vivo replication and reversion to a wild type of a neutralization-resistant antigenic variant of hepatitis A virus. *J. Infect. Dis.* **161,** 7–13.
92. Lemon, S. M., Binn, L. N., and Marchwicki, R. H. (1983) Radioimmunofocus assay for quantitation of hepatitis A virus in cell culture. *J. Clin. Microbiol.* **17,** 834–839.
93. Lemon, S. M., Chao, S. F., Jansen, R. W., Binn, L. N., and LeDuc, J. W. (1987) Genomic heterogeneity among human and nonhuman strains of hepatitis A virus. *J. Virol.* **61,** 735–742.
94. Lemon, S. M., Jansen, R. W., and Newbold, J. E. (1985) Infectious hepatitis A particles produced in cell culture consist of three distinct types with different buoyant densities in CsCl. *J. Virol.* **54,** 78–85.
95. Lemon, S. M. and Stapleton, J. T. (1994) Prevention of hepatitis A, in *Viral Hepatitis* (Zuckerman, A. J. and Thomas, H. C., eds.), Churchill Livingstone, London, pp. 61–79.
96. Lorenz, D., Barker, L., and Stevens, D. (1970) Hepatitis in the marmoset: saguinus mystax. *Proc. Soc. Exp. Biol. Med.* **135,** 348–354.
97. Ludmerer, K. M. and Kissane, J. M. (1984) Fulminant hepatic failure in a 21-year old man. *Amer. J. Med.* **76,** 718–724.
98. MacCallum, F. O. (1947) Homologous serum jaundice. *Lancet* **2,** 691–692.
99. MacCallum, F. O. and Bradley, W. H. (1944) Transmission of infective hepatitis to human volunteers. *Lancet* **2,** 228.
100. Malcolm, B. A., Chin, S. M., Jewell, D. A., Stratton-Thomas, J. R., Thudium, K. B., Ralston, R., and Rosenberg, S. (1992) Expression and characterization of recombinant hepatitis A virus 3C proteinase. *Biochemistry* **31,** 3358–3363.
101. Mao, J. (1990) Development of live, attenuated hepatitis A vaccine (H2-strain). *Vaccine* **8,** 523–524.
102. Martin, A., Escriou, N., Chao, S.-F., Girard, M., Lemon, S. M., and Wychowski, C. (1995) Identification and site-directed mutagenesis of the primary (2A/2B) cleavage site of the hepatitis A virus polyprotein: functional impact on the infectivity of HAV RNA transcripts. *Virology* **213,** 213–222.
103. Mathiesen, L. R., Feinstone, S. M., Wong, D. C., Skinhoej, P., and Purcell, R. H. (1978) Enzyme-linked immunosorbent assay for detection of hepatitis A antigen in stool and antibody to hepatitis A antigen in sera: comparison with solid-phase radioimmunoassay, immune electron microscopy, and immune adherence hemagglutination assay. *J. Clin. Microbiol.* **7,** 184–193.
104. Maynard, J. E. (1963) Infectious hepatitis at Fort Yukon, Alaska—report of an outbreak, 1960–1961. *Amer. J. Public Health* **53,** 31–39.
105. McCaustland, K. A., Bond, W. W., Bradley, D. W., Ebert, J. W., and Maynard, J. E. (1982) Survival of hepatitis A virus in feces after drying and storage for one month. *J. Clin. Microbiol.* **16,** 957–958.

106. McDonald, S. (1908) Acute yellow atrophy of the liver. *Edinburgh Med. J.* **15,** 208.
107. McLinden, J., Stapleton, J., and Rosen, E. (1991) Anti-HAV neutralizing antibodies made to antigens produced by recombinant baculoviruses, in *Vaccines 91: Modern Approaches to New Vaccines* (Ginsberg, H., Brown, F., Lerner, R. A., and Chanock, R., eds.), Cold Spring Harbor, New York, pp. 239–244.
108. Melnick, J. L. (1982) Classification of hepatitis A virus as enterovirus type 72 and of hepatitis B virus as hepadnavirus type 1. *Intervirology* **18,** 105–106.
109. Mijch, A. M. and Gust, I. D. (1986) Clinical, serologic and epidemiologic aspects of hepatitis A virus infection. *Semin. Liver Dis.* **6,** 42–45.
110. Miller, M. J. (1993) Summary of current nomenclature, taxonomy, and classification of various microbial agents. *Clin. Infect. Dis.* **16,** 597–615.
111. Murphy, P., Ping, L. H., and Lemon, S. M. (1993) Peptide scanning demonstrates the presence of a linear epitope located near the carboxy terminus of VP1 of hepatitis A virus. *8th Intern. Symp. Viral Hepatitis Liver Disease*, 102.
112. Najarian, R., Caput, D., and Gee, W. (1985) Primary structure and gene organization of human hepatitis A virus. *Proc. Natl. Acad. Sci. USA* **82,** 2627–2631.
113. Neefe, J. R., Stokes, J., and Gellis, S. S. (1945) Homologous serum hepatitis and infectious (epidemic) hepatitis: experimental study of immunity and cross immunity in volunteers. *Amer. J. Me*d. Sci. **210,** 561–575.
114. Neefe, J. R. and Stokes, J., Jr. (1945) An epidemic of infectious hepatitis apparently due to a water borne agent. *JAMA* **128,** 1063.
115. Niu, M. T., Polish, L. B., Robertson, B. H., Khanna, B. K., Woodruff, B. A., Shapiro, C. N., Miller, M. A., Smith, J. D., Gedrose, J. K., Alter, M. J., and Margolis, H. S. (1992) Multistate outbreak of hepatitis A associated with frozen strawberries. *J. Infect. Dis.* **166,** 518–524.
116. Norkrans, G. (1978) Clinical, epidemiological and prognostic aspects of hepatitis A, B and "non-A, non-B." *Scand. J. Infect. Dis.* **17,** s1–s44.
117. Normann, A., Graff, J., Gerritzen, A., Brackmann, H.-H., and Flehmig, B. (1992) Detection of hepatitis A virus RNA in commercially available factor VIII preparation. *Lancet* **340,** 1232–1233.
118. Ostermayr, R., von der Helm, K., Gauss-Muller, V., Winnacker, E. L., and Deinhardt, F. (1987) Expression of hepatitis A virus cDNA in Escherichia coli: antigenic VP1 recombinant protein. *J. Virol.* **61,** 3645–3647.
119. Palmenberg, A. C. (1990) Proteolytic processing of picornaviral polyprotein. *Ann. Rev. Microbiol.* **44,** 603–623.
120. Parry, J. V. and Mortimer, P. P. (1984) The heat sensitivity of hepatitis A virus determined by a simple tissue culture method. *J. Med. Virol.* **14,** 277–283.
121. Peterson, D. A., Hurley, T. R., and Hoff, J. C. (1983) Effect of chlorine treatment on infectivity of hepatitis A virus. *Appl. Environ. Microbiol.* **45,** 223–227.
122. Pickering, L. K. (1987) Infections in day care. *Ped. Infect. Dis.* **6,** 614–617.
123. Ping, L.-H., Jansen, R. W., Stapleton, J. T., Cohen, J. I., and Lemon, S. M. (1988) Identification of an immunodominant antigenic site involving the capsid protein VP3 of hepatitis A virus. *Proc. Natl. Acad. Sci. USA* **85,** 8281–8285.
124. Ping, L.-H. and Lemon, S. M. (1992) Antigenic structure of human hepatitis A virus defined by analysis of escape mutants selected against murine monoclonal antibodies. *J. Virol.* **66,** 2208–2216.
125. Porter, A. G. (1993) Picornavirus nonstructural proteins: emerging roles in virus replication and inhibition of host cell functions. *J. Virol.* **67,** 6917–6921.

126. Powdrill, T. F. and Johnston, J. M. (1991) Immunologic priming with recombinant hepatitis A virus proteins produced in e. coli. *J. Virol.* **65,** 2686–2690.
127. Provost, P. J. and Hilleman, M. R. (1979) Propagation of human hepatitis A virus in cell culture in vitro. *Proc. Soc. Exp. Biol. Med.* **160,** 213–221.
128. Purcell, R. H., Feinstone, S. M., Ticehurst, J. R., Daemer, R. J., and Baroudy, B. M. (1984) Hepatitis A virus, in *Viral Hepatitis and Liver Disease* (Vyas, G. N., Dienstag, J. L., and Hoofnagle, J. H., eds.), Grune and Stratton, Orlando, pp. 9–22.
129. Rakela, J., Lange, S. M., Ludwig, J., and Baldus, W. P. (1985) Fulminant hepatitis: Mayo clinic experience with 34 cases. *Mayo. Clin. Proc.* **60,** 289–292.
130. Reves, R. R. and Pickering, L. K. (1992) Impact of child day care on infectious diseases in adults. *Inf. Dis. Clin. N. Am.* **6,** 239–250.
131. Robertson, B. H., Jansen, R. W., Khanna, B., Totsuka, A., Nainan, O. V., Siegl, G., Widell, A., Margolis, H. S., Isomura, S., Ito, K., Ishizu, T., Moritsugu, Y., and Lemon, S. M. (1992) Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. *J. Gen. Virol.* **73,** 1365–1377.
132. Robertson, B. H., Jia, X.-Y., Tian, H., Margolis, H. S., Summers, D. F., and Ehrenfeld, E. (1993) Antibody response to nonstructural proteins of hepatitis A virus following infection. *J. Med. Virol.* **40,** 76–82.
133. Rosen, E., Stapleton, J. T., and McLinden, J. (1993) Synthesis of immunogenic hepatitis A virus particles by recombiant baculoviruses. *Vaccine* **11,** 706–712.
134. Rosenblum, L. S., Villarino, M. E., Nainan, O. V., Melish, M. E., Hadler, S. C., Pinsky, P. P., Jarvis, W. R., Ott, C. E., and Margolis, H. S. (1991) Hepatitis A outbreak in a neonatal intensive care unit: risk factors for transmission and evidence of prolonged viral excretion among preterm infants. *J. Infect. Dis.* **164,** 476–482.
135. Ross, B. C. and Anderson, D. A. (1991) Characterization of hepatitis A virus capsid proteins with antisera raised to recombinant antigens. *J. Virol. Meth.* **32,** 213–220.
136. Ross, B. C., Anderson, D. A., and Gust, I. D. (1991) Hepatitis A virus and hepatitis A infection. *Adv. Virus Res.* **39,** 209–254.
137. Rueckert, R. R. (1996) Picornaviridae: the viruses and their replication, in *Fields Virology* (Fields, B. N., Knipe, D. M., Howley, P. M., Chanuck, R. M., Melnick, J. L., Monath, T. P., Roizman, B., and Straus, S. E., eds.), Lippincott–Raven, Philadelphia, PA, pp. 609–654.
138. Rueckert, R. R. and Wimmer, E. (1984) Systematic nomenclature of picornavirus proteins. *J. Virol.* **50,** 957–959.
139. Sagliocca, L., Mele, A., Gill, O. N., and Cappeta, G. (1988) A village outbreak of hepatitis A: acquaintance network and inapparent pre-school transmission compared. *Eur. J. Epidemiol.* **4,** 470–472.
140. Schenzle, D., Kietz, K., and Frosner, G. G. (1979) Antibody against hepatitis A in seven European countries. II. Statistical analysis of cross-sectional surveys. *Amer. J. Epidemiol.* **110,** 70–76.
141. Schoenbaum, S. C., Baker, O., and Jezek, Z. (1976) Common-source epidemic of hepatitis due to glazed and iced pastreis. *Amer. J. Epidemiol.* **104,** 74–80.
142. Schultheiss, T., Kusov, Y. Y., and Gauss-Muller, V. (1994) Proteinase 3C of hepatitis A virus (HAV) cleaves the HAV polyprotein P2-P3 at all sites including VP1/2A and 2A/2B. *Virology* **198,** 275–281.
143. Schultheiss, T., Sommergruber, W., Kusov, Y. Y., and Gauss-Muller, V. (1995) Cleavage specificity of purified recombinant hepatitis A virus 3C proteinase on natural substrates. *J. Virol.* **69,** 1727–1733.

144. Shapiro, C. N., McQuillan, G. M., Robertson, B., Hadler, S. C., and Margolis, H. S. (1990) Seroepidemiology of hepatitis A infection in the United States. *1990 Intern. Symp. Viral Hepatitis Liver Disease Abstr.* **22,** 44.
145. Shieh, Y.-S. C., Baric, R. S., Sobsey, M. D., Ticehurst, J., Miele, T. A., DeLeon, R., and Walter, R. (1991) Detection of hepatitis A virus and other enteroviruses in water by ssRNA probes. *J. Virol. Meth.* **31,** 119–136.
146. Siegl, G. and Frosner, G. G. (1978) Characterization and classification of virus particles associated with hepatitis A: I. size, density, and sedimentation. *J. Virol.* **26,** 40–47.
147. Siegl, G., Frosner, G. G., Gauss-Muller, V., Tratschin, J. D., and Deinhardt, F. (1981) The physicochemical properties of infectious hepatitis A virion. *J. Gen. Virol.* **57,** 331–341.
148. Siegl, G. and Lemon, S. M. (1990) Recent advances in hepatitis A vaccine development. *Virus Res.* **17,** 75–92.
149. Siegl, G., Weitz, M., and Kronauer, G. (1984) Stability of hepatitis A virus. *Intervirology* **22,** 218–226.
150. Sjogren, M. H., Purcell, R. H., McKee, K., Binn, L., Macarthy, P., Ticehurst, J., Feinstone, S., Caudill, J., See, A., Hoke, C., Bancroft, W., and D'Hondt, E. (1992) Clinical and laboratory observations following oral or intramuscular administration of a live attenuated hepatitis A vaccine candidate. *Vaccine* **10,** S135–S138.
151. Sjogren, M. H., Tanno, H., Fay, O., Sileoni, S., Cohen, B. D., Burke, D. S., and Feighny, R. J. (1987) Hepatitis A virus in stool during clinical relapse. *Ann. Int. Med.* **106,** 221–226.
152. Skinhoj, P., Mikkelsen, F., and Hollinger, F. B. (1977) Hepatitis A in Greenland: importance of specific antibody testing in epidemiologic surveillance. *Amer. J. Epidemiol.* **105,** 140–147.
153. Stapleton, J. T. (1992) Passive immunization against hepatitis A. *Vaccine* **10,** S45–S47.
154. Stapleton, J. T. (1995) Host immune response to hepatitis A virus. *J. Infect. Dis.* **171,** s9–14.
155. Stapleton, J. T., Frederick, J., and Meyer, B. (1991) Hepatitis A virus attachment to cultured cell lines. *J. Infect. Dis.* **164,** 1098–1103.
156. Stapleton, J. T., Jansen, R., and Lemon, S. M. (1985) Neutralizing antibody to Hepatitis A virus in immune serum gobulin and in the sera of human recipients of immune serum globulin. *Gastroenterology* **89,** 637–642.
157. Stapleton, J. T. and Lemon, S. M. (1987) Neutralization escape mutants define a dominant immunogenic neutralization site on hepatitis A virus. *J. Virol.* **61,** 491–498.
158. Stapleton, J. T. and Lemon, S. M. (1994) Hepatitis A and hepatitis E, in *Infectious Diseases, A Modern Treatise of Infectious Process* (Hoeprich, P. D., Jordan, M. C., and Ronald, A. R., eds.), J. B. Lippencott Company, Philadelphia, pp. 790–800.
159. Stapleton, J. T., Raina, V., Winokur, P. L., Walters, K., Klinzman, D., Rosen, E., and McLinden, J. H. (1993) Antigenic and immunogenic properties of recombinant hepatitis A virus 14S and 70S subviral particles. *J. Virol.* **67,** 1080–1085.
160. Stapleton, J. T., Rosen, E., and McLinden, J. (1991) Detection of hepatitis A virus capsid proteins in insect cells infected with recombinant baculoviruses encoding the entire hepatitis A virus open reading frame, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), Williams & Wilkins, Baltimore, MD, pp. 50–54.

161. Stewart, D. R., Morris, T. S., Purcell, R. H., and Emerson, S. U. (1997) Detection of antibodies to the nonstructural 3C proteinase of hepatitis A virus. *J. Infect. Dis.* **176,** 593–601.
162. Summers, P. L., Dubois, D. R., Houston Cohen, W., MacArthy, P. O., Binn, L. N., Sjogren, M. H., Snitbhan, R., Innis, B. L., and Eckels, K. H. (1993) Solid-phase antibody capture hemadsorption assay for detection of hepatitis A virus immunoglobulin M antibodies. *J. Clin. Microbiol.* **31,** 1299–1302.
163. Szmuness, W., Dienstag, J. L., and Purcell, R. H. (1977) The prevalence of antibody to hepatitis A antigen in various parts of the world. *Amer. J. Epidemiol.* **106,** 391–398.
164. Szmuness, W., Dienstag, J. L., Purcell, R. H., Harley, E. J., Stevens, C. E., and Wong, D. C. (1976) Distribution of antibody to hepatitis A antigen in urban adult populations. *N. Engl. J. Med.* **295,** 755–759.
165. Tabor, E. (1984) Clinical presentation of hepatitis A, in *Hepatitis A* (Gerety, R. J., ed.), Academic, London, pp. 47–53.
166. Tanno, H., Fay, O. H., Fojman, J. A., and Palazzi, J. (1988) Biphasic form of hepatitis A virus infection: a frequent variant in Argentina. *Liver* **7,** 53–57.
167. Taylor, G. M., Goldin, R. D., Karayiannis, P., and Thomas, H. C. (1992), *in situ* hybridization studies in hepatitis A infection. *Hepatology* **16,** 642–648.
168. Tedeschi, V., Purcell, R. H., and Emerson, S. U. (1993) Partial characterization of hepatitis A viruses from three intermediate passage levels of a series resulting in adaptation to growth in cell culture and attenuation of virulence. *J. Med. Virol.* **39,** 16–22.
169. Ticehurst, J., Cohen, J. I., Feinstone, S. M., Purcell, R. H., Jansen, R. W., and Lemon, S. M. (1989) Replication of hepatitis A virus: new ideas from studies with cloned cDNA, in *Molecular Aspects of Picornavirus Infection and Detection* (Semler, B. L. and Ehrenfeld, E., eds.), *Am. Soc. Microbiol.* Washington, pp. 27–50.
170. Ticehurst, J. and Purcell, R. H. (1993) Hepatitis A virus and diagnostic techniques for hepatitis A, in *Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections* (Schmidt, N. J. and Emmons, R., eds.), American Public Health Assoc., Washington.
171. Ticehurst, J. R., Feinstone, S. M., Chestnut, T., Tassopoulos, N. C., Popper, H., and Purcell, R. H. (1987) Detection of hepatitis A virus by extraction of viral RNA and molecular hybridization. *J. Clin. Microbiol.* **25,** 1822–1829.
172. Tözün, N., Forbes, A., Anderson, M. G., and Murray-Lyon, I. M. (1991) Safety of alcohol after viral hepatitis. *Lancet* **337,** 1079–1080.
173. Updike, W. S., Tesar, M., and Ehrenfeld, E. (1991) Detection of hepatitis A virus proteins in infected BS-C-1 cells. *Virology* **185,** 411–418.
174. Vallbracht, A., Gabriel, P., Maier, K., Hartmann, F., and Flehmig, B. (1986) Cell-mediated cytotoxicity in hepatitis A virus infection. *Hepatology* **6,** 1308–1314.
175. Vallbracht, A., Maier, K., Stierhof, Y. D., Wiedmann, K. H., Flehmig, B., and Fleischer, B. (1989) Liver-derived cytotoxic T cells in hepatitis A virus infection. *J. Infect. Dis.* **160,** 209–217.
176. van den Anker, J. N., Sukhai, R. N., and Dumas, A. M. (1988) Relapsing hepatitis in a child associated with isolation of hepatitis A virus antigen from the liver. *Eur. J. Ped.* **147,** 333.
177. Villarejos, V. M., Provost, P. J., and Ittensohn, O. L. (1976) Seroepidemiologic investigations of human hepatitis caused by A, B., and a possible third agent. *Proc. Soc. Exp. Biol. Med.* **152,** 524–528.

178. Weitz, M., Baroudy, B. M., Maloy, W. L., Ticehurst, J. R., and Purcell, R. H. (1986) Detection of a genome-linked protein (VPg) of hepatitis A virus and its comparison with other picornaviral VPgs. *J. Virol.* **60,** 124–130.
179. Weitz, M. and Siegl, G. (1993) Hepatitis A virus: Structural and molecular virology, in *Viral Hepatitis—Scientific Basis and Clinical Management* (Zuckerman, A. J. and Thomas, H. C., eds.), Churchill Livingstone, Edinburgh, U.K., pp. 21–34.
180. Widell, A. (1988) *Hepatitis A Virus Virological Studies*, doctoral dissertation, University of Lund, Sweden **1,** 3–12.
181. Wiedermann, G., Ambrosch, F., Kollaritsch, H., Hofmann, H., Kunz, C., D'Hondt, E., Delem, A., André, F. E., Safary, A., and Stéphenne, J. (1990) Safety and immunogenicity of an inactivated hepatitis A candidate vaccine in healthy adult volunteers. *Vaccine* **8,** 581–584.
182. Winokur, P. L., McLinden, J. H., and Stapleton, J. T. (1991) The hepatitis A virus polyprotein expressed by a recombinant vaccinia virus undergoes proteolytic processing and assembly into virus-like particles. *J. Virol.* **65,** 5029–5036.
183. Winokur, P. L. and Stapleton, J. T. (1992) Immunoglobulin prophylaxis for hepatitis A. *Clin. Infect. Dis.* **14,** 580–586.
184. Wunschmann, S., Vallbracht, A., Flehmig, B., Winokur, P., Klinzman, D., and Stapleton, J. T. (1997) Cytolytic T lymphocyte epitopes are present on hepatitis A virus structural proteins, in *Viral Hepatitis and Liver Disease* (Rizzeto, M., ed.), in press.
185. Xu, Z.-Y., Li, Z.-H., Wang, J.-X., Xiao, Z.-P., and Dong, D.-X. (1992) Ecology and prevention of a shellfish-associated hepatitis A epidemic in Shanghai, China. *Vaccine* **10(Suppl. 1),** S67-S68.
186. Zachoval, R., Kroener, M., Brommer, M., and Deinhardt, F. (1990) Serology and interferon production during the early phase of acute hepatitis A. *J. Infect. Dis.* **161,** 353–354.
187. Zajac, A. J., Amphlett, E. M., Rowlands, D. J., and Sangar, D. V. (1991) Parameters influencing the attachment of hepatitis A virus to a variety of continuous cell lines. *J. Gen. Virol.* **72,** 1667–1675.
188. Zuckerman, A. J. (1983) The history of viral hepatitis from antiquity to the present, in *Viral Hepatitis: Laboratory and Clinical Science* (Deinhardt, F. and Deinhardt, J., eds.), Marcel Dekker, New York, pp. 3–32.

3

Hepatitis B Virus

Biology, Pathogenesis, Epidemiology, Clinical Description, and Diagnosis

Girish N. Vyas and T. S. Benedict Yen

1. Introduction

The clinical, epidemiological, pathological, and immunological attributes of viral hepatitides share many clinical features despite their remarkably different etiologies, which include multiple unrelated hepatitis viruses, viz. HAV, HBV, HCV, HDV, HEV, and perhaps HGV. This group of viral agents has a dichotomous mode of epidemiological transmission, viz., enteric for HAV and HEV, which does not establish persistent infection, and parenteral for HBV, HCV, HDV, and HGV, which tend to establish persistent infection or chronic carrier state. The etiology of acute or chronic liver disease caused by these diverse agents cannot be established on the basis of clinical signs and symptoms, or abnormal liver function tests, for example, elevated alanine aminotransferase (ALT). Since Blumberg's Nobel Prize-winning discovery of Hepatitis B surface antigen (HBsAg, originally termed Australia antigen) in 1965, enzyme immunoassays (EIA) for the immunological markers of viral hepatitides in serum have remained the mainstay of laboratory diagnosis. The cloning of HBV DNA and complete nucleotide sequencing of the cloned DNA in 1979 opened new approaches to understanding the biology of HBV as a blood-borne pathogen that has not been propagated in cultures. Amplification of viral DNA by the polymerase chain reaction (PCR), another Nobel Prize-winning discovery, has enabled us to most sensitively and directly detect HBV DNA in serum, cells, and tissues, rather than indirectly detecting HBV infection by host immune response to the viral antigens. Thus, during the past decade, rapid progress has been achieved in our understanding of the molecular biology of HBV, epide-

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

Table 1
Immunological and virological characteristics of hepatitis B virus (HBV)

Characteristics	HBV
Family	*Hepadnaviridae*
Virion size and shape	42 nm, spherical, with 27 nm capsids
Envelope	Large, middle, small surface proteins lipids
Nucleocapsid	
Genome	Circular partially dsDNA, 3.2 kb
Proteins	Core protein, polymerase, ?host proteins
Replication	Reverse transcription of positive-strand RNA intermediate
Antigens	HBsAg, HBeAg, HBcAg
Antibodies	Anti-HBs, HBe, HBc, HBc IgM

miology, pathogenesis, diagnosis, evaluating treatment, and prophylaxis. Since the laboratory diagnosis, prophylaxis, and treatment of HBV have been covered more extensively elsewhere in this volume, we have succinctly summarized immunobiology, molecular biology, pathogenesis, epidemiology, and clinical diagnosis of HBV infection, which have been embodied in dozens of monographs and several thousand original research papers from which the authors have selectively cited the references most useful in further reading.

2. Viral Life Cycle

2.1. Description of Virion and Subviral Particles

Hepatitis B virus (HBV) is a member of the family Hepadnaviridae, which includes various closely related viruses that infect birds and mammals *(10,14)*. The duck HBV (DHBV) and woodchuck HBV (WHBV) have proven to be useful models for elucidating the replication and persistence of HBV infection. All these viruses show hepatotropism, to a greater or lesser degree, and all show narrow host-range specificity. Thus, HBV infects only human beings and chimpanzees, while none of the animal Hepadnaviridae infects human beings. HBV is an enveloped DNA virus approx 42 nm in diameter. The characteristics of the virus are summarized in Table 1, and the viral genome is diagrammed in Fig. 1. The viral envelope consists of host-derived lipids and three forms of the viral surface protein. All three surface proteins share the same C-terminal domain, which is the only domain present in the small (or major) surface protein. The middle surface protein has a 55 residue N-terminal extension called the preS2 domain. The large surface

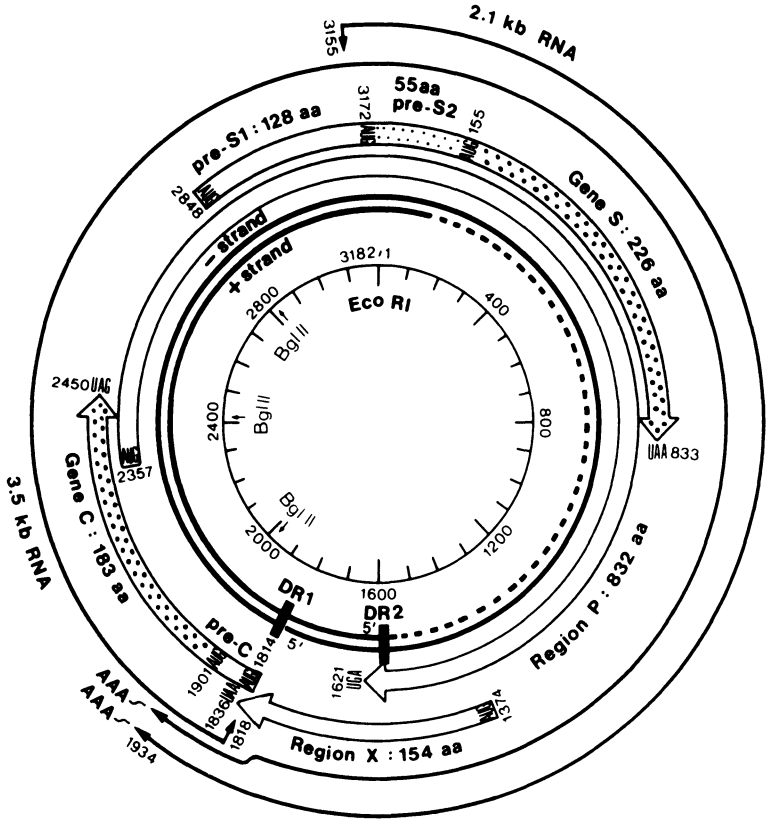


Fig. 1. Genetic map of HBV, showing the four open-reading frames and the two major transcripts. The minor preS1 and X transcripts are not shown. DR1 and DR2 refer to direct repeat 1 and direct repeat 2. (Courtesy of Dr. Pierre Tiollais, Pasteur Institute, Paris, France.)

protein has, in addition, to the pre-S2 domain, a 119 or 108 residue (depending on subtype) N-terminal extension called the pre-S1 domain. Inside the envelope is a nucleocapsid approx 27 nm in diameter, comprising approx 200 copies of the viral core protein and probably a single copy of the genome that is approx 3.2 kb in length. Unique among animal virus families, the viral DNA is a partially double-stranded circular molecule, with the viral polymerase protein covalently attached to the 5' end of one strand. In addition, the DHBV particle has recently been shown to contain a small amount of the cytosolic heat shock protein Hsc70, probably on the inner surface of the envelope, as well as Hsp90 and p23, two other chaperone proteins *(16)*. Whether HBV similarly contains cellular heat shock proteins has not been

examined. Finally, a protein kinase activity has been observed associated with HBV particles, but its identity has not been determined, nor is it clear that this represents an integral component of the virion.

Besides virion particles (also known as Dane particles), numerous subviral particles (also called surface antigen particles) circulate in the serum of infected people. In fact, these particles usually greatly outnumber virions (by up to 6 orders of magnitude) and constitute the so-called Australia antigen first detected by Blumberg as a marker for HBV infection. Subviral particles contain only lipids and viral surface proteins, and are devoid of the nucleocapsid. They also contain little or no large surface protein, which appears to carry the receptor-binding site (*see* Subheading 2.2.). Thus, subviral particles are not only noninfectious; they are also incapable of interfering with infection by virions. By negative staining, they appear as spherical or filamentous particles approx 22 nm in diameter, with no discernable internal structure. Mild detergent treatment of the filaments causes their disaggregation into spherical particles, raising the possibility that one form may be derived from the other, either in the infected cell or in the serum.

2.2. Receptor Binding and Internalization

Since subviral HBV particles contain large amounts of middle and small surface proteins, but almost no large surface protein, whereas virions contain all three forms, it seemed likely that the large surface protein carries the determinant for binding to the cellular receptor for HBV. Indeed, all evidence points to the residues unique to the large surface protein (known as the preS1 domain) as the receptor binding domain. For example, antibodies to preS1 block the binding of HBV virions to liver membranes and to a well-differentiated human hepatoma cell line (HepG2). Similarly, antibodies to preS1 block infection of duck hepatocytes by DHBV in culture. Nevertheless, conclusive evidence in this regard awaits identification of the cellular receptor for HBV. Unfortunately, despite the efforts of many laboratories, the receptor for HBV remains elusive. Although numerous candidates have been identified and characterized in greater or less detail, none has fulfilled all of the criteria necessary for its identification as the HBV receptor. However, two groups have recently characterized a 180-kDa cell-surface glycoprotein that binds specifically to the duck virus and whose binding is blocked by neutralizing antibodies to the duck preS1 domain *(25,39)*. Molecular cloning of the cDNA reveals this protein to be a carboxypeptidase. This protein may not be the primary receptor for DHBV, since it is found on many different cell types (e.g., fibroblasts) that are not susceptible to DHBV infection, but it may be a coreceptor. Alternatively, it may be that events downstream to receptor binding determines cellular tropism of DHBV. Positive identifi-

cation and cloning of the receptor(s) for HBV would greatly facilitate research in this field, since there is no efficient cell-culture-based infection system.

Like all other enveloped viruses, HBV presumably must fuse its envelope with a cellular membrane in order for the nucleocapsid to gain entry into the cytosol. The mechanism of this fusion is unknown, but recent data indicate that it probably takes place in an early (nonacidified) endosomal compartment *(23)*. The molecular events following release of the nucleocapsid into the cytosol are also entirely unknown, but it is clear that eventually the genome must reach the nucleus, become uncoated, and be deproteinated and repaired into a supercoiled covalently closed circular (CCC) episome. The repair process probably does not require any viral factors, since deproteinated genomic DNA can be directly cloned into plasmid vectors in bacteria. It is also likely that, as in other viral systems, a protein component of the nucleocapsid is involved in nuclear import. Indeed, both the core and polymerase proteins have been shown to have nuclear localization signals, although the importance of these signals for nuclear import of virion DNA has not been demonstrated.

2.3. *Gene Expression*

Once the genomic DNA has been converted into CCC DNA, it becomes competent for transcription by host RNA polymerase II. Four promoters have been well characterized, while at least one other promoter has been tentatively identified *(52)*. All promoters are located on one strand of the DNA, and there is only a single polyadenylation signal. Therefore, all HBV transcripts share the same 3' end. Interestingly, there is not a one-to-one correspondence between promoters and open-reading frames. This arrangement allows for the synthesis of perhaps as many as nine primary translation products from a small genome at widely different levels.

The core promoter is located just upstream of the polyadenylation signal. Therefore, one might expect the resulting transcripts to be extremely short. However, the polyadenylation signal is ignored the first time it is traversed by the elongating polymerase and is used only on the second pass. The basis for this read-through phenomenon is twofold. First, the polyadenylation signal is noncanonical and hence is intrinsically inefficient. Second, the propinquity of the 5' end of the transcript further diminishes usage of this signal. During the second pass, however, polyadenylation becomes efficient, not only because the 5' end is now quite distant, but also because of the existence of RNA elements that increase the usage of the signal. These elements are present only in the read-through transcript, because they are encoded by DNA sequences upstream of the core promoter.

Additional, more complex control elements may be present in DHBV that also result in 3' end formation only on the second pass *(18)*. Consequently, the core promoter transcripts contain a small terminal redundancy and are slightly longer than one genome-equivalent. This feature, which also is found in retroviral transcripts, is critical for HBV genome replication (*see* Subheading 2.5.).

The core promoter gives rise to two major classes of transcripts, whose fates are quite different. The longer, less abundant transcripts start upstream of the first ATG codon in the core open-reading frame and are translated into a 215-residue protein called (inappropriately) the precore protein. Hence, these long transcripts are known as the precore transcripts. The shorter transcripts start downstream of the first ATG codon in the core open-reading frame and hence cannot give rise to precore protein. Instead, translation starts at the next in-frame ATG codon, resulting in a protein (core protein) that is 29 residues shorter than the precore protein. (It should be emphasized again that core protein is not derived from the precore protein.) These transcripts are also translated into the polymerase protein, even though this open-reading frame starts far downstream of the core initiation codon. It appears that a small proportion of the ribosomes scanning down the core transcripts from the 5' end do not recognize the core protein initiation codon, probably because it does not fully match the Kozak consensus sequence. Instead, by a process still poorly understood, they manage to evade a series of interfering ATG codons and finally initiate translation at the first ATG codon in the polymerase open-reading frame. Not surprisingly, very low amounts of the polymerase protein are synthesized in the infected cell. In addition to serving as the translational template for two different proteins, the short transcripts are also the precursor to progeny viral genomes (*see* Subheading 2.4.). Hence, these transcripts are usually called the pregenomic RNA. It should be noted that all known components of the nucleocapsid (core protein, polymerase, and pregenomic RNA) are derived from the same transcript. This is probably not an accidental arrangement, since it would allow the various components to be synthesized at a fixed (and presumably optimal) ratio.

The core promoter contains numerous *cis*-elements that bind various cellular transcription factors, both hepatocyte-enriched and ubiquitous, which result in preferential but not exclusive expression in hepatocytes. The relative importance of each element is still being evaluated. In addition, directly upstream of the core promoter is the viral enhancer II, which also binds a variety of hepatocyte-enriched and ubiquitous transcription factors. Some of these factors have a negative influence on core promoter activity, especially in nonhepatocytes, and may be an additional factor in limited expression of this promoter outside of the hepatocyte *(3)*. Finally, enhancer

I, which is further upstream, also strongly activates the core promoter in a hepatocyte-specific manner. Because of a combination of these factors, the core promoter is strongly expressed only in hepatocytes and a few other cell types.

It was thought until recently that the core promoter functions as an indivisible unit, and, as a result, the ratio of the precore and pregenomic transcripts is fixed. However, it is now clear that there is independent regulation of the two sets of transcripts by various *cis*-acting DNA elements. For example, the ubiquitous factor Sp1 increases the amount of only pregenomic transcripts, and the hepatocyte-enriched factor HNF4 selectively suppresses precore RNA transcription *(54)*. In addition, naturally occurring core promoter mutants show selective defects in precore mRNA synthesis *(2)*. Since these transcripts serve entirely different purposes, these findings raise the possibility that the ratio of these transcripts can be regulated during the infectious cycle.

The preS1 promoter (also called SI promoter) lies just upstream of the surface open-reading frame and gives rise to 2.3-kb-long transcripts that are translated into the large surface protein. This promoter shows strong hepatocyte-specificity, since its activity is highly dependent on the hepatocyte-enriched transcription factor HNF1. The middle and small surface proteins, despite being N-terminally truncated forms of the large surface protein, are not derived from this protein by proteolytic processing. Rather, just like the core protein, they are primary translation products derived from shorter transcripts. However, in this case, these transcripts are derived from an entirely separate promoter far downstream of the preS1 promoter. This S promoter (also called preS2 or SII promoter) gives rise to multiple mRNA species with 5' end heterogeneity. These ends straddle the ATG-initiating codon for the middle surface protein. Thus, the largest transcripts code mainly for the middle surface protein, while the other transcripts code only for the small surface protein. Because most of the transcripts start downstream of the middle surface protein initiating codon, and because this codon is in a poor context for translational initiation, more small surface protein is synthesized than middle surface protein. The S promoter by itself is not strictly cell-type specific, showing approx 10 times higher activity in hepatocytes than in nonhepatocytes. In the context of the entire HBV genome, it shows much stricter hepatocyte-specificity, presumably because of its activation by enhancer I, which shows high-level activity only in hepatocytes.

The amount of preS1 transcripts is normally much lower than the amount of S transcripts, both in infected livers and in cultured cells transfected with the whole HBV genome, and this difference results in the relatively low synthesis of large surface protein. Interestingly, the preS1

promoter per se is almost as strong as the S promoter when used individually to drive heterologous reporter genes. Therefore, there must be additional factors that account for the striking difference in steady-state transcript levels. Indeed, it has been shown that sequences in the S promoter can negatively regulate preS1 transcripts at the posttranscriptional level (C. C. Lu, personal communication). Preliminary experiments indicate that transcriptional elongation is blocked by these sequences. Thus, mutation or deletion of a portion of the S promoter not only decreases the amount of S transcripts but also increases the amount of preS1 transcripts. The resulting change in surface protein expression has important consequences not only for the virus but also for the host cell (*see* below).

The X promoter is just downstream of enhancer I, and its 0.7-kb transcripts can be translated into the X protein, which is a transcriptional transactivator (*see* below). However, while the X promoter seems to be highly active in transfected hepatoma cells, the amount of X transcripts in infected livers is below the sensitivity of Northern blotting. The basis for this difference is unknown. It is possible that hepatoma cells are lacking in (or have too much of) a factor that controls the X promoter or that X protein negatively regulates its own transcription in normal hepatocytes. Another unusual feature of the X transcripts is that in transfected cells, approx one-half of them terminate at the polyadenylation signal only at the second pass; that is, like the core promoter transcripts, they are greater than genome-length. Whether a similar phenomenon occurs in vivo is not clear, since these long transcripts are difficult to resolve from the core promoter transcripts by Northern blotting. Indeed, if these transcripts constitute a majority of the X transcripts in vivo, this would provide an alternative explanation for the lack of detectable 0.7-kb X transcripts in infected livers.

Finally, transcripts initiating from within the X open-reading frame have been found in transfected cells, which can give rise to truncated X proteins initiating at internal in-frame ATG codons. Since unique functions have been ascribed to these small X proteins, at least in transfected cells, these transcripts may well play a role in the HBV life cycle, although there is no evidence regarding their presence in the infected liver.

Posttranscriptional regulation of gene expression has recently been described for HBV. Two groups independently found that the steady-state amount of the S transcripts in the cytoplasm decreases substantially (>five-fold) when the X gene region is deleted, placed in the reverse orientation, or placed downstream of the polyadenylation signal, without any significant effect on nuclear transcript levels or the RNA half-life in the cytoplasm *(17,19)*. Therefore, this region appears to function at the RNA level to allow export of S transcripts from the nucleus to the cytoplasm. Indeed, this region,

which is named the posttranscriptional regulatory element (PRE), can partially replace the rev-response element of human immunodeficiency virus type1 (HIV-1), which has been shown to effect mRNA export. The PRE does not depend on any viral gene products for its function and has been shown to bind cellular proteins in a specific manner. Thus, the PRE presumably utilizes a cellular pathway to facilitate RNA export. The identity of these cellular proteins, and the mechanism of their mode of action, remain to be determined.

2.4. Viral Proteins

The core protein is a cytosolic protein that forms a stable dimer even at very low concentrations. At higher concentrations, these dimers self-assemble to form nucleocapsids. During the infective cycle, these capsids contain the HBV pregenomic RNA as well as the associated polymerase. However, if core protein is expressed without these other components, nucleocapsids are still formed, although these contain an apparently random mixture of cellular mRNAs. Since nucleocapsids readily form in bacteria and yeast, it is unlikely that any cellular proteins are involved in their formation. The C-terminal tail of core protein contains a large number of arginine residues and hence is highly basic, and comprises the RNA-binding domain. Unlike the capsid (gag) proteins of retroviruses, core protein does not show sequence-specific binding to RNA, and the specificity of encapsidation of the HBV pregenomic RNA appears to be conferred by the polymerase protein.

The C-terminal tail of core protein also functions as a nuclear localization signal, to allow import of core protein into the nucleus. The functional importance of this import is unclear, although core particles can be found in large numbers in the nucleoplasm of some infected hepatocytes in human liver biopsies. One possibility is that this import function is necessary for bringing the infecting genomic DNA into the nucleus in nonmitotic cells (such as normal hepatocytes). Another possibility, not mutually exclusive of the first, is that core protein may have an additional function in the nucleus, since core protein can bind DNA as well as RNA. It is also unclear whether core protein enters the nucleus as a dimer or as a particle. While data favoring the former possibility have been produced in transgenic mice *(12)*, core particles are small enough to fit through nuclear pores and have indeed been found within the nuclear pores of yeast cells *(49)*.

The precore protein has a signal sequence at the amino-terminus and hence is directed to the ER cotranslationally. A significant portion of the precore protein then is translocated entirely into the ER lumen, where it is processed and secreted as the e antigen (HBeAg), which can be found circu-

lating in the serum of people with active HBV replication in the liver. The remainder of the precore protein has two fates, at least in cultured cells. Some remain as a transmembrane protein and get transported to the cell surface, while the rest can slip back into the cytosol. A portion of this cytosolic precore protein can also be transported into the nucleus. Therefore, precore protein and its derivatives are found in multiple intra- and extracellular compartments. Unfortunately, the precise function of none of these forms is known, despite the fact that all hepadnaviruses express the precore protein. Precore mutants of HBV arise frequently during natural infections, and both the duck and woodchuck hepadnaviruses productively infect their respective hosts even when the precore open-reading frame is mutated to prevent protein expression. Clearly, precore protein is not necessary for viral replication per se, but its conservation suggests that expression of this protein confers a selective advantage on hepadnaviruses. One possibility that is supported by data from both infected people and woodchucks is that precore protein favors the establishment of chronic infections *(30)*. Thus, mothers who are e antigen positive are much more likely than e antigen negative mothers to have children who show chronic infection. Similarly, in a woodchuck experiment, all five neonates infected with a precore-minus woodchuck hepadnavirus mutant were able to clear the virus, in contrast to 20 cohorts infected with the wild-type virus, only six of which cleared the virus. Since chronically infected individuals are the major source of transmission of HBV, precore deficient viruses would quickly die out in nature because of the absence of secondary infections. How precore protein may confer this property on HBV is unknown. It has been suggested that perhaps e antigen acts to suppress the immune response to HBV, which is responsible for viral clearance. In addition, precore protein has been recently shown to downregulate HBV replication, probably by forming heteromultimers with core protein and interfering with nucleocapsid formation. Since defective interfering particles that attenuate replication of RNA viruses are known to facilitate persistent infections, it is possible that precore protein performs a similar function for HBV. Interestingly, HBV mutants that make little or no precore protein arise with high frequency during chronic infection, and the presence of these mutants has been associated with greater disease severity (*see* Subheading 5.).

The polymerase protein is the other virally encoded protein component of the nucleocapsid. It is a bipartite protein, consisting of an N-terminal polymerase domain and a C-terminal reverse transcriptase and RNase H domain. There is a fairly large intervening domain that serves no apparent function and hence has been named the spacer. This domain overlaps the preS1 and preS2 regions of the surface open-reading frame, so it is possible

that the sole reason for its existence is to allow expression of middle and large surface proteins. Since the core protein shows no sequence specificity in its binding to RNA, the polymerase protein, either alone or in combination with core protein, must contain the recognition domain for HBV pregenomic RNA. Interestingly, the polymerase shows a strong tendency to act in *cis*; that is, it preferentially reverse-transcribes the same RNA from which it has been translated. This result suggests that perhaps a translation intermediate of polymerase (in terms of primary, secondary, or tertiary structure) is the form that actually binds the pregenomic RNA. This interpretation is strengthened by the observations that polymerase protein in vitro cannot act on exogenously added substrates and that cytosolic chaperone proteins are necessary for duck hepadnaviral replication.

The RNA element that is recognized by polymerase is a stem-loop structure with a bulge, named "epsilon," which is present near the 5' end of pregenomic RNA. Because of the terminally redundant nature of this RNA, however, it is also present at the 3' end. Therefore, it is also present at the 3' end of all HBV transcripts, which contain a common 3' end. Yet only the pregenomic RNA is packaged. The basis for this selectivity is as yet unclear. An even finer level of selectivity is also present, in that precore transcripts, which are identical to pregenomic RNA except for a short 5' extension, are also not encapsidated. Data from duck hepadnavirus studies indicate that the crucial difference is that the epsilon sequence is translated (as part of the precore protein) in the precore transcripts but not in the pregenomic RNA. Apparently, the transit of ribosomes prevents polymerase recognition of epsilon, either by direct physical blockage or by changing the secondary structure.

As stated above, three forms of the surface protein are synthesized by HBV. All three are targeted to the ER by an internal signal sequence, where they assume the configuration of a transmembrane protein. These proteins are linked by disulfide bonds into homo- or heterodimers, which then self-aggregate into large polymers *(45)*. The polymers can be linked by additional disulfide bonds in some cell lines, although these additional bonds are not necessary for morphogenesis. The N-terminal region of the large surface protein, including the preS1 and preS2 domains and a portion of the S domain, remains on the cytosolic face of the ER, where it is available for interaction with nucleocapsids containing single-stranded or partially double-stranded HBV DNA. Recent data indicate that segments of both the preS1 and S domains bind to the nucleocapsid *(32)*. The nucleocapsid is then enveloped by the surface proteins and host lipids and buds into the lumen, to be secreted by the constitutive secretory pathway.

Unlike the envelope proteins of most other viruses, the middle and small surface proteins are secretion-competent in the absence of the nucleo-

capsid or any other HBV proteins. They form subviral or surface protein particles, in a morphogenetic pathway similar to that of complete virions. These particles then rapidly exit the cell via the constitutive secretory pathway. In contrast, the large surface protein by itself never gets secreted out of the cell. While it is capable of extruding into the lumen of the distal ER or intermediate compartment to form intraluminal subviral particles, these particles accumulate in these intracellular compartments, probably because they are held back by cellular chaperones such as calnexin *(47)*. Mixed particles of large surface protein with the other forms behave according to the relative amounts of the various components. Particles with a small proportion (less than 20%) of large surface protein are secreted, but particles with larger amounts are not.

The overproduction of the large surface protein, leading to intracellular accumulation of surface protein particles, is not an uncommon event during chronic HBV infection. Indeed, the presence of these cells (called "ground glass cells" because of the histological appearance of their cytoplasm) was used by pathologists as an early marker for chronic hepatitis B, before the availability of specific diagnostic reagents. Ultrastructurally, ground glass cells contain filamentous surface protein particles within dilated smooth vesicles, representing smooth ER and/or an intermediate compartment. Chisari et al. *(6)* have shown that transgenic mice that overexpress large surface protein contain numerous ground glass cells in the liver, confirming that this overexpression is the primary cause of ground glass cell formation. Overexpression of large surface protein also has a dramatic impact on cellular gene expression and physiology that has implications for pathogenesis of HBV-related diseases (*see* Subheading 3.). The cause of the overexpression during human infections is as yet unknown. It is likely that viral mutants are involved, since there is a high frequency of mutations in the S promoter region that results in large surface protein overexpression in cultured cells transfected with these mutants.

It appears that the preS1 domain of large surface protein, which bears the apparent site of interaction with the cellular HBV promoter, is exposed on the surface of large surface protein particles. Therefore, it is not surprising that large surface protein particles are not secreted, as they would presumably bind to the receptor and act as defective interfering particles. The interesting question is why subviral particles comprising small and middle surface proteins are secreted in such large amounts, since for essentially all other viral families, envelope proteins are not efficiently secreted by themselves. It is possible that these particles bind up host antibodies to surface proteins and hence act as decoy particles. Another intriguing possibility,

raised by recent preliminary data, is that subviral particles actually increase the efficiency of HBV infection, perhaps by altering host cell physiology (H. Will, personal communication).

Another unusual aspect of the large surface protein is that its N-terminus changes its orientation, relative to the membrane, at some stage between completion of translation and its extrusion into the lumen *(29)*. When first translated, the N-terminus is present on the luminal side of the ER membrane, which is topologically equivalent to the inside of the viral envelope. Yet, by the time either viral particles or large surface protein particles form, at least 50% of the N-termini are exposed on the particle surface, that is, on the outside of the envelope. The mechanism of this posttranslational translocation is unknown, but there is a clear need for it to occur for the viral life cycle. The N-terminus of the large surface protein is necessary both for virion morphogenesis (i.e., interaction between the envelope proteins and capsid particles) and for binding to the putative HBV receptor. For the first function, it must be on the cytosolic face of the membrane, since nucleocapsids reside in the cytosol. Yet for the second function, it must be on the opposite face, since the receptor can only bind to sequences present on the virion surface. Both are not mutually exclusive, thus accounting for at least 50% of the N-termini exposed on the particle surface. Further studies of this phenomenon are warranted, since it may provide new insight into the biogenesis of cellular membrane proteins.

The final protein product of HBV is the X protein, so named because of the mystery surrounding its role in the HBV life cycle. While it is capable of *trans*-activating a wide variety of heterologous promoters in cultured cells and probably also in vivo, it *trans*-activates HBV gene transcription only modestly and only in selected cell lines *(53)*. Furthermore, the duck hepadnavirus does not carry an X gene homolog. Nevertheless, studies with the woodchuck hepadnavirus have clearly demonstrated that the X protein performs an essential function, since any mutation that prevents X protein synthesis blocks even the slightest trace of infection in woodchucks. What this function might be is largely a matter of speculation. Activation of viral gene expression is certainly a strong candidate, but may not be the full answer. Thus, while studies in transgenic mice have shown a significant decrease in HBV gene expression in hepatocytes in the absence of X protein, the block is far from absolute, unlike the block in the replication of X-minus woodchuck hepadnaviruses. Other possible roles for X protein include activation of cellular genes necessary for viral replication, repair of the gaps in the viral genome *(35)*, and even perturbation of proteosome function *(20)*. Firm answers await further detailed analyses in transgenic mice as well as a more tractable animal model of HBV infection.

Further complicating the picture is the likelihood that multiple forms of X protein are synthesized. Data from several groups have shown that one or possibly two smaller forms of X protein can be translated either from the full-length X transcript or from short transcripts derived from an internal X promoter, and that these forms have different *trans*-activation functions from full-length X protein. The role, if any, of these other forms of X protein during natural infection is unknown and can only be clarified by studies of additional X mutants in woodchucks.

2.5. *Genome Replication*

After encapsidation of the pregenomic RNA by the polymerase and core proteins, reverse transcription of the RNA commences. The polymerase makes a short oligodeoxynucleotide *in situ* at the epsilon site, in a unique self-priming reaction that uses a tyrosine residue as the donor of the initiating hydroxyl group. Thereafter, a series of complicated translocations ensue, to ensure that a circular double-stranded DNA is synthesized as the final product. Briefly, the polymerase and the covalently attached oligodeoxynucleotide move to a sequence known as direct-repeat 1 (DR1) near the 3' end of the pregenome, by virtue of a short stretch of homology between epsilon and DR1. Elongation of the nascent minus strand DNA then continues until the exhaustion of template at the 5' end of the pregenome. Simultaneously, the RNaseH activity of polymerase degrades all but the very 5' end of the pregenome. This small RNA molecule then serves as the primer for plus strand DNA synthesis after moving to the 5' end of the minus strand DNA. It can hybridize to this portion of the DNA because of the presence of another copy of the direct repeat, called direct repeat 2 (DR2). The polymerase then synthesizes the plus strand DNA, using first the 5' end of minus strand DNA as template, then jumping to the 3' end to allow synthesis of a complete copy. This last feat is possible because of the short stretch of terminal redundancy at the ends of minus strand DNA, resulting from the placement of the core promoter immediately upstream of the polyadenylation signal. The endproduct of all these molecular acrobatics is a nicked, circular double-stranded DNA with the polymerase protein attached to the 5' end of the minus strand DNA and an RNA primer at the 3' end of the plus strand DNA.

Because the HBV polymerase is mechanistically quite different from eucaryotic DNA polymerases, it is an attractive target for therapeutic compounds to block viral replication in chronically infected individuals. Indeed, many different nucleoside analogs have been shown to have antiviral activity. Unfortunately, in clinical trials, one turned out to be highly toxic because of cross-inhibition of the mitochondrial DNA polymerase. Nevertheless, it

is likely that nontoxic and effective HBV polymerase inhibitors or antiviral drugs such as lamivudine (3TC) have clinical utility.

Recently, it has been shown that the RNA binding and priming activity of the duck hepadnaviral (and probably also HBV) polymerase protein depends on its association with Hsp90, p23, and perhaps other chaperone proteins. At least some of these proteins become incorporated into the nucleocapsid and, hence, the virion particle. It is likely that these chaperones also assist the polymerase in switching between various metastable conformations necessary for its diverse functions (RNA binding, priming, strand elongation, template switching, RNA primer translocation, etc.). Therefore, compounds that block the function of these chaperones may constitute another class of viral inhibitors of possible clinical utility.

As soon as the nucleocapsid forms, it has the theoretical capacity to interact with the surface proteins in the ER to bud into the lumen. However, recent experiments have shown that only nucleocapsids with at least the minus strand DNA synthesis completed are efficiently secreted *(11)*. Thus, there must be a signal (called by some a maturation signal) that is transmitted from the polymerase and/or attached DNA, indicating that the minus strand has been synthesized. The nature of this signal is unknown, but the reason for its existence is probably to make the virion genome more stable, since RNA is much more labile than DNA. Indeed, HBV is more hardy and transmissible than retroviruses, such as HIV-1, and part of this difference may be attributable to the difference in genome chemistry. Since the synthesis of the plus strand DNA is not necessary for the maturation signal, the plus strand DNA in virions is usually incomplete, presumably because of the exhaustion of nucleotide precursors in the virion. These features of HBV replication explain the unusual structure of the genomic DNA found in virions.

A proportion of the nucleocapsid particles do not bud at the ER to form virions, but instead transport the viral DNA back into the nucleus of the host cell. This recycling pathway is important in maintaining infection, as otherwise, cell division and perhaps nuclease degradation would slowly eliminate the episomal viral DNA. It is estimated that each infected cell maintains 10–20 copies of CCC DNA in the nucleus of the typical infected hepatocyte.

HBV replication does not involve integration of the viral genome into the host chromosome, but such integrations, almost invariably associated with large deletions or rearrangements of the genome, commonly occur in infected hepatocytes, even early in infection. The integrated viral DNA can still be transcriptionally active, giving rise to viral proteins (usually the surface proteins, since the surface gene is most frequently left intact) even after episomal DNA has been eliminated. However, over extended periods of time, for unknown reasons, many of these integrants frequently become transcrip-

tionally silent, and HBV gene expression ceases. The role of such integrants is possibly insignificant in human hepatocellular carcinoma (HCC) (*see* Subheading 3.4.).

3. Clinical Disease

3.1. Introduction

HBV usually causes mild or even no disease symptomology with acute infections, but a substantial proportion of infected people fails to clear the virus and become chronically infected *(14)*. These individuals, often termed chronic carriers of HBV, are then at risk for the development of chronic hepatitis, cirrhosis, liver failure, and even HCC. While a highly effective vaccine is now available (*see* below), this is of no assistance for the estimated 300 million people who are already infected worldwide, of whom at least 1 million die annually of HBV-induced liver diseases. In addition, only a small percentage of people at risk get vaccinated, because of economic and sociological reasons. For example, in the United States alone, more than 200,000 new infections still occur every year. Therefore, HBV continues to be a major health problem. The fact that HBV is a major sexually-transmitted infection is underappreciated in the United States. Acute HBV infection is fully resolved in 90–95% of the infected adults, who remain immune for their lifetime. In contrast, as many as 80% of the babies born to HBV-infected mothers are perinatally infected and remain persistently infected. The spectacular success of the HBV vaccine in preventing the perinatal transmission of HBV has justifiably raised expectations that universal vaccination will lead to a dramatic decline in HBV infection and consequent decline in chronic liver disease and HCC. Unfortunately, the high cost of HBV vaccine is an impediment to worldwide implementation of massive immunization programs.

3.2. Acute Infection

While symptomatic acute hepatitis can occur following exposure to HBV (after an incubation period of 45–120 d), the incidence is relatively low. It is also dependent on the age of the person. Thus, for preschool children, it is extremely rare for signs or symptoms to be apparent. With increasing age, the incidence increases correspondingly, and for adults, approx 25% can show nonspecific symptoms such as fatigue, anorexia, myalgia, low-grade fever, and/or signs of hepatic dysfunction, principally jaundice and hepatomegaly. Very rarely, the hepatitis is fulminant, with acute liver failure leading to hepatic encephalopathy, hepatorenal syndrome, and bleeding diathesis. Mortality is high (>75%), and again shows a positive correlation

with age. Extrahepatic manifestations of acute HBV infection are mainly the result of antibody-antigen complex formation and can be seen in up to 20% of infected people. The main disorders include a serum sickness-like syndrome, polyarteritis nodosum, and papular acrodermatitis of childhood. An immunomodulatory role or immunopathogenetic role of immune complexes has been implicated in progression of acute HBV infection to persistent infection. However, without critical molecular analysis of the viral antigens and isotypes of antibodies in immune complexes purified from the serial serum samples of patients with acute hepatitis with and without resolving HBV infection, the significance of immune complexes in modulation of the host response largely remains enigmatic and anecdotal.

Although HBV caused a significant proportion (20–25%) of acute icteric posttransfusion hepatitis (PTH) prior to introduction of routine screening of donated blood for HBsAg in 1971, non-A, non-B PTH was recognized as the major cause of PTH, with propensity to develop serious chronic liver disease. The discovery of HCV as the major cause of non-A, non-B PTH and introduction of routine anti-HCV screening of donated blood has dramatically declined PTH. As a result of contemporary screening of blood donations for HBsAg, anti-HBc, anti-HCV, and ALT, PTH has been virtually eliminated. It is estimated that occult transmission of HBV infection may be occurring in 0.15% of transfused patients, and a similar transmission rate may exist for HCV because of the viremic window period before seroconversion that is detected by EIA screening of donors' blood *(26a)*. Therefore, blood-borne virus infections in donated blood may possibly be detected by gene amplification technology beginning about the year 2000.

Acute HBV infection is clinically indistinguishable from other viral etiologies of acute hepatitis, and only laboratory diagnosis by serological tests for other viral agents can help differentiate the multiple etiologies of acute HBV infection (discussed elsewhere in this volume).

3.3. Chronic HBV Infection

Following acute infection, most infected people mount a successful immune response against HBV and clear the virus from their liver. There is also long-term seropositivity for antibodies against both the core and surface proteins. The former provides a marker for previous exposure, while the latter indicates the presence of protective immunity against new infections. However, a substantial minority of HBV-infected individuals fail to clear the virus and become chronically infected (defined as surface antigenemia persisting for greater than 6 mo). These people also produce anticore antibodies, but have no antisurface antibodies. Interestingly, there is a strong

negative correlation of the likelihood of chronicity with the age at infection. Thus, a large majority (approx 80%) of infants infected at or before birth become chronically infected. On the other hand, less than 10% of infected adults develop chronic infection. It is these chronically infected people who have become, by far, the major source of infection for further spread of HBV in the population.

Many of the chronically infected people are asymptomatic and also show little or no chemical signs of hepatitis. Biopsy shows either histologically normal liver or mild mononuclear infiltration of the portal tracts without piecemeal necrosis (so-called chronic persistent hepatitis). These people usually have good prognosis, with 5-yr survival of 97%. However, approximately one quarter of those with chronic HBV infection show active hepatitis, which may be asymptomatic, but frequently gives rise to anorexia, malaise, and chronic fatigue. The serum ALT and gamma-glutamyltransferase levels are moderately-to-markedly elevated, with variable increase in serum bilirubin. When biopsied, these people show the presence of piecemeal necrosis, lobular inflammation, and acidophile bodies (apoptotic hepatocytes) to variable degrees. With prolonged chronic hepatitis B of sufficient severity, cirrhosis can ensue. The 5-yr survival of these people depends on the severity and chronicity of hepatitis, ranging from 90% for people with mild hepatitis to 50% for those with cirrhosis.

3.4. Hepatocellular Carcinoma

Chronic hepatitis B is a major risk factor for the development of HCC worldwide. For people in Taiwan, the risk of HCC is almost 200 times higher in people with HBV markers than those without. The risk ratio seems significantly lower in Western countries. Whether this difference is because of genetic differences, the younger age of infection in Taiwan (usually perinatal, versus adult in the West), or environmental cocarcinogens such as aflatoxin, is unclear. In any case, HCC is a major cause of death for people with chronic hepatitis B, and conversely, HBV is a major cause of HCC. Worldwide, more than 500,000 people die each year of HCC, with a large majority causally related to HBV.

4. Pathogenesis

4.1. Hepatitis

Because many people who contract HBV infection show no evidence of hepatic dysfunction, and because even among patients with acute hepatitis B the disease manifests itself only several weeks after high-level viremia, it appears that HBV by itself is noncytopathic. This conclusion has been confirmed by studies in transfected cultured cells and in animal models.

Instead, the host immune response plays a major role in causing hepatocellular necrosis. The consensus in the field is that class I MHC-restricted cytotoxic T-lymphocytes (CTLs) with specificity against HBV core and/or precore protein are important for viral clearance *(7)*. Classically, therefore, it has been thought that the CTLs, perhaps together with cytokines and recruited, nonantigen specific leukocytes, kill infected hepatocytes and thereby clear the virus.

Recently, however, transgenic mouse studies have demonstrated that it may not be necessary for CTLs to kill all infected hepatocytes in order for the virus to be cleared *(13)*. These remarkable observations utilized transgenic mice that contain the entire HBV genome and hence produce not only viral gene products but also progeny virions that are secreted into the serum. These mice are, of course, tolerant to all viral antigens and do not show any pathology in the liver or other organs. However, passive transfer of HBV surface-antigen specific CTLs causes an acute hepatitis. The hepatitis is self-limited. Interestingly, soon after CTL transfer, and persisting for many weeks thereafter (though not permanently), HBV transcripts can no longer be detected in the liver, and HBV gene products disappear from the hepatocytes and serum. All this occurs despite the fact that the transgene remains intact and hence competent for transcription. Further analysis has revealed that this effect occurs at the posttranscriptional level and is mediated by several cytokines, including gamma interferon and tumor necrosis factor-alpha. Therefore, in infected people, it is likely that the host immune response can clear HBV from infected hepatocytes without killing the majority of infected host cells (since, unlike in the transgenic mouse, the HBV genome is episomal and would presumably slowly get degraded if not replenished by replication). These results explain why experimentally infected woodchucks can clear the virus and still survive, despite infection of essentially all their hepatocytes. A similar phenomenon must occur in human beings (*see* Fig. 2). This would appear to be an important protective mechanism for the host, so that HBV infection would not routinely lead to fulminant hepatitis. On the other hand, a partially effective immune response may not totally clear the virus, yet would decrease viral antigen expression sufficiently to dampen the immune response temporarily. When the immune response wanes, the remaining viral genomes can resume an active infection, and again elicit an immune response. Therefore, this effect may also allow viral persistence, and may explain the cycles of active disease between periods of quiescence seen in many people with chronic hepatitis B. In any case, the ability of cytokines to shut down HBV gene expression and replication raises the hope that these reagents, or modified forms thereof, may be used in the future to cure chronic HBV infection without causing massive hepatic damage.

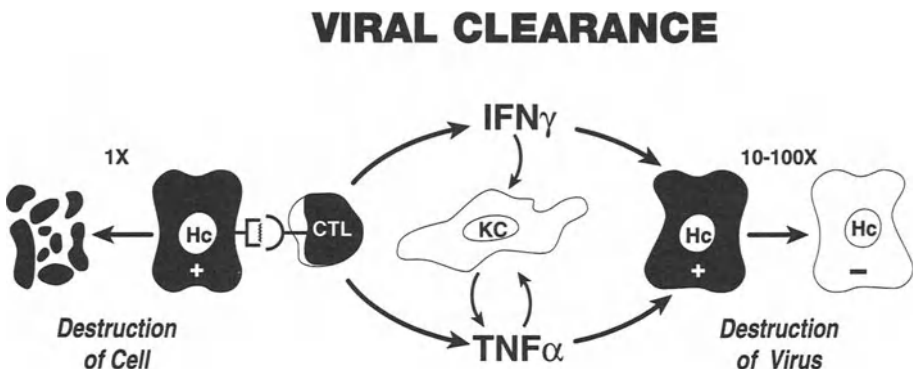


Fig. 2. Model of how HBV is cleared from the infected liver. Cytotoxic T-lymphocytes (CTLs) that recognize viral antigens expressed on the hepatocyte (Hc) directly destroy a minority of infected hepatocytes. However, the release of cytokines such as interferon-gamma (IFN) and tumor necrosis factor-alpha (TNF), with signal amplification by Kupffer cells (KC), leads to destruction of HBV in the majority of infected cells without cytotoxicity. (Courtesy of Dr. Francis V. Chisari, Scripps Research Institute, La Jolla, CA.)

4.2. *Hepatocellular Carcinoma (HCC)*

The molecular basis for oncogenesis during chronic HBV infection is still a subject of debate. In the woodchuck, it is clear that insertional activation of the N-myc proto-oncogene by the hepadnaviral genome is important for HCC formation. However, no consistent pattern of sites of genomic integration in the human chromosome has been demonstrated for HBV, and it seems unlikely that this mechanism is important for the generation of the majority of HBV-related HCCs. Instead, attention has been focused on the X protein, since, as a transcriptional *trans*-activator, it can presumably influence host cell gene expression and hence proliferation and/or differentiation. Indeed, one group has reported that transgenic mice expressing high levels of X protein develop HCC *(22)*. However, these mice were derived from a strain that shows fairly high levels of spontaneous HCC development. Furthermore, other groups have generated X-transgenic mice that do not develop HCC, even in old age. Significantly, at least two of these latter mouse strains do show increased susceptibility to hepatic carcinogenesis induced by other agents (chemical carcinogens in one case, N-*myc* proto-oncogene in the other) *(36,37)*. Therefore, X protein may be a cofactor in

carcinogenesis but is probably by itself insufficient for inducing HCC. This interpretation fits with the observation that most asymptomatic chronic carriers, who presumably express the X protein in their hepatocytes for many years, do not develop HCC.

What may be the cofactor(s) that assist X protein in oncogenesis? One possibility is environmental carcinogens, whose involvement can explain the variation in HBV-associated HCC incidence in different geographic areas. Another possibility is the cycles of hepatocellular injury, death, and regeneration during chronic hepatitis B. Since inflammatory cells can secrete mutagenic compounds, such as reactive oxygen species, and since mitogenesis itself can predispose to mutagenesis, this is a plausible scenario that also fits the observation that HBV-infected people with cirrhosis (presumably reflecting many years of active inflammation) have much higher incidence of HCC than asymptomatic carriers. A third possibility is that other HBV gene products can cooperate with X protein in oncogenesis. Indeed, a truncated form of the HBV middle surface protein *trans*-activates transcription, and even the normal large surface protein, if overexpressed, activate both viral and host gene transcription by causing ER stress *(48)*. While truncation of the middle surface protein rarely occurs during natural infections, overexpression of large surface protein is frequently seen in chronic infection, resulting in the formation of "ground glass" hepatocytes in the liver. Furthermore, Chisari et al. *(6)* have shown that transgenic mice that overexpress large surface protein suffer from chronic hepatitis and then develop HCC. While this observation may be explained by the recurrent injury, death, and regeneration occurring in the liver, it is instructive to note that other (non-HBV) transgenic mice with chronic hepatitis do not develop HCC, even late in life *(24,40)*. Therefore, it is likely that overexpression of large surface protein can also play a specific role in carcinogenesis, at least in the transgenic mouse model. The importance of large surface protein in human oncogenesis remains to be defined. Finally, a recent report found increased microsatellite instability in HBV-related cirrhosis *(33)*. Since genomic instability is a hallmark of malignancies, this is an area that deserves further exploration.

In summary, our knowledge of the basis for HCC formation during chronic HBV infection is still incomplete. It is likely that multiple mechanisms are involved, with a complex interplay of viral factors (X protein, large surface protein, insertional mutagenesis), host response (immune destruction and hepatocyte regeneration, genomic instability), and environmental insults (chemical carcinogens), not all of which may operate in the genesis of any single case of HCC.

5. Immunobiology of HBV and Host Response

Several viral antigens and host antibodies are useful as serological markers in the diagnosis of HBV infection *(14)*. The HBsAg determinants are carried by the surface proteins. The e antigen (HBeAg) arises from a post-translational cleavage of precore protein and generally reflects a higher level of HBV replication. HBcAg reactivity reflects the presence of intact core protein. In HBV infection, the humoral immune response of the host to viral antigens leads to corresponding antibodies, viz. anti-HBs, anti-HBc, and anti-HBe in the serum. The serological diagnosis of HBV infection is established principally by the detection of circulating HBsAg, supplemented by a combination of other serological markers used in the differential diagnosis of acute vs chronic infection or prior exposure with resolution of infection.

5.1. HBsAg and Anti-HBs

A unique transcriptional property of HBV-infected cells, the synthesis and secretion of more than 1000-fold excess of the S gene product (20-nm virion-free particles), permits serological detection of HBsAg in the serum. High levels of circulating HBsAg, high viremia, HBeAg, and abnormal liver function tests are encountered in patients with histologic evidence of chronic liver disease. Similar concentrations of HBsAg and HBeAg with normal ALT are also encountered among HBV-infected healthy blood donors without any histologic lesions. Therefore, HBV is considered noncytopathic, and the pathogenetic significance of high viremia in liver disease remains uncertain. Testing for HBsAg alone may fail to detect as many as 5–10% of the acute cases, because either the host immune response may have already cleared HBsAg before the onset of clinical symptoms, or the level of HBsAg may be below the detection limit of current methods *(51)*.

There are four major serological subtypes of HBsAg: adw, ayw, adr, and ayr. Although the subtypes are useful as epidemiologic tracers of infection, they are of no pathogenetic significance. Resolution of any HBV infection culminates in production of anti-HBs, a long-lasting antibody response against the "a" determinant(s) of HBsAg shared by 10 different serotypes and conferring lifelong immunity against every serotype of HBV *(1,26,41)*. In contrast, a failure of immune response to HBsAg "a" determinant establishes persistent infection and leads to a spectrum of conditions with/without disease. Most remarkably, the chronic carrier state is characterized by the invariable presence of HBsAg and anti-HBc and the notable absence of anti-HBs, which connotes a specific immunologic tolerance to HBsAg "a" determinant. The precise immune mechanisms underlying the persistence of HBV infection are poorly defined.

5.2. HBsAg-negative Chronic Liver Disease

In chronic liver disease caused by HBV infection, liver biopsy tissues procured for histologic diagnosis are often available for hepatocellular DNA analyses, immunohistochemical detection of HBV gene products, and *in situ* hybridization to quantitate the level of viral replication at a single cell level. Southern blot analyses revealing the replicative forms of HBV DNA in the liver tissues of HBsAg-negative chronic liver disease patients were reported by us *(9)*; this observation is confirmed by the PCR analyses performed with hepatocellular DNA from several HBsAg-negative patients undergoing liver transplantation at the University of California, San Francisco *(46)*. The presence of HBV DNA in the liver tissue of HBV-seronegative patients can be confirmed by a simultaneous molecular analysis of *in situ* PCR-amplified HBV DNA and the immunohistochemical detection of the viral antigens.

5.3. HBsAg Escape Mutants

Because the overlapping S and P genes are translated in different open reading frames, we proposed in 1981 the concept of replication-competent HBV DNA arising from point mutations in the DNA sequence encoding the nine amino acid residues 139–147, which determine the immunoreactivity of the "a" determinant of HBsAg *(41,44)*. Such genetic variants with amino acid substitutions in the nine residues of the HBsAg could bestow a conformational change adequate to escape the otherwise neutralizing anti-HBs. Although recombinant HBsAg used in a monoclonal vaccine against HBV induces potent anti-HBs response and provides protection against HBV infection of all serotypes, Carman et al. have reported a vaccine-induced escape mutant of HBV in successfully vaccinated children who had simultaneously circulating HBsAg and anti-HBs *(5)*. Sequence analysis demonstrated that an escape mutant from one of the children had undergone a glycine-to-arginine substitution at position 145. This substitution may have caused a conformational change in an external loop of HBsAg so that the vaccine-induced anti-HBs could not bind to the mutant HBsAg, thus allowing HBV to replicate in the presence of a normal antibody response against the HBV envelope protein. Similar mutants have been found in Japan, Singapore, and the Gambia, suggesting that the immune escape mutant of HBV can occur worldwide *(15,27,28)*.

Similar escape mutants may arise following immunotherapy after orthotopic liver transplantation. Because HBV reinfection causes frequent loss of the allograft and reduced patient survival, immunotherapy with hepatitis B immune globulin (HBIg) has been used to reduce the rate of recurrence. However, continued immunotherapy after 12 mo of treatment leads to escape mutants in 20% of the patients, with frequent mutation in the codon

for arginine at amino acid 145 in the B cell epitope of HBsAg *(4,38)*. Lamivudine, an inhibitor of HBV polymerase, has been used as an alternative to prevent reinfection, but treatment failures have occurred as a result of escape mutants frequently induced in the YMDD locus of the polymerase gene *(31)*. Considering the distinct mechanisms of antiviral action of HBIg and lamivudine, a combination therapy is logical and is in an early stage of clinical trials.

5.4. HBeAg and Anti-HBe

The C gene of HBV contains two in-frame translation initiation codons (ATGs). When the protein synthesis is initiated at the first ATG, a 25-kDa precore protein is generated. On entry into the endoplasmic reticulum and proteolytic cleavage at both the C-terminus and the N-terminus, the degraded composite protein reveals an HBeAg determinant that has been used as a marker of virus replication, as a correlate of patient's infectivity, and is an indicator of the severity of liver disease. Generally, seroconversion from HBeAg positivity to anti-HBe suggests a decline in virus replication and/or consequent improvement in liver disease. Simultaneous occurrence of HBeAg and anti-HBe is uncommon and transient during seroconversion. However, the diagnostic interpretation of the patterns of HBeAg and anti-HBe in HBV infection has recently undergone a significant reevaluation as a result of the identification of precore mutants *(30)*. Molecular analyses demonstrated that individuals with anti-HBe may in fact have viremia with mutated HBV DNA having a single base pair substitution at position 1986 in the precore region, converting the triplet codon at that site into a stop codon and disabling production of HBeAg. Many patients with this mutant form of HBV in their serum have mild disease. However, reports also suggest that the precore mutants cause severe hepatitis and frequent occurrence of fulminant hepatitis. Similarly, mutants with mutations in the core promoter that synthesize decreased amounts of precore protein also have been implicated in severe hepatitis among patients with anti-HBe in Japan but not in the United States. This controversy only highlights our inadequate understanding of the immunopathologic mechanisms underlying hepatocellular injury in viral hepatitis and liver disease. The pre-core mutants appear to be more common in the Mediterranean and Far Eastern countries than in the United States. Thus, a proper interpretation of HBeAg and anti-HBe patterns is not well defined.

5.5. Anti-HBc as a Marker of HBV Infection

HBcAg is not detectable in the serum throughout the natural history of HBV infection. It is detected only after purification of HBV particles and

removal of the lipid envelope with a detergent. Anti-HBc is detectable in virtually all patients with HBV infection within 1–2 wk after the appearance of HBsAg and just before the onset of symptoms. During the core window of the acute stage, between the loss of serum HBsAg and appearance of anti-HBs, IgM anti-HBc and HBV DNA may be the only detectable markers. Some patients with acute or chronic HBV infection have only anti-HBc without detectable HBsAg in serum *(50,51)*. Detection of IgM anti-HBc is the standard for diagnosis of acute hepatitis, although lower titers of IgG anti-HBc are present concurrently. The presence of IgM anti-HBc in serum diluted 1:5000 may indicate recent acute hepatitis B. The absence of IgM anti-HBc in 1:5000 dilution of serum excludes a diagnosis of acute hepatitis B with virtual certainty *(21)*. Although widely used in Korea and Japan, this test algorithm is not routinely used in laboratory practice in the United States. Total anti-HBc is detectable in almost all patients with acute or chronic HBV infection. The titer and percentage of IgG anti-HBc continue to rise during early convalescence. However, there is no significant evidence to show that anti-HBc offers any immune protection. Isolated anti-HBc reactivity in serum may occur in three different settings:

1. False positive reactions are caused by weak signals of anti-HBc positivity because of the presence of IgM-like components in the test specimen.
2. Some patients with chronic HBV infection have detectable anti-HBc and undetectable HBsAg in serum.
3. Some cases have a prolonged window period in which HBsAg has disappeared, and the only detectable markers are anti-HBc and anti-HBs, which often take several months to years to become detectable.

5.6. Anti-HBc Screening of Blood Donors

Anti-HBc testing was introduced in the United States as a surrogate marker to identify units of donated blood that posed an increased risk of transmitting non-A, non-B hepatitis, AIDS, and occult HBV infection *(42)*. Before the discovery of HCV and the development of specific tests, anti-HBc screening for blood donors was estimated to reduce the incidence of posttransfusion non-A, non-B hepatitis in United States by up to 40%. Our own studies on detection of HBV DNA show that cryptic HBV infections exist in subjects whose only serologic marker of HBV is a high titer of anti-HBc *(50)*. However, with adequate HBsAg and anti-HCV screening already available, whether an additional anti-HBc test for the prevention of transfusion-transmitted HBV and HCV infection is required or not remains a controversial issue. In Taiwan, an area of high HBV prevalence, PCR revealed HBV DNA in 7 out of 85 *(8%)* serum samples that were HBsAg-negative in a radioimmunoassay but contained only anti-HBc *(34,43)*. Because HBV

DNA was not detected by PCR in 158 HBsAg-negative but anti-HBc-positive blood units in the United States, Douglas et al. concluded that anti-HBc testing for potential hepatitis B infectivity is an inefficient means of screening blood donors in areas of low endemicity *(8)*. However, a 1995 consensus panel statement issued by the National Heart, Lung, and Blood Institute recommended that anti-HBc be retained to screen donated blood in order to prevent HBsAg-negative HBV transmissions, and because it might also prevent some window-period HIV transmissions.

In summary, the host-virus interaction has been fairly well defined by serological markers of HBV infection and immunity. However, our understanding of the biological mechanisms underlying establishment of persistent HBV infection remains rudimentary despite the recent progress made in our understanding of the pathogenesis of liver disease, largely through a series of systematic investigations by Chisari and his colleagues *(7)*. PCR is increasingly employed in molecular approaches to laboratory diagnosis and monitoring efficacy of newer therapeutic agents, such as lamivudine *(38)*. The observation of naturally evolving mutants under immune pressure of anti-HBs in vaccinated subjects, or in liver transplant patients treated with HBIg, poses a question whether the widely used monoclonal recombinant HBsAg vaccine should be augmented by other serotypes or not. In coming years, we should learn a lot more about HBV biology and its interaction with the host, especially persistent HBV infection and its oncogenic sequelae.

Acknowlegment

This work was supported in part by NIH Grant HL 53384 and UCSF Liver Center Grant P30-DK26743.

References

1. Bhatnagar, P. K., Papas, E., Blum, H. E., Milich, D. R., Niecki, D., Karel, M. J., and Vyas, G. N. (1982) A synthetic analogue of hepatitis B surface antigen sequence 139–147 produces immune response specific for the common a determinant. *Proc. Natl. Acad. Sci. USA* **79,** 4400–4404.
2. Buckwold, V. E., Xu, Z., Chen, M., Yen, T. S., and Ou, J. H. (1996) Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J. Virol.* **70,** 5845–5851.
3. Buckwold, V. E., Chen, M., and Ou, J. H. (1997) Interaction of transcription factors RFX1 and MIBP1 with the gamma motif of the negative regulatory element of the hepatitis B virus core promoter. *Virology* **227,** 515–518.
4. Carman, W. F., Trautwein, C., van Deursen, F. J., Colman, K., Dornan, E., McIntyre, G., Waters, J., Klum, V., Müller, R., Thomas, H. C., and Manns, M. P. (1996) Hepatitis B virus envelope variation after transplantation with and without hepatitis B immune globulin prophylaxis. *Hepatology* **24,** 489–493.

5. Carman, W. F., Zanetti, A. R., Karayannis, P., Waters, J., Manzillo, G., Tanzi, E., Zuckerman, A. J., and Thomas, H. C. (1990) Vaccine-induced escape mutant of hepatitis B virus. *Lancet* **336,** 325–329.
6. Chisari, F. V., Klopchin, K., Moriyama, T., Pasquinelli, C., Dunsford, H. A., Sell, S., Pinkert, C. A., Brinster, R. L., and Palmiter, R. D. (1989) Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. *Cell.* **59,** 1145–1156.
7. Chisari, F. V. (1997) Cytotoxic T cells and viral hepatitis. *J. Clin Invest.* **99,** 1472–1477.
8. Douglas, D. D., Taswell, H. F., Rakela, J., and Rabe, D. (1992) Absence of hepatitis B virus DNA detected by polymerase chain reaction in blood donors who are hepatitis B surface antigen negative and antibodies to hepatitis B core antigen positive from a United States population with a low prevalence of hepatitis B serological markers. *Transfusion* **33,** 212–216.
9. Figus, A., Blum, H. E., Vyas, G. N., De Virgilis, S., Cao, A., Lippi, M., Lai, E., and Balestrieri, A. (1984) Hepatitis B virus nucleotide sequences in patients with non-A, non-B chronic liver disease. *Hepatology* **4,** 364.
10. Ganem, D. (1996) Hepadnaviridae and their replication, in *Virology*, 3rd. ed., vol. 2 (Fields, B. N. and Knipe, D. M., eds.), Raven, New York, pp. 2703–2737.
11. Gerelsaikhan, T., Tavis, J. E., and Bruss, V. (1996) Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. *J. Virol.* **70,** 4269–4274.
12. Guidotti, L. G., Martinez, V., Loh, Y. T., Rogler, C. E., and Chisari, F. V. (1994) Hepatitis B virus nucleocapsid particles do not cross the hepatocyte nuclear membrane in transgenic mice. *J. Virol.* **68,** 5469–5475.
13. Guidotti, L. G., Ishikawa, T., Hobbs, M. V., Matzke, B., Schreiber, R., and Chisari, F. V. (1996) Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity* **4,** 25–36.
14. Hollinger, F. B. (1996) Hepatitis B Virus, in *Virology*, 3rd. ed., vol. 2 (Fields, B. N., Knipe, D. M., and Howley, P. M., eds.), Raven, New York, pp. 2739–2807.
15. Howard, C. R., Karthigesu, V. D., Allison, L. M. C., Fortuin, M., Mendy, M., and Whittle, H. C. (1994) Hepatitis B virus variants with altered a determinants causing infections in immunized children, in *Viral Hepatitis and Liver Disease* (Nishioka, K., Suzuki, H., Mishiro, S., and Oda, T., eds.), Springer-Verlag, Tokyo, pp. 252–255.
16. Hu, J., Toft, D. O., and Seeger, C. (1997) Hepadnavirus assembly and reverse transcription require a multi-component chaperone complex which is incorporated into nucleocapsids. *EMBO J.* **16,** 59–68.
17. Huang, J. and Liang, T. J. (1993) A novel hepatitis B virus (HBV) genetic element with Rev response element-like properties that is essential for expression of HBV gene products. *Mol. Cell. Biol.* **13,** 7476–7486.
18. Huang, M. and Summers, J. (1994) pet, a small sequence distal to the pregenome cap site, is required for expression of the duck hepatitis B virus pregenome. *J. Virol.* **68,** 1564–1572.
19. Huang, Z. M. and Yen, T. S. (1995) Role of the hepatitis B virus posttranscriptional regulatory element in export of intronless transcripts. *Mol. Cell. Biol.* **15,** 3864–3869.
20. Huang, J., Kwong, J., Sun, E. C., and Liang, T. J. (1996) Proteasome complex as a potential cellular target of hepatitis B virus X protein. *J. Virol.* **70,** 5582–5591.
21. Japanese Red Cross non-A, non-B hepatitis research group (1991) Effect of screening for hepatitis B virus core antibody on incidence of post-transfusion hepatitis, *Lancet* **338,** 1040–1041.
22. Kim, C. M., Koike, K., Saito, I., Miyamura, T., and Jay, G. (1991) HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature* **351,** 317–320.

23. Kock, J., Borst, E. M., and Schlicht, H. J. (1996) Uptake of duck hepatitis B virus into hepatocytes occurs by endocytosis but does not require passage of the virus through an acidic intracellular compartment. *J. Virol.* **70,** 5827–5831.
24. Ku, N. O., Michie, S., Oshima, R. G., and Omary, M. B. (1995) Chronic hepatitis, hepatocyte fragility, and increased soluble phosphoglycokeratins in transgenic mice expressing a keratin 18 conserved arginine mutant. *J. Cell. Biol.* **131,** 1303–1314.
25. Kuroki, K., Eng, F., Ishikawa, T., Turck, C., Harada, F., and Ganem, D. (1995) gp180, a host cell glycoprotein that binds duck hepatitis B virus particles, is encoded by a member of the carboxypeptidase gene family. *J. Biol. Chem.* **270,** 15,022–15,028.
26. Lee, H. S., Ulrich, P. P., and Vyas, G. N. (1991) Mutations in the S-gene affecting the immunologic determinants of the envelope protein of hepatitis B virus. *J. Hepatol.* **13**, S97–101. 1981, 227–237.
26a. Murphy, E. L., Busch, M. P., Jong, M., Cornett, P., and Vyas, G. N. (1998) A prospective study of the risk of transfusion-acquired viral infections. *Transfusion Medicine* **8,** 173–178.
27. Okamoto, H., Yano, K., Nozaki, Y., Matsui, A., Mayazaki, H., Yamamoto, K., Tsuda, F., Machida, A., and Mishiro, S. (1992) Mutations within the S gene of hepatitis B virus transmitted from mothers to babies immunized with hepatitis B immune globulin and vaccine. *Ped. Res.* **32,** 264–268.
28. Oon, C.-J., Lim, G.-K., Ye, Ye Z., Goh, K. T., Tan, K. L., Yo, Yo, S. L., Hopes, E., Harrison, T. J., and Zuckerman, A. J. (1995) Molecular epidemiology of hepatitis B virus vaccine variants in Singapore. *Vaccine* **13,** 699–702.
29. Ostapchuk, P., Hearing, P., and Ganem, D. (1994) A dramatic shift in the transmembrane topology of a viral envelope glycoprotein accompanies hepatitis B viral morphogenesis. *EMBO J.* **13,** 1048–1057.
30. Ou, J. H. (1997) Molecular biology of hepatitis B virus e antigen. *J. Gastroenterol Hepatol,* in press.
31. Perrillo, R., Rakela, J., Martin, P., Levy, G., Schiff, E., Wright, T., Dienstag, J., Gish, R., Villeneuve, J., Caldwell, S., Brown, N., and Self, P., Lamivudine Transplant Study Group (1996) Lamivudine for hepatitis B after liver transplantation (abstract). *Hepatology* **24,** 182A.
32. Poisson, F., Severac, A., Hourioux, C., Goudeau, A., and Roingeard, P. (1997) Both pre-S1 and S domains of hepatitis B virus envelope proteins interact with the core particle. *Virology* **228,** 115–120.
33. Salvucci, M., Lemoine, A., Azoulay, D., Sebagh, M., Bismuth, H., Reyns, M., May, E., and Debuire, B. (1996) Frequent microsatellite instability in post hepatitis B viral cirrhosis. *Oncogene* **13,** 2681–2685.
34. Shih, L. N., Sheu, J. C., Wang, J. T., Huang, G. T., Yang, P. M., Lee, H. S., Sung, J. L., Wang, T. H., and Chen, D. S. (1990) Serum hepatitis B virus DNA in healthy-negative Chinese adults evaluated by polymerase chain reaction. *J. Med. Virol.* **32,** 257.
35. Sitterlin, D., Lee, T. H., Prigent, S., Tiollais, P., Butel, J. S., and Transy, C. (1997) Interaction of the UV-damaged DNA-binding protein with hepatitis B virus X protein is conserved among mammalian hepadnaviruses and restricted to transactivation-proficient X-insertion mutants. *J. Virol.* **71,** 6194–6199.
36. Slagle, B. L., Lee, T. H., Medina, D., Finegold, M. J., and Butel, J. S. (1996) Increased sensitivity to the hepatocarcinogen diethylnitrosamine in transgenic mice carrying the hepatitis B virus X gene. *Mol Carcinogenet.* **15,** 261–269.

37. Terradillos, O., Billet, O., Renard, C. A., Levy, R., Molina, T., Briand, P., and Buendia, M. A. (1997) The hepatitis B virus X gene potentiates c-myc-induced liver oncogenesis in transgenic mice. *Oncogene* **14,** 395–404.
38. Terrault, N. A., Zhou, S., McGory, R. W., Pruett, T. L., Lake, J. R., Roberts, J. P., Ascher, N. L., and Wright, T. L. (1998) Incidence and clinical consequences of surface and polymerase gene mutations in liver transplant recipients on hepatitis B immunoglobulin. *Hepatology* **28,** 555–561.
39. Tong, S., Li, J., and Wands, J. R. (1995) Interaction between duck hepatitis B virus and a 170-kilodalton cellular protein is mediated through a neutralizing epitope of the pre-S region and occurs during viral infection. *J. Virol.* **69,** 7106–7112.
40. Toyonaga, T., Hino, O., Sugai, S., Wakasugi, S., Abe, K., Shichiri, M., and Yamamura, K. (1994) Chronic active hepatitis in transgenic mice expressing interferon-gamma in the liver. *Proc. Natl. Acad. Sci. USA* **91,** 614–618.
41. Vyas, G. N. (1980) Molecular immunology of the hepatitis B surface antigen (HBsAg), in *Hepatitis B Vaccine* (Maupas, P. and Guesry, P., eds.), Elsevier/North-Holland Biomedical, Amsterdam.
42. Vyas, G. N. and Perkins, H. A. (1982) Non-B posttransfusion hepatitis associated with hepatitis B core antibodies in donor blood. *N. Engl. J. Med.* **306,** 749–750.
43. Wang, J. T., Wang, T. H., Sheu, J. C., Shih, L. N., Lin, J. T., and Chen, D. S. (1991) Detection of hepatitis B virus DNA by polymerase chain reaction in plasma of volunteer blood donors negative for hepatitis B surface antigen. *J. Infect. Dis.* **163,** 397–399.
44. Waters, J. A., Kennedy, M., Voet, P., Hauser, P., Petre, J., Carman, W., and Thomas, H. C. (1992) Loss of the common "a" determinant of hepatitis B surface antigen by a vaccine-induced mutant. *J. Clin. Invest.* **90,** 2543–2547.
45. Wounderlich, G. and Bruss, V. (1996) Characterization of early hepatitis B virus surface protein oligomers. *Arch. Virol.* **141,** 1191–1205.
46. Wright, T. L. and Lau, J. Y. N. (1993) Clinical aspects of hepatitis B virus infection. *Lancet* **342,** 1340–1344.
47. Xu, Z., Bruss, V., and Yen, T. S. (1997a) Formation of intracellular particles by hepatitis B virus large surface protein. *J. Virol.* **71,** 5487–5494.
48. Xu, Z., Jensen, G., and Yen, T. S. B. (1997b) Activation of hepatitis B virus S promoter by the viral large surface protein via induction of stress in the endoplasmic reticulum. *J. Virol.*, in press.
49. Yamaguchi, M., Miyatsu, Y., Mizokami, H., Matsuoka, L., and Takeo, K. (1996) Translocation of hepatitis B virus core particles through nuclear pores in transformed yeast cells. *J. Electron. Microsc.* (Tokyo) **45,** 321–324.
50. Yang, G., Ulrich, P. P., Aiyer, R. A., Rawal, B. D., and Vyas, G. N. (1993) Detection of hepatitis B virus in plasma using flow cytometric analyses of polymerase chain reaction-amplified DNA incorporating digoxigenin–11–dUTP. *Blood* **81,** 1083–1088.
51. Yang, G. and Vyas, G. N. (1996) Immunodiagnosis of viral hepatitides A to E and non-A to -E (Minireview) *Clin. Diag. Lab. Immunol.* **3,** 247–256.
52. Yen, T. S. B. (1993) Regulation of hepatitis B virus gene expression. *Semin. Virol.* **4,** 33–42.
53. Yen, T. S. B. (1996) Hepadnaviral X protein: review of recent progress. *J. Biomed. Sci.* **3,** 20–30.
54. Yu, X. and Mertz, J. E. (1996) Promoters for synthesis of the pre-C and pregenomic mRNAs of human hepatitis B virus are genetically distinct and differentially regulated. *J. Virol.* **70,** 8719–8726.

4

Hepatitis C Virus

Biology, Pathogenesis, Epidemiology, Clinical Description, and Diagnosis

Mauro Bendinelli, Maria Linda Vatteroni, Fabrizio Maggi, and Mauro Pistello

1. Introduction

Prior to the late 1980s, the problem of the etiology of non-A, non-B (NANB) hepatitis was completely blurred. In the early 1980s, the pioneering work of several groups had clearly demonstrated the viral nature of the disease by injecting blood of infected individuals into chimpanzees, hence permitting a number of interesting findings. However, all attempts to characterize the virus and to develop specific diagnostic tests had failed. Clinical and experimental observations had led to the postulation of the existence of at least two distinct viruses, while electron microscopy had suggested a variety of virus-like particles. Attention had eventually focused on a particle with dimensions and inactivation sensitivity similar to those of togaviruses. In this climate, the cloning in *E. coli* by Choo et al. *(1)* of the genome of a virus that in the subsequent few months was shown to represent the most common etiologic agent of posttransfusion and community-acquired (also defined as sporadic or cryptogenetic) NANB hepatitis signified an extremely important breakthrough as well as an innovative approach to the demonstration of viral agents. Most of the information discussed below stems from this breakthrough.

2. The Virus

Lack of important experimental tools, such as sensitive in vitro culture systems and suitable methods for analyzing the viral antigens, has so far prevented a satisfactory understanding of the morphology and physico-

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

chemical properties of the HCV virion. Even more limited is knowledge of the replicative cycle. Great progress has instead been made in the understanding of the viral genome and this, together with the study of the encoded proteins in prokaryotic and eukaryotic expression systems, has permitted remarkable insight into the characteristics and putative functions of the viral proteins.

2.1. Taxonomy

Early physico-chemical properties had suggested that HCV might be a togavirus *(2)*. More recently, accumulating information on virion morphology and comparative analysis of the nucleotide (nt) sequences have led to recognition of considerable genetic and biological analogies to the members of the family *Flaviviridae* and especially to those grouped in the genus *Pestivirus (3)*. Similarities include: short stretches of nt at the 5' untranslated terminal region (UTR; ref. *4*), the size of the precursor polyproteins and their hydrophobic profile except in the envelope region *(5)*, and certain amino acid domains interspersed in the NS3 and NS5 proteins and located in similar positions of the polypeptide chains *(6)*. However, there are also significant differences; for example, in the number and processing of viral proteins and possibly in the characteristics of specific replicative steps. Hence, recently, it has been proposed that HCV be classified in a new genus of flaviviruses tentatively termed *Hepacivirus (7)*.

Interestingly, HCV also shares properties with picornaviruses (i.e., the organization of the 5'-UTR and possibly translation regulation; ref. *8*) and with selected plant viruses such as the potyviruses. This has led to speculations that HCV might represent an evolutionary link between plant and animal viruses *(9)*.

2.2. Host Range, Cell Tropism, and In Vitro Propagation

Humans are the only host species found to be naturally infected by HCV. The only animals that are consistently susceptible to experimental HCV infection are chimpanzees, which develop a persistent viremia and signs of hepatitis. Early experiments using these primates to propagate the virus provided extremely important information on various aspects of HCV *(10–13)*. Also the first cloning of the viral genome was achieved starting from pooled chimpanzee plasma *(1)*.

Clinical and experimental data indicate the marked hepatotropism of HCV. Thus, in vivo, hepatocytes are currently believed to represent the major targets of virus replication *(14–16)*. The possibility that HCV replicates in other cell types is still controversial. Evidence that peripheral lymphocytes and monocytes carry the viral genome and replicate the virus is not univocal *(17–21)*.

Attempts to grow HCV in vitro have been numerous, but to date, successes have been modest. By using the reverse-transcriptase polymerase chain reaction (RT-PCR), evidence of low-grade viral replication has been obtained in primary human and chimpanzee liver cells *(22–24)*, in hepatocarcinoma cell lines *(25,26)*, and in several human hematopoietic cell lines including MOLT-4 *(27)*, HPB-Ma *(28)*, H9 *(29)*, MT-2 *(30,31)*, CE *(32)*, and TOFE *(33)*. In general, however, the virus titers produced have not only been low but have also fluctuated markedly, with a tendency of the virus to disappear within days or weeks. In addition, with few exceptions *(24,34)*, it has proved difficult or impossible to pass the infection serially, and no obvious cytopathic effects have been observed. Even though long-term infected cultures have been described recently *(25,35,36)*, further efforts are clearly needed to establish efficient means for reliable in vitro culture of HCV *(37)*.

2.3. *The Viral Particle*

Irrefutable evidence in the early 1980s that the agent of non-A, non-B hepatitis was viral led to an interesting series of observations. The new agent was small in size, inactivated by chloroform, and was termed, "tubular forming agent," as it induced characteristic tubular structures in the hepatocytes of infected chimps *(38)*. Subsequently, filtration and density gradient analysis studies strengthened the early impression that the virus was akin to togaviruses and flaviviruses *(39,40)*. Figure 1 shows the probable morphological aspect of HCV as perceived currently, based on analogy with related viruses and on recent studies that have finally allowed a reasonable visualization of the virion *(41,42)*. The round-shaped particle is 55–65 nm in diameter and possesses a lipoprotein envelope with small projections (approx 6 nm), which surrounds a 30–35-nm nucleocapsid of probable icosahedral symmetry *(43)*. Consistent with the presence of glycoprotein peplomers in the envelope, the virus has been shown to bind to sugar-binding lectins *(44)*. Particle density is highly variable, ranging from 1.14 to 1.16 g/mL. Such a low and variable density might reflect the fact that the viral particle interacts nonspecifically with lipoproteins or other plasma components *(45)* and/or specifically with antiviral immunoglobulins, thus forming circulating immune complexes *(46,47)*. Alternatively, this might reflect the presence of defective virus particles. In addition to the complete virions, the plasma of infected patients has been shown to contain 30–35-nm particles composed of nucleocapsid proteins. Thus HCV may circulate in plasma in different forms *(41,48)*.

There has been limited study of the serological properties of HCV. For example, the lack of reliable serum neutralization assays has not permitted

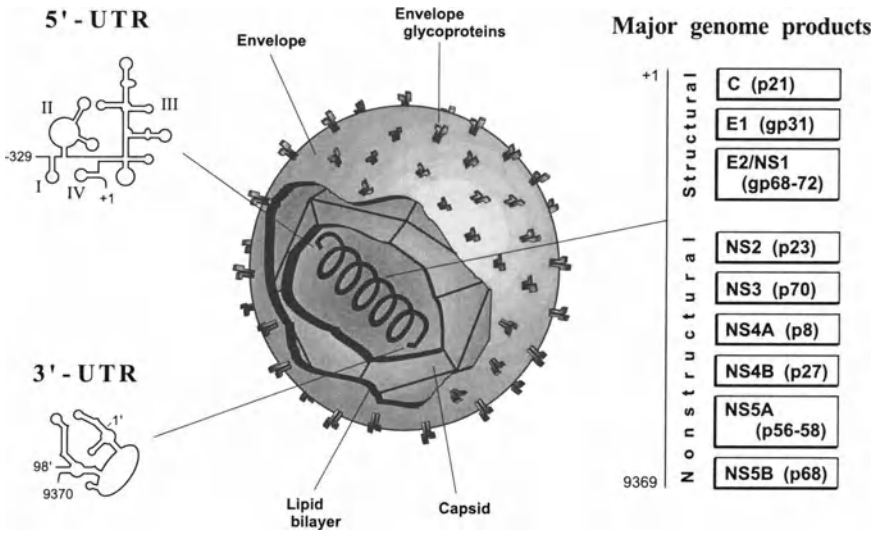


Fig. 1. Molecular anatomy of the HCV virion. The predicted secondary structure models of the 5' and 3' UTR and the genomic products from structural and nonstructural domains of the HCV genome are represented. Roman numbers in the 5'UTR indicate the structural domains, nt positions 1' to 98' in the 3' UTR indicate the highly conserved terminal segment. The coding genes and the corresponding products are boxed (numbers within parenthesis indicate the size of each protein or glycoprotein). Glycoprotein gp68–72 can be also present in its cleaved form: E2A and P7. Nt positions correspond to the prototype strain HCV-J of subtype 1b (EMBL accession number D90208; ref. *52*).

careful investigation of possible serotypes, even though analysis of infected patient sera with synthetic peptides has demonstrated the existence of serological differences between isolates belonging to different genotypes (*see* Subheading 2.6.).

Similar to other enveloped viruses, HCV infectivity is relatively unstable at 4°C and stable at –70°C. Inactivation of infected plasma requires 100°C for 5 min, 80°C for 72 h *(49)*, or pasteurization *(50)*; infectivity is also destroyed by ether, chloroform, β-propiolactone, or formalin (1:1000 for 96 h or 1:2000 for 72 h at 37°C; ref. *51*).

2.4. The Genome and the Proteins Encoded

The genome of HCV is single-stranded linear RNA of approx 9500 nt and has positive (messenger) polarity *(6,52–55)*. Most of the molecule represents a long translational open reading frame (ORF) with a coding poten-

Table 1
HCV Proteins

Protein	Genomic region	Amino acid position	Major functions
p21	Core	1–191	Capsid protein
gp31	E1	192–383	Envelope glycoprotein
gp68–72	E2/NS1	384–809	Envelope glycoprotein
E2-A	E2/NS1	384–746	Envelope glycoprotein
p7	E2/NS1	747–809	No known
p23	NS2	810–1026	NS2/NS3 metallo-protease component
p70	NS3	1027–1657	Serine-protease; helicase; NS2/NS3 metallo-protease component
p8	NS4A	1658–1711	*See* Fig. 3
p27	NS4B	1712–1972	Membrane-associated replicase component?
p56–58	NS5A	1973–2420	Not known
p68	NS5B	2421–3011	RNA-dependent RNA polymerase

tial of 3011–3033 amino acids and consisting of a structural domain that codes for components of the viral particle and a nonstructural domain that encodes proteins not found in the virion but participate in virus biosynthesis *(4,56)*. The two extremities of the RNA molecule (UTR) may vary in length and have no known coding functions; however, they possess defined secondary structures (Fig. 1) and have essential regulatory functions.

Post- or cotraslationally, the large polyprotein product of the viral genome undergoes an ordered series of proteolytic cleavages, and at least ten different functional protein species are formed *(57,58)*. Functions and properties of these proteins (Table 1) have been deduced from analogy with the corresponding proteins of other viruses and from cDNA expression in a variety of systems.

There are at least three proteins that are believed to have structural functions, though direct confirmation through demonstration of their presence in purified virions is still lacking. These are the capsid (core) protein and the envelope, E1 and E2, glycoproteins. The core protein or p21 is coded for by gene C and has been seen to form multimers and interact physically with the viral RNA to form the nucleocapsid *(43,59–61)*. Basic properties, resulting from a high content (approx 20%) of Arg and Lys residues, the

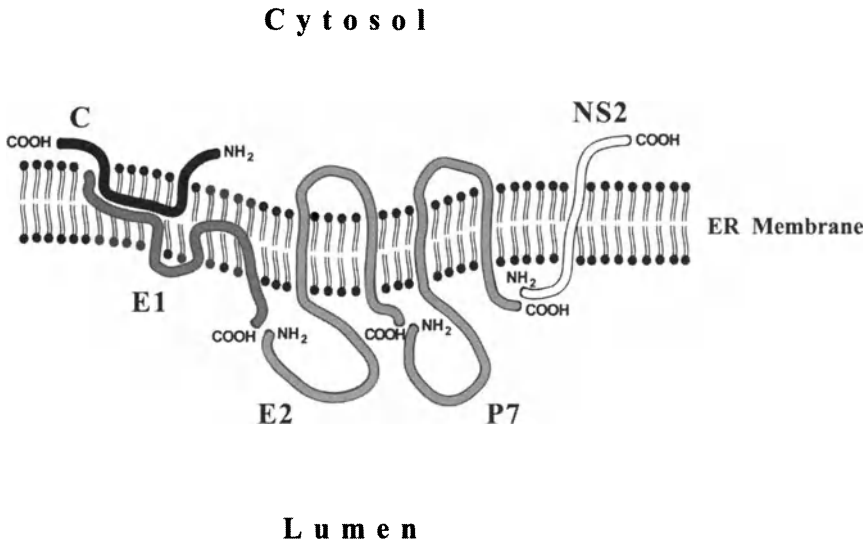


Fig. 2. Suggested spatial distribution of the structural (C, E1, E2 and P7) and nonstructural (NS2) proteins of HCV in the lipid bilayer of the endoplasmic reticulum membrane. Numbers indicate the amino acid position of intramembrane domains (modified from refs. *63,79*).

presence of sites of potential phosphorylation *(62)*, and an elevated number of conserved linear B-cell epitopes represent characteristic features of this protein *(63)*. Data showing that p21 can bind ribosomal subunits *(60)* and inhibit HBV replication in vitro *(64)* have suggested that the core protein might also play some as yet undefined regulatory role in viral replication and pathogenesis. More recently, the core protein has been shown to interact with the E1 protein as well as with certain cellular components but not with the E2 protein of HCV *(65–67)*. The product of the genome region E1 (gp31) is a highly glycosylated protein (5–6 putative glycosylation sites; ref. *68*), which, despite being smaller and lacking significant sequence homology, appears to be functionally analogous to the envelope proteins of related viruses. The presence of E1 in the envelope is also suggested by numerous stretches of hydrophobic amino acids that might serve to anchor the molecule to the cell membrane (Fig. 2). The third structural protein (E2 or gp68–72) is coded by gene E2/NS1 and was initially thought to be nonstructural in function. It is also highly glycosylated, containing about ten putative glycosylation sites, is anchored to the cell membrane through a C-terminal signal sequence *(69,70)*, has sequence motifs that suggest complex secondary and tertiary structure, and apparently associates to the E1 glycoprotein to

form an heterodimer (Fig. 2) thought to be involved in virus attachment to cell receptors *(71–74)*. At the N-terminal end of gp68–72 there is a segment (amino acids 384–414) characterized by a high degree of diversity among different isolates *(75,76)* and therefore known as hypervariable region 1 (HVR1). In addition, certain genotypes, such as subtype 1b possess, next to HVR1, a second shorter hypervariable region (HVR2; ref. *77*). These HVR have features that are reminiscent of the V3 region of HIV surface glycoprotein (gp120). They have been proposed as the likely location of neutralization epitopes that possibly are implicated in the immunoselection processes that contribute to keep HCV in persistent equilibrium with its host (*see* Subheading 3.3.).

A fourth product of the structural portion of the viral genome has been recognized recently. This is a small protein (p7) that results from an alternative cleavage of the precursor polyprotein at the C-terminal of the E2 region. This region contains two cleavage sites used with different frequencies. The cleavage site used more frequently leads to formation of full-length gp68–72, while cleavage at both sites leads to formation of protein p7 as well as of a smaller E2 protein (protein 2A). The role of p7 in viral replication is still unknown, but there is some evidence that it might become associated via several hydrophobic residues to the cell membrane (Fig. 2), while its suggested role in virion assembly has been recently disputed *(78–81)*.

The products of the nonstructural domain of the viral genome (NS2, NS3, NS4, and NS5) are less well understood *(82)*. Recent studies indicate that the protein coded by gene NS2 (or p23) is highly hydrophobic and might in fact represent a transmembrane protein with autocatalytic activity (Fig. 2). It has also been suggested that during particle maturation, this protein might form a bridge between the envelope glycoproteins and the nucleocapsid *(83)*. A zinc-dependent metalloprotease is encoded in the region spanning NS2 and the N-terminal end of NS3 *(84–86)*. The product of gene NS3 (p70) is a protein that appears to contain two different domains with clearly distinct functions. The N-terminal part of the molecule has serine protease activity and appears to be responsible for processing the C-terminal segment of the precursor polyprotein *(87,88)*. Furthermore, the C-terminal portion of the NS3 protein has considerable homologies with enzymes possessing helicase activity, has been shown to have unique RNA helicase functions and is presumed to be involved in unfolding of viral RNA during the early stages of genome replication *(89–91)*.

The products of genes NS4 and NS5 are processed into two smaller proteins. The two proteins resulting from gene NS4 (designated NS4A [p8] and NS4B [p27]) are highly hydrophobic and are probably membrane-bound. Recent results suggest that they exert a key function in the regulation of precursor polyprotein processing and in the synthesis of certain viral pro-

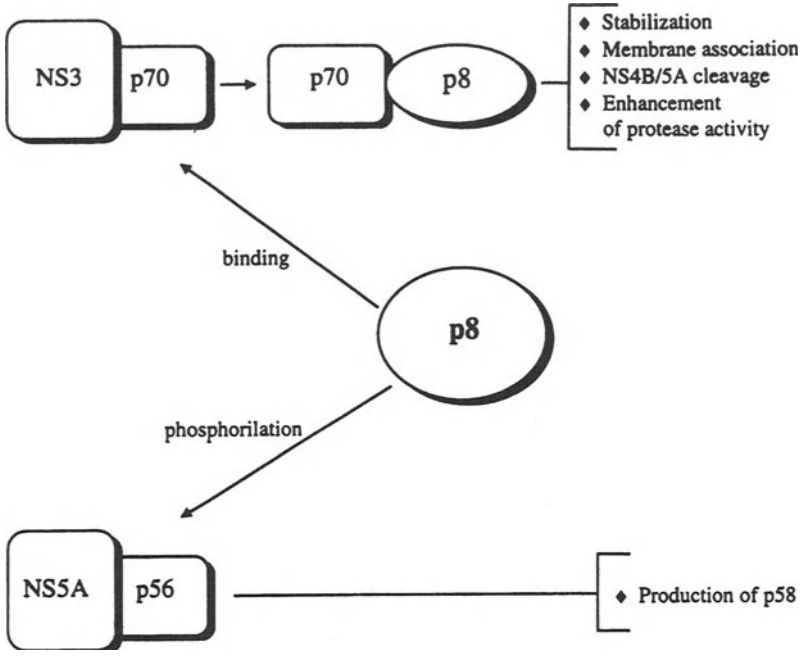


Fig. 3. Schematic representation of the multiple functions attributed to the NS4A protein p8 and effects on the target proteins NS3 and NS5 (modified from ref. *97*).

teins. More specifically, p8 appears to be multifunctional (Fig. 3): by binding the NS3 protease, it would mediate the docking of this enzyme onto the endoplasmic reticulum, increase its stability, and permit or augment its catalytic efficiency *(92–96)*. In addition, it is assumed to regulate the production and phosphorylation of the NS5 protein *(97,98)*. The two products of gene NS5 are known as NS5A (p56–p58) and NS5B (p68). Protein NS5A is highly phosphorylated *(99)* and presents an elevated degree of diversity in different HCV isolates *(100,101)*. Its function(s) is still unknown, although recent data have suggested that it might represent a potent transcriptional activator *(102)* and a determinant of HCV sensitivity to interferon (IFN) treatment *(103,104)* possibly through repression of the IFN-induced protein kinase (PKR; ref. *105*). NS5B is instead a highly conserved membrane-associated phosphoprotein *(106)*, which, based on motif similarities with other viral replicases and more direct evidence, is considered the RNA-dependent RNA polymerase *(107,108)*.

As already mentioned, the 5' and 3' UTR (Fig. 1) contain important regulatory elements involved in controlling genome replication and expression. The UTR at the 5' end may vary in length between 324 and 341 nt but is otherwise highly conserved among viral isolates *(5,109)*. This region possess a complex secondary structure composed of four stable domains preceding the initiator AUG, and its overall functions appear especially important as suggested by the numerous regulatory elements it contains. These include:

1. four nt stretches homologous to similar sequences found in the 5'-UTR of pestiviruses, one of which is an activator of translation *(54,110)*;
2. several small ORF that are not known to be translated *(6,8)*;
3. a 27-nt-long hairpin structure that might participate in regulation of genome expression and bind cellular factors potentially implicated in HCV replication *(8,111)*; and
4. a large stem-loop, proximal to the initiator AUG codon of the polyprotein, considered to be an internal ribosomal entry site (IRES; refs. *112–114*).

Contrary to what previously was believed, recent studies have demonstrated that the 3'-UTR also has a complex organization, consisting of a short highly variable nt sequence, a poly-U stretch, a polypyrimidinic stretch, and a terminal 98-nt segment (Fig. 1). The latter is highly conserved, capable of a stable stem-and-loop secondary structure and of binding several cell proteins and is probably involved in the initiation of viral replication *(115–120)*.

2.5. Replication

We still know essentially nothing about the mechanism(s) of virus entry into cells, the receptor(s) involved, and early replicative steps. Thanks to its supposed ability to bind certain plasma proteins, HCV has been proposed to exploit the receptors by which such proteins attach to the cell surface *(121,122)*. A precedent for a similar mechanism is found in the phenomenon known as antibody-mediated enhancement, by which certain viruses complexed with antibodies (notably some flaviviruses) may enter into cells via IgG receptors.

As direct information is very limited, present perception of the intracellular replicative steps of HCV relies mainly on what is known about the replication of related positive-strand RNA viruses, especially pestiviruses and flaviviruses, and on what can be inferred from the deduced structure of the viral proteins and their expression in mammalian systems. Viral biosynthesis and the whole replicative process are thought to occur entirely in the cytoplasm. Replication of the viral genome proceeds through the synthesis

of complementary antigenomic (minus stranded) RNA strands, which presumably serve as templates for the synthesis of progeny-positive strand genomes and have repeatedly been detected in HCV-infected cells by PCR and *in situ* hybridization *(123)*. These steps are presumed to require the combined activity of two viral enzymes: the NS3 helicase, involved in unfolding of the replicative fork and the RNA polymerase NS5, responsible for actual *de novo* synthesis of viral RNA *(4)*. Infected cells have been shown to contain two different species of positive-sense viral RNA: a longer one with the 5' end folded to form a hairpin, which would represent the true genomic RNA, and a shorter, polyadenylated one that would represent the true messenger RNA *(8,54,109,110,124,125)*. The latter RNA would function as a single large monocistronic messenger, leading to the production of a single polyprotein, which is then cleaved to yield all of the mature viral proteins.

Cleavage of the viral precursor polyprotein is carried out mainly by viral proteases but cellular proteases are also involved. In particular, processing of the nonstructural portion of the polyprotein is effected by two different viral proteases: the NS3 serino-protease cleaves the junctions NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B *(126–130)*, while the viral metallo-protease cleaves the NS2/NS3 junction *(46,84,85)*. Host-cell signal peptidases located in the lumen of the endoplasmic reticulum are responsible mainly for cleaving the structural region of the polyprotein *(131)*.

Studies on the subcellular localization of HCV proteins expressed in mammalian cells suggest that they are mainly associated with the endoplasmic reticulum where glycosylation and translocation to the lumen take place *(61,71)*. Only NS3 and NS5A would appear to be free in the cytoplasm *(132)*, while there is no clear evidence for a nuclear localization of viral proteins. When deleted of its hydrophobic C-terminal portion, the core protein p21 has been shown to reach the nucleus *(133–135)*; however, there are no indications that such a truncated form of the protein is produced in the course of normal replication. The implications of the localization of the viral proteins for the subsequent steps of virion assembly and release from the cell remain is to be determined. The entire assembly process is still obscure. Also obscure is the mechanism(s) of virus release from infected cells. It is likely that the virus buds through the plasma membrane while the nucleocapsid is being formed. Such a process would be compatible with cell survival, but the consequences of HCV replication for the integrity of host-cell structure and physiology are unknown. In a recent immunoelectron microscopy study of in vitro infected cells, virus-like particles were mostly found within cytoplasmic vesicles, suggesting the possibility that HCV morphogenesis is vesicle-oriented *(42)*.

2.6. Genetic Heterogeneity

Analysis of the genome of numerous HCV isolates has evidenced a high degree of variation in the nt sequence, similar to that of other positive-strand RNA viruses (up to one-third of the nt may be different in the most divergent strains). However, genetic heterogeneity is not evenly distributed across the genome but is especially elevated in the envelope coding regions E1 (47–31%) and E2/NS1 (43–29%) with peak prevalence in the HVR1 segment of the latter molecule (50% variation and more among isolates; ref. *136*). Variability is instead lower in the 5'-UTR (<10%; ref. *137*), in the core region, (12–19%) and in the NS3 region (20–30%; ref. *138*), probably reflecting the important functions exerted by these regions, which do not tolerate extensive variation.

Similar to other RNA viruses, genetic heterogeneity is believed to originate from poor fidelity of RNA replication *(137,139)*. Overall, the average rate of mutation for the entire genome has been calculated at 0.144% base substitutions per nt position per year, similar to all viruses with single-strand RNA genomes *(53,140,141)*. A certain degree of variation is found not only among different isolates but also within the viral sequences detectable in a given patient, i.e., the virus is present in the infected host in the form of a quasispecies, with one or more predominant sequences and several minor variant(s). Under the pressure of immune effectors and possibly other environmental factors, the minor variants have the potential to become the predominant ones, thus providing the virus with great plasticity *(139)*. Interestingly, in the envelope region, many mutations are nonsynonymous, that is, they lead to modifications in the amino acid sequence of the encoded proteins, indicating that this protein has less structural constraints and is under stronger selection pressures than other viral proteins.

Based on genetic similarities, HCV obtained from patients worldwide have been classified into distinct genotypes that form separate branches in phylogenetic trees, and several partially different grouping systems have been proposed. Table 2 presents the classification system initially proposed by Simmonds and coworkers *(138,142)* and subsequently accepted by many workers in the area *(143)*. According to such strategy, isolates are grouped into a same type when their nt sequence homology in the NS5B gene is 72% or higher and in a same subtype when homology is 86% or higher. At present, the Simmond's classification encompasses six different types (defined by Arabic numbers), most of which contain several subtypes (indicated by lowercase letters), but there are still considerable uncertainties (*see* Table 2). In addition, there are probably more genotypes to be discovered as more HCV isolates from different parts of the world are characterized. In the absence of

Table 2
Classification and Nomenclature of HCV Genotypes

Type	Subtype	Representative isolates	References
1	1a	HCV-1	6
	1b	HCV-J	52
	1c	EG-28	142
2	2a	HC-J6	54
	2b	HC-J8	125
	2c	S83	382
	2d	NE92	383
	2e	JK020	384
	2f	JK081	384
3	3a	E-b1	385
	3b	Tb	385
	3c	NE048	386
	3d	NE274	386
	3e	NE145	386
	3f	NE125	386
	10a[a]	JK049	384
4	4a	EG-13	143
	4b	Z1	387
	4c	Z6	387
	4d	DK13	387
	4e	Z5	387
	4f	Z8	387
	4g	GB549	383
	4h	GB438	383
5	5a	SA-1	137
6	6a	HK-2	137
	6b	Th580	383
	7a[a]	VN540	388
	7b	VN235	388
	7c	Th271	383
	7d	Th846	383
	8a[a]	VN507	387
	8b	VN405	387
	9a[a]	VN004	388
	9b	Th555	383
	9c	Th553	383
	11a[a]	JK046	384

[a]Isolates previously classified as types 10 or 7, 8, 9, and 11 have been reclassified within type 3 and type 6, respectively, according refs. *389–391*.

traditional methods for grouping isolates (e.g., serum neutralization assays), HCV genotyping has become current practice. Although final definition of a genotype rests on more-or-less extensive sequence analysis, several rapid methods for HCV genotyping have been proposed, and some are also available in commercial kit form. Methods in use include (sub)type-specific RT-PCR, hybridization of RT-PCR products with specific probes, and restriction fragment polymorphism analysis. Methods based on the detection of type-specific antibody in patients' sera using synthetic peptides (serotyping) have also been proposed *(144–146)*. The reliability of such methods and the epidemiological and pathobiological implications of HCV genotypes are presently the subject of active investigation (*see* below). Interestingly, as a result of multiple exposures, patients can be coinfected or superinfected with different serotypes (*see* below) but recombination between different genotypes appears to occur rarely, if at all *(147)*.

3. The Infection

3.1. Host Penetration and Invasion

Despite recent progress, our understanding of the life cycle of HCV in the infected host is extremely limited. As extensively discussed in Subheading 5., person-to-person transmission of HCV generally occurs via parenteral exposure or intimate contact with infected bodily fluids. In the latter circumstance, the precise portal(s) of entry into the host is/are not known, also whether or not the virus undergoes primary amplification locally at the site of entry and/or in the draining lymph nodes is unclear. The route by which the virus reaches the liver is unknown; however, HCV can circulate freely and massively in plasma and infects migratory cells such as lymphocytes and monocytes. This suggests possible routes for host invasion, although no virus was detected in these cells of acutely infected individuals *(148)*. In the liver of chimpanzees injected with infected blood, viral RNA was detected as early as 2 d postinoculum, thus preceding detection of viremia by days *(11)*. Ability to enter the body through mucosal surfaces would most likely imply that HCV infects cell types other than hepatocytes and blood mononuclear cells. *In situ* hybridization, PCR, and immunofluorescence studies have provided evidence of viral components in hepatocytes, Kupffer cells, B and T lymphocytes and macrophages, and immunohistochemistry has demonstrated viral antigens in biliary epithelial cells and in the salivary gland epithelium *(16,123,149–152)*. Interestingly, HCV quasispecies composition in the liver, peripheral blood mononuclear cells and plasma has been found to differ *(153–157)*, a finding that might suggest the existence of additional sites of viral replication.

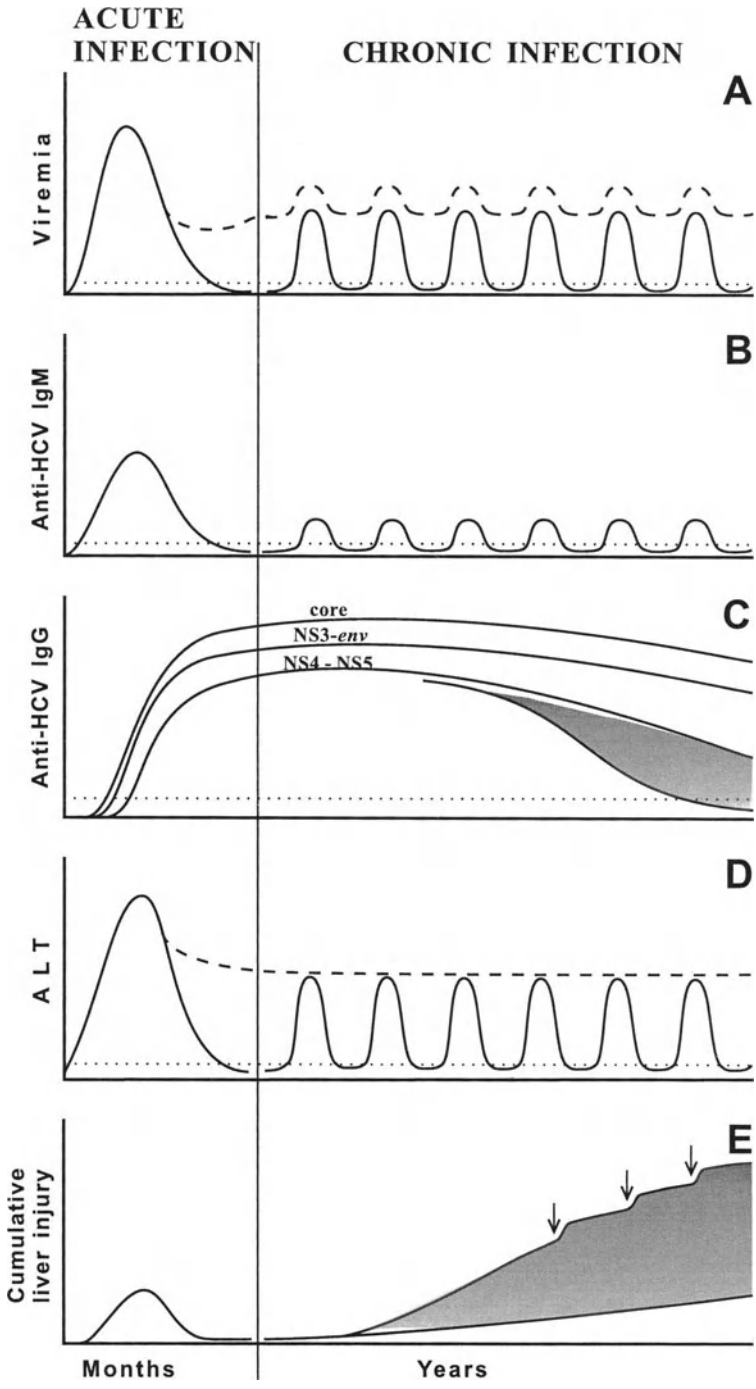
In chimpanzees, viremia usually becomes detectable within days postexposure, but detection can be intermittent, suggesting the existence of distinct bursts of viral replication *(158–161)*. Also, in patients transfused with infected blood, viremia develops early and is usually demonstrable within 1–3 wk, thus preceding biochemical abnormalities and clinical symptoms by at least 3–5 wk and antiviral antibody by 4–6 wk *(162–166)*. As determined by quantitative PCR and branched DNA assays, viremia also reaches high levels (10^8 genomes/mL or more) before clinical symptoms develop *(167–169)*.

HCV has a remarkable tendency to persist. Greater than 80% of infections result in the establishment of a chronic viremia, even though clinical symptoms and biochemical abnormalities are absent. The plasma viral loads detected in the course of persistence are extremely variable in individual patients, ranging between 10^2 and 10^{10} genomes/mL and may undergo considerable fluctuations over time. Viremia may also become undetectable for protracted periods *(162,167,170–173)*. These variations presumably reflect fluctuations in the number of infected cells and in host factors that affect viral clearance as well as in the replication efficiency of the viral quasispecies (Fig. 4).

Recently, interesting clues regarding the kinetics of HCV replication during chronic infection were obtained by measuring the effects of IFN alpha treatment on viremia levels of responder patients. By exploiting mathematical models similar to those used for studying viral kinetics in HIV-infected

---

Fig. 4. *(See opposite page)* Schematic view of the possible courses of HCV infection and disease. Dotted lines represent the lower limit of sensitivity of detection methods or upper normal values (ALT). **(A)** Viremia concentration peaks in the first months (acute infection) and then declines or, in a limited number of patients, becomes undetectable. Most primary infections do not resolve, and viremia persists at variably elevated levels (chronic infection), often with marked fluctuations in titer. **(B)** Antiviral IgM antibodies develop during acute infection and last a few months but in a considerable proportion of patients may be detected also during chronic infection, though generally in discontinuous fashion. **(C)** IgG antibodies to different viral antigens have different kinetics: Anticore antibodies usually are the first to appear and remain detectable throughout infection, whereas anti-NS4 and anti-NS5 antibodies develop somewhat later and may become undetectable within years from acute infection. **(D)** During acute infection, ALT peak soon after viremia. During chronic infection, ALT may remain in the normal range for prolonged periods but usually are variably elevated and often show wide fluctuations that are believed to follow viremia fluctuations. **(E)** Liver damage associated with acute infection is generally moderate. During chronic infection, hepatic lesions usually evolve slowly but in some patients may progress more rapidly. Liver failure is observed only in a limited proportion of cases, especially when other noxae coexist (arrows).


ACUTE INFECTION
CHRONIC INFECTION
A
Viremia
B
Anti-HCV IgM
C
core
NS3-env
NS4 - NS5
Anti-HCV IgG
D
A L T
E
Cumulative liver injury
Months
Years

patients, it has been estimated that HCV half-life in the plasma is a few hours. The number of virions produced and cleared per day was calculated to be at least 6.7×10^{10} virions, with considerable variations in individual patients (range, 0.2 to 43.8×10^{10} virions per day). No correlation with infecting HCV genotype, serum aminotransferase (ALT) levels, or histopathology of the liver was noted *(174,175)*. These results suggest that during persistence in the host, HCV is produced continuously and very actively, possibly as a result of multiple rounds of replication in *de novo* infected cells. Thus, the dynamics of the virus-host equilibrium reached by HCV and HIV in chronically infected subjects appear to be similar. Further data are, however, necessary to substantiate this conclusion, which may have important implications for therapy design and monitoring. Further studies are also needed to establish the full range of tissues that contribute to maintaining the viremia.

3.2. *Anti-HCV Immune Responses*

Immune responses to HCV have been the subject of intensive scrutiny in recent years, as assays for measuring HCV-specific antibody and cell-mediated immunity have gradually become available. Infection evokes a vigorous immune response, which is believed to modulate viremia levels *(176)* and probably contributes to keep the extension of hepatocyte invasion within limits, as suggested by evidence showing that in immunosuppressed chimpanzees, infection has a particularly aggressive course *(177)*. In most cases, however, the immune response is not successful at overcoming the infecting virus. The well-documented occurrence of superinfections in humans and chimpanzees sequentially exposed to the same or different strains of HCV demonstrates the weakness of the protective immunity resulting from infection *(178–180)*.

3.2.1. *Antigenic Determinants of HCV*

Our present understanding of HCV antigens is based on the use of recombinant proteins and synthetic oligopeptides deduced from viral nt sequences. Immunodominant conserved B-cell epitopes reactive with patient sera have been identified in the core protein and, to a lesser extent, in the NS3, NS4, and NS5 proteins *(181–184)*. Linear and conformational B-cell epitopes have also been found in the envelope proteins E1 and E2/NS1 *(71,185–187)* but tend to be more variable. The E2/NS1 protein reportedly contains several continuous B-epitopes in its N-terminal region, at least five of which are isolate specific *(76,188)*. Envelope epitopes are thought to be involved in protective immunity as suggested by vaccination experiments in the chimpanzee (*see* Subheading 6.). In fact, the presence HVR1 suggests that E2/NS1 is the target of potent selective forces that especially model those parts of the molecule that are free of structural and functional con-

straints. It is noteworthy that this region shares considerable homologies to regions of the envelope proteins of pestiviruses (gp53/55) and HIV (V3 loop) that contain important neutralization epitopes. However, the patterns of reactivities to E1 and E2/NS1 peptides may vary considerably in individual patients, depending on the stage of infection. Similar variations have also been observed in infected and vaccinated chimpanzees. Interestingly, in this species, reactivity to rare epitopes in the E2 region was seen only in a few cases of self-limiting infection *(187,189–191)*.

T-cell epitopes have been identified in peptides derived from different regions of the viral genome *(192–196)*. Some of the T-cell epitopes found in the core protein are partially superimposed to B-cell epitopes, thus emphasizing the immunodominant nature of this molecule. Interestingly, T-cell epitopes recognized by liver-derived lymphocytes appear to differ from those recognized by peripheral lymphocytes (*see* Subheading 3.2.3.).

3.2.2. Antibody Responses

In diseased individuals, antiviral antibodies are usually first demonstrable within a few weeks of clinical onset (Fig. 4). In some patients, however, there may be a longer seronegative phase (window period). Commercially available kits for detection of anti-HCV antibody have been redesigned several times and with considerable success, in the attempt to reduce the length of the window period and increase sensitivity. Efforts to identify epitope-specific antibodies that might serve as markers of infectious activity and as prognostic tools have instead been less fruitful.

3.2.2.1. IgM Antibodies

The production of IgM antibodies has been extensively investigated as a possible marker for recent infections and reactivations, but the results obtained are difficult to compare because of the use of different methods and antigens (Fig. 4). On the other hand, commercial assays for this class of antibody are still under evaluation *(197)*. The proportion of acutely infected patients exhibiting IgM antibodies has ranged between 50 and 90% in different studies, but IgM antibodies specific for structural and nonstructural HCV proteins have also been detected in 50–70% of patients with chronic hepatitis; these are generally believed to represent an indication of active virus replication *(198,199)*. IgM levels have been seen to correlate directly with the levels of ALT and viremia *(198,200,201)* and have been reported to be highly prevalent in individuals infected with genotype 1b HCV, which appears to be associated with severe liver damage *(202)*. The presence of IgM antibodies is also considered predictive of a poor responsiveness to IFN, while a reduction in their titer following treatment has been shown to correlate with a drop in viremia *(199,200,202,203)*.

3.2.2.2. IgG Antibodies

Determination of anti-HCV IgG antibody is usually reliable and can be performed with numerous in-house or commercial assays. Currently available commercial kits (third-generation assays) include ELISA, immunoblotting, and other assays and generally contain conserved antigens derived from several structural and nonstructural proteins. IgG antibodies to the envelope, core, and NS3 proteins usually become detectable after serum ALT and viremia have peaked *(10,160,163–165,204)* and remain demonstrable for protracted periods or indefinitely. Antibodies to NS4 and NS5 tend to develop later (Fig. 4). Furthermore, antibodies to NS4 may disappear following treatment or spontaneous recovery *(168,205)*. This, together with the late development of antiNS4 antibodies, explains at least in part the poor sensitivity of first-generation serological assays based solely on the NS4 antigen c100-3.

Using third-generation kits, no consistent correlation has generally been observed between the presence/titers of IgG to specific HCV antigens and infecting subtype, stage of infection, level of viremia, or prognosis *(206–210)*. Moreover, a direct correlation was observed in some studies between course of infection and presence of antibodies to specific HCV oligopeptides *(211)*.

3.2.2.3. Neutralizing Antibodies

Despite the difficulties encountered in growing HCV in vitro, attempts to measure neutralizing activity in the sera of infected patients have been performed. So far information is scarce. According to one study *(212)*, neutralizing antibodies were isolate specific and inhibited only the isolates obtained prior to serum collection, suggesting the continuous emergence of neutralization-resistant viral variants. In a subsequent study *(74)*, the neutralizing activity was inhibited by preincubating sera with a fusion protein containing the HVR1 domain of the corresponding HCV, thus indicating that this domain does indeed contain protective epitope(s). The isolate specificity of the HVR1 neutralization epitope has been recently confirmed using a hyperimune rabbit serum raised against an HVR1 synthetic peptide *(212a)*. Data pointing to the same conclusion have been obtained in the chimpanzee *(213)*. Epitopes involved in in vitro neutralization have been detected also outside the HVR1 *(214)*. Recently, an assay has been devised to measure the binding on HCV envelope proteins to MOLT-4 lymphoid cells and binding inhibition by immune sera that might represent a correlate of neutralization. With this assay, binding-inhibitory activity was detected in sera of infected individuals and chimpanzees and, at higher titers, in the sera of chimpanzees vaccinated with envelope glycoproteins *(215)*.

3.2.3. Cell-Mediated Immune Responses

Parameters of HCV-specific cell-mediated immunity studied in the course of infection include T helper (Th) and cytotoxic T lymphocyte (CTL) activities. Th activity has been investigated by measuring the ability of peripheral and liver infiltrating lymphocytes to proliferate when incubated in the presence of various HCV recombinant proteins and synthetic peptides. Infected patients have evidenced various degrees of response; however, blastogenesis was often especially pronounced against core and NS4 antigens *(192–196,216,217)*. In one study, comparison of the epitope specificity of antiNS4 Th cell clones obtained from peripheral and intrahepatic lymphocytes showed differences suggestive of a compartmentalization of anti-HCV Th responses *(217)*. Whether and to what extent Th lymphocytes infiltrating the liver contribute to local antiviral defenses or to hepatocellular injury is not known. Based on the types of lymphokines produced, they were classified as Th0 or Th1 *(218,219)*. Studies have detected markedly elevated lymphoproliferative responses to core protein antigens in patients with mild liver damage and in responders to IFN-alpha, but similar responses were present also in patients with active hepatitis *(194,196,216)*. Subjects with self-limited acute hepatitis have been reported to produce higher lymphoproliferative responses to several HCV antigens compared with patients that developed chronic hepatitis. In the former patients, activated T lymphocytes were mainly of type 1 or 0, which are deemed important for protection, whereas in the latter patients Th2 lymphocyte activation was particularly evident *(194,220–222)*.

HCV-specific MHC I-restricted CTL activity has been investigated using recombinant vaccinia virus-infected autologous target cells expressing various HCV antigens. These studies have permitted identification, in the circulation and liver of infected patients and chimpanzees, of CTL specific to short amino acid sequences deduced from structural and nonstructural regions of the viral genome in association with several HLA antigens *(194,195,223–225)*. CTL responses, however, varied considerably in individual patients with no apparent correlation with disease activity *(226)*. In one study, patients with chronic hepatitis, but not patients that had apparently recovered, exhibited circulating CTL, which recognized HCV antigens binding HLA-A2.1 molecules *(227)*. However, patients with active chronic hepatitis from HCV, lacking detectable CTL activity, have repeatedly been observed *(224,228)*. Recently, an inverse correlation was found between an HLA-B44-restricted CTL activity to core epitopes and viremia load *(228,229)*, thus suggesting that CTL can modulate HCV replication. A degree of liver compartmentalization has also been reported for CTL activity *(224)*.

3.3. Mechanisms of HCV Persistence

As discussed above, HCV infection is followed by a robust immune response to multiple viral epitopes but, nevertheless, in the large majority of patients infection is not eradicated. Understanding the mechanisms whereby HCV avoids immune elimination would be extremely useful for devising strategies capable of preventing or curing chronic infection. Similar to many other persistent viral infections, numerous mechanisms have been proposed, but none has been definitely proven to represent the key event for persistence.

Great importance is presently given to the plasticity of the viral genome which, as discussed in Subheading 2.6., arises from a high rate of mutation and is maximum in the envelope protein coding regions. Nt sequence variations in the N-terminal segment of E2/NS1 gene are known to occur in the course of chronic infection and produce rates of amino acid substitutions ranging from 0.5 to 1.7 amino acids/mo *(75,188,230–234)*. In one study of 12 patients with chronic hepatitis followed for 1 yr, 90% of the mutations observed were nonsynonymous *(235)*. The frequency of sequence variations differs in individual patients and tends to be low during the early stages of infection to become higher in subsequent stages. The emergence of new viral variants is especially frequent in patients with high or fluctuating ALT values and may coincide with disease recurrences or remain clinically silent *(188,232,236–241)*. Also, the complexity of HCV quasipecies (i.e., the number of different viral sequences contemporaneously present in a given individual) was reported to be especially high in patients with severe liver damage *(233)*. However, much work is still needed to understand the relationship of HCV quasispecies variations with persistence and pathogenesis *(145,154–157,242,243)*.

The multiple B-cell epitopes present at the N-terminal segment of E2/NS1, which includes the HVR1 and probably contains neutralization determinants, appear to be particularly prone to immune selection. The sequential emergence of variations in these epitopes, followed by synthesis of antibodies specific for the newly emerged sequences, has been described in the course of infection *(74,188,231,244–246)*. Anedoctal descriptions of agammaglobulinemic patients that underwent no HVR1 changes for prolonged periods have been considered further evidence for the importance of immunity in driving HCV evolution *(247)*. The observation that not all the amino acid substitutions induced antibodies that were detectable with the corresponding synthetic peptides suggests that such changes affect conformational B- or T-cell epitopes *(231)*. As discussed in Subheading 3.2.1., the E2/NS1 region also contains epitopes recognized by CTL, and changes in

amino acid composition might nullify this defense mechanism as well as neutralizing antibodies. CTL escape variants have been described in infected chimpanzees where the epitope involved was, however, in NS3 *(248)*. In chronically infected humans, sequence variations of the core protein have been described that might favor escape from cell-mediated immunity *(249)*.

Thus, HCV persistence might be allowed, or at least facilitated, by the continuous emergence of antigenic variants of the virus selected for by the action of immune effectors (despite the exuberant viral replication that occurs during initial infection, variants begin to develop after a certain interval), as evoked also for other viruses *(250)*. Conversely, self-limited infections would result from the immune response winning the race against HCV diversification. The emphasis presently given to this hypothesis is probably justified; however, it should not lead to overlooking that evidence arguing against the immune escape mechanism of persistence also exists *(147)*. This includes the fact that HCV plasticity is similar to that of many other RNA viruses that are readily eliminated from the host after acute infection. Other factors that might contribute to the establishment and maintenance of HCV persistence have so far received little experimental attention. These include direct cell-to-cell diffusion of the virus, the production of defective interfering particles, an inherently low sensitivity of the virus to neutralization and other antiviral effects of antibody possibly related to the extensive glycosylation of envelope proteins, shielding of potentially protective viral determinants by host cell components incorporated into or covering the viral envelope, antibody-mediated enhancement of infection, dowregulation of viral replication, and so forth. The ability to infect and impair cells of the immune system may also play a role; alterations of monocyte functions have been described in infected individuals *(251)*.

4. The Diseases

Diseases associated with HCV are primarily a spectrum of liver pathologies ranging from acute hepatitis to cirrhosis and hepatocellular carcinoma (HCC; Fig. 5). In addition, HCV has been suggested as a possible cause of several extrahepatic illnesses, but the etiologic link with these affections remains uncertain.

4.1. Acute and Chronic Hepatitis

Information about duration of incubation and other aspects of the acute phase of infection derives from documented cases of posttransfusion hepatitis and from experimental infection of chimpanzees. In these circumstances, the interval between exposure to the virus and onset of clinical manifesta-

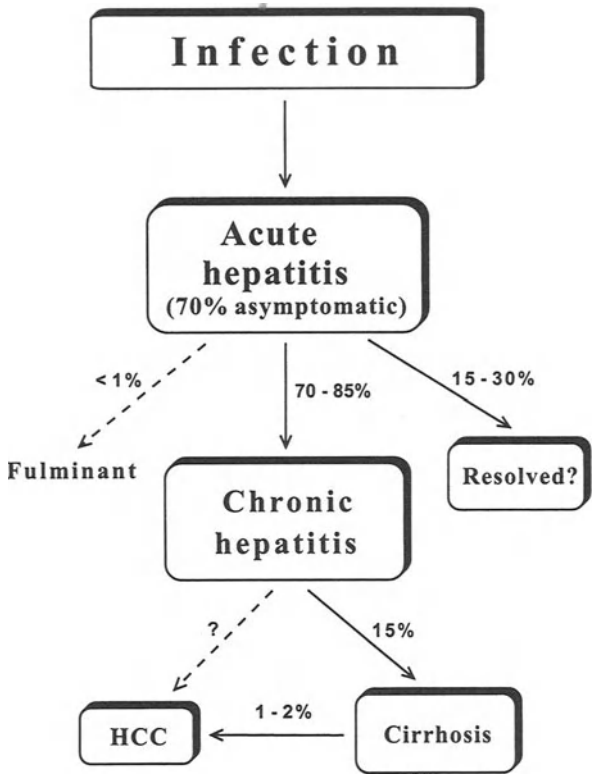


Fig. 5. Clinical outcomes of HCV infection.

tions ranges between 4 and 20 wk with a mode of 7–8 wk *(163,252)*. Seventy to 80% of posttransfusion infections are subclinical and, when clinically evident, are usually mildly symptomatic and anicteric, thus indicating that acute HCV infection is generally compatible with maintenance of liver function. Usually the major symptoms consist of weakness, malaise, nausea, and anorexia, showing no clearly distinctive features compared with other viral hepatitis. Hepatosplenomegaly is infrequent.

Clinical evaluation of the early stages of community-acquired infection is more difficult. Symptomatic cases appear to be more frequent than in posttransfusion cases (in one study, icterus was observed in 70% of individuals with sporadic acute hepatitis; ref. *168*) but this may reflect, in part, the fact that generally only symptomatic individuals seek medical advice, since patients with community-acquired infection tend to show milder liver lesions than patients with posttransfusion infection when biopsied at the time

of first diagnosis *(170)*. However, the dose of virus to which an individual is exposed is certainly important in determining clinical outcome and may explain why acute hepatitis C tends to exhibit a milder course in intravenous drug addicts than in posttransfusion patients *(253)*. ALT values usually remain lower in acute HCV disease than in acute hepatitis from HAV or HBV, infrequently exceeding 1000 U/L. Peak ALT levels are seen between 8 and 12 wk from initiation of infection, and fluctuate considerably or show a polyphasic behavior, though single peaks or plateau patterns are also observed *(163,166,168,252,254,255)*.

In a large proportion of patients, symptoms of acute hepatitis gradually resolve spontaneously within a few months. Resolution of symptoms and ALT normalization, however, do not mean that the virus has been cleared, since persistent or intermittent viremia is the most frequent occurrence (*see* Subheading 3.1,). Fulminant acute hepatitis C is rare, though it tends to be less so in certain Asian countries and in patients coinfected with other hepatotropic viruses *(256,257)*.

Since the acute phase is generally benign, the importance of HCV infection is mainly because of its remarkable propensity to persist and produce chronic liver injury *(258)*. Hepatitis is considered chronic when plasma ALT remains above normal levels for >6 mo after the acute phase. This evolution is observed in greater than 50% and 70% of posttransfusion and sporadic hepatitis cases, respectively *(168,170,259–262)*. No biochemical, virological, or serological markers predictive of a chronic evolution of the disease have been identified.

The behavior of ALT levels in plasma during chronic infection reflects the development of liver lesions. Soon after the acute phase and for a period that can last 2–3 yr, ALT levels tend to decrease progressively. Subsequently, for periods of up to 10–15 yr, ALT levels usually remain normal or are only slightly elevated; nevertheless, liver function worsens progressively, albeit slowly *(261)*. This period is usually symptom free or accompanied by nonspecific manifestations of moderate liver dysfunction. In the great majority of cases, therefore, the gradual deterioration of hepatic tissue produced by HCV has no obvious clinical counterpart *(136,166)*. Patients often become aware of the infection during this stage and incidentally, when an HCV antibody test is performed during medical controls done for other reasons. However, with duration of infection, symptoms become progressively greater and the ALT profile increasingly more abnormal.

In the course of chronic infection ALT levels are intermittently or continuously elevated and, in the latter case, can show a fluctuating pattern (*252,255,261*; Fig. 4). Increases and decreases of ALT levels may be proceeded by similar fluctuations of viremia *(12,160,162,163,232)*. Viremia

fluctuations in the absence of appreciable ALT changes have also been described and have been interpreted as a possible indication of an extrahepatic HCV replication *(159,163)*. On the other hand, long-term follow-up has also led to the recognition of viremic subjects that show ALT levels within the normal range for protracted periods. Whether these patients can be considered healthy carriers is still debated. In most such patients, biopsy evidences degrees of liver damage ranging from persistent or active chronic hepatitis to cirrhosis *(262–265)*. Patients with normal histology have also been described *(266)* and usually have low viremia titers *(263)*. In a recent study *(267)*, a large proportion of 167 viremic patients exhibiting normal ALT levels during periods of observation of 9–26 mo infection were found to be infected with type 2 HCV.

During the course of chronic infections, episodes of acute hepatitis ("flare-ups") can be observed and may be caused by reactivation of the underlying infection or to reinfections. The latter occurrence has been described not only in repeatedly exposed chimpanzees but also in hemophiliacs and other patients who require frequent infusion of blood derivatives *(161,178,268,269)*.

4.2. Cirrhosis and Hepatocarcinoma (HCC)

The most serious long-term sequelae of HCV infection are cirrhosis and HCC. Chronic infection with HCV is, actually, being recognized as a more important factor in HCC pathogenesis than infection with other hepatitis viruses at least in certain geographical areas, contributing approx 50% of cases *(270)*. Follow-up of well documented cases of posttransfusion infection has clearly documented the slow evolution of HCV-associated pathology: clinical signs of chronic hepatitis usually develop after 10–14 yr, those of cirrhosis after 16–18 yr, and HCC after 23–30 yr. In a few documented cases, HCC required up to 50 yr or more to develop *(255,261)*. However, in a few patients, disease may take a more aggressive course; thus, cases of cirrhosis that developed 15–18 mo after acute hepatitis have been described *(252,258)*.

It has been estimated that cirrhosis occurs in 20–40% of chronic hepatitis patients followed for 10 yr or more and HCC in approx 10% of patients with cirrhosis. For unknown reasons, HCC prevalence is especially high in Asia *(168,252,255,261)*. Evolution toward cirrhosis and HCC is generally believed to be especially frequent in patients that show histological signs of chronic active hepatitis since clinical onset. There is no doubt, however, that even clinically silent persistent hepatitis can evolve in chronic active hepatitis and then cirrhosis. Though HCC usually develops in cirrhotic livers, cases occurring in the absence of cirrhosis have been described *(271)*.

Several factors have been evaluated as possible predictive markers of evolution toward cirrhosis and HCC, including HCV genotype, viremia load, duration of infection, age and immunological situation at the time of infection, ALT behavior, coinfection with hepatitis B virus, and alcoholism, but no firm conclusions are yet possible *(166,272–275)*.

4.3. Liver Histopathology

The lesions found in the liver of acutely and chronically infected patients are not pathognonomic for HCV infection, consisting essentially of inflammation and hepatocellular necrosis. The major usefulness of biopsy findings is in the staging of liver damage severity and in evaluating how lesions progress with time or respond to therapy *(276)*. Histologically, a large proportion of acute hepatitis infections do not resolve but evolve with time into a chronic hepatitis of increasing severity that can progress to cirrhosis and HCC (Figs. 4 and 5). The severity of liver damage may be graded with Knodell's "histologic activity index" which considers the following four types of lesions: periportal necrosis (score from 1 to 10), intralobular degeneration and focal necrosis, portal inflammation, and fibrosis (each one with a score from 1 to 4) *(277,278)*. In the forms of hepatitis caused by other agents, this index usually correlates reasonably well with peripheral markers of liver damage and with clinical symptoms. This, however, is much less so in hepatitis C. At biopsy, viremic blood donors with normal ALT levels have evidence of all possible types of lesions, ranging from chronic persistent hepatitis to cirrhosis. To compound the matter, the indexes obtained from multiple biopsies of one patient can differ considerably, as a possible result of intrahepatic differences in virus-host equilibrium. A single biopsy, therefore, may not be representative of overall activity of infection and liver damage. In general, however, at the time of diagnosis, many patients present with signs of mild chronic hepatitis or with lesions that make it difficult to distinguish between persistent and active chronic hepatitis *(50)*.

Features that are frequently observed in chronic hepatitis C include lymphoid aggregates or follicles in the portal areas (present in approx 80% of biopsies), biliary duct lesions, and steatosis (present in more than 50% of biopsies). Biliary duct lesions consist of infiltration of inflammatory cells in the basal membrane, stratification and loss of polarity of epithelial cells, nuclear picnosis, degeneration, and mitotic activity or in combinations of these findings, and may lead to disappearance of the biliary ducts *(50,279–282)*. Even in the presence of cirrhosis or HCC, there are no histopathological features specific of an underlying HCV infection, except for the possible observation of sporadic lymphoid aggregates *(50,280)*.

4.4. Mechanisms of HCV-Induced Liver Damage

The mechanisms whereby HCV infection damages the liver are poorly understood. Currently, the prevailing opinion is that hepatic tissue injury is mainly immunologically mediated. This view is based on circumstantial evidence showing that

1. HCV is not obviously cytopathic in vitro at least for certain cell types.
2. Biochemical alterations indicative of hepatocyte necrosis develop after a considerable interval from viremia peak.
3. Treatment of infected humans and chimpanzees with corticosteroids and other immunosuppressants can reduce the biochemical abnormalities even though viremia titers increase *(177,283,284)*.
4. Occasionally, sudden withdrawal of immunosuppressive treatments has resulted in reactivation of chronic hepatitis and fulminant liver failure *(285)*.
5. Liver transplant patients may have extremely high viremia levels as a consequence of immunosuppressive therapy and graft reinfection and nevertheless remain asymptomatic *(286)*.

Moreover, the characteristics of HCV-sensitized lymphocytes found in infected patients and recruited into the liver (*see* Subheading 3.2.3.) are compatible with this view.

A role in liver damage has also been attributed to autoimmunity, based essentially on the presence in infected individuals of antibodies to a presumed nuclear self-epitope defined as GOR, which shares significant homology with a segment (amino acids 5–23) of the core protein and on the role HCV would appear to play in type 2b autoimmune hepatitis *(205,287,288)*. This form of autoimmune hepatitis is usually observed in adult males and is associated with the presence of anti-HCV antibodies. Contrary to what is observed in type 2a autoimmune hepatitis, antiliver/kidney microsomal LKM1 autoantibodies are low in titer *(289)* and are regarded as secondary to the liver damage induced by HCV. However, antiLKM1 autoantibodies are found only in some HCV-infected individuals, and their production is thus considered to require additional, presently unknown concomitant factors *(290)*. Reportedly, the prevalence of HCV-associated autoimmune hepatitis is especially high in certain European areas, an occurrence that does not seem to depend on differences in the geographical distribution of HCV types *(136,291–294)*.

While immunopathological mechanisms of hepatocyte damage are most likely of paramount importance, there are also indications suggesting a direct hepatopathogenic effect of the virus. Though not a constant finding, high levels of viremia have repeatedly been described in patients with severe liver damage *(173,263,295,296)*, which might reflect a more rapid viral rep-

lication as well as a better ability to escape immune control. Moreover, the course of HCV infection may be unusually progressive in persons with congenital or acquired immunodeficiencies and in immunosuppressed chimpanzees, and administration of antilymphocyte globulin to liver transplant patients has been seen to exacerbate impairment of the engrafted organ *(177,297–299)*. Finally and possibly more significantly, prolonged replication of HCV in cultured hepatoma cells has recently been associated with altered cellular behavior *(25)*.

The observation of especially severe degrees of liver damage in patients infected with subtype 1b HCV *(267,271,300,301)* might also be considered an indication of the importance of viral factors in pathogenesis. However, as discussed in Subheading 5., the prevalence of this genotype in Europe directly correlates with patients' age and duration of infection. Thus, the observed association between severe liver lesions and genotype 1b might simply reflect an elevated diffusion of this genotype in Europe 20 yr ago. The other genotypes would appear to have been introduced into Europe more recently, and hence available data would not be sufficiently informative regarding their pathogenicity. Cirrhosis has been observed in infection with all genotypes.

On the other hand, liver transplant recipients infected with genotype 1b have shown a greater frequency of posttransplant acute hepatitis and a greater tendency to develop chronic active hepatitis compared with similar patients infected with other genotypes *(302)*. Furthermore, type 3 has been associated with an especially aggressive form of chronic liver disease *(303)*. These and other data would suggest that viral determinants exist that control the pathological outcome of infection. The genome of type 1b contains two hypervariable domains in the E2/NS1 region, while the other types contain only one, and this might render type 1b especially fit at evading the host's immune surveillance *(77)*. Whether genotype 1b causes higher levels of viremia than other viral genotypes is, however, uncertain *(171,273,304–306)*. HCV quasispecies complexity tends to be especially high in patients with severe liver damage and in those who do not respond to IFN therapy and appears to be related to the type of equilibrium the virus reaches with the immune system rather than to duration of infection *(233,235,307–309)*. In summary, whether HCV genotype and quasispecies complexity influence clinical outcome and whether their investigation has clinical utility are important questions that deserve further scrutiny *(154,155,310–312)*.

The molecular mechanisms that lead from HCV infection to HCC are not known. Because HCV uses no DNA intermediate for replicating, integration of the viral genome, totally or inpart, into host's chromosomes is extremely unlikely. Recently, HCV quasispecies compositions have been

shown to differ in cancerous and noncancerous liver tissues but the pathogenetic significance of this finding remains to be clarified *(313)*. Continuous regeneration of hepatocytes destroyed by the infectious process may be an important driving force in tumorigenesis and may act in concert with other factors. A role for the HCV core protein in cell transformation has been suggested *(314,315)*. Also, NIH 3T3 cells were transformed by transfection with the 5' half of the NS3 gene *(316)*.

There is little doubt that HCV infection is capable of producing the full range of liver diseases discussed above in the absence of other liver noxae. However, as might be expected, the coexistence of other hepatopathogenic factors, such as infection by other hepatitis viruses and excessive alcohol consumption, have been associated with a more severe course *(317)*. Coinfection with HIV may also negatively influence the course of HCV infection *(298,318–320)*, whereas the opposite does not seem to occur *(321)*. The genetic constitution (major histocompatibility complex) of the host and old age at the start of infection may also influence the severity of HCV-induced disease *(322–324)*.

4.5. Extrahepatic Diseases

Evidence has implicated HCV as an etiologic or precipitating agent in a number of extrahepatic diseases. Conditions for which a link with HCV infection appears more likely are mixed essential cryoglobulinemia, porphyria cutanea tarda, and membrano-proliferative glomerulonephritis *(325–327)*.

Cryoglobulins (proteins that tend to precipitate when sera are kept at temperatures below 37°C and pH 7) are present in normal sera but when they reach abnormal concentrations may cause a systemic vasculitis that manifests itself with purpura, arthralgia, hepatosplenomegaly, and fatigue. Over half of HCV-infected patients have detectable serum levels of cryoglobulins, which are mostly polyclonal in nature but may contain a monoclonal component *(328)*. On the other hand, a large proportion of individuals with type II mixed essential cryoglobulinemia (i.e., presenting both polyclonal and oligoclonal cryoglobulins) have anti-HCV antibody and HCV RNA in their sera *(329)*. Anti-HCV IgG and HCV RNA are especially abundant in the cryoprecipitate associated with monoclonal IgM with RF activity *(328,330)*. Recently, a clonal expansion of intrahepatic B-cells with monoclonal and oligoclonal patterns has been described in HCV infected patients *(331)*. Using immunohistochemical methods, HCV-like antigens have been detected in skin biopsies as well as in liver biopsies of HCV-infected patients with mixed essential cryoglobulinemia. Viral antigens were observed both in the walls and within the lumen of vessels. In the latter location, HCV-like

antigen activity was associated with inflammatory infiltrates and deposits of hyalin material, thus suggesting that the virus might be directly involved in the genesis of lesions associated with type II cryoglobulinemia *(332,333)*. Cryoglobulinemia occurrence seems to be unrelated to HCV genotype and load *(206,334)*.

Porphyria cutanea tarda is a familial or sporadic alteration of heme metabolism caused by deficit of uroporphyrinogen decarboxylase that, in the presence of cofactors, becomes clinically evident with photosensitivity, various skin alterations, and liver dysfunctions. Sixty to 80% of patients have anti-HCV antibodies and/or HCV RNA in plasma *(325,335)*. It has been suggested that HCV infection is one of the factors that precipitate the clinical manifestations. Biopsy of the liver has shown alterations ranging from chronic persistent hepatitis to cirrhosis and hepatocarcinoma *(336)*.

Membrano-proliferative glomerulonephritis is believed to be triggered by the deposition of immune complexes in glomerular capillaries, with consequent gradual impairment of glomerural function. It has been described in patients with chronic HCV infection and circulating cryoglobulins *(337,338)*, and HCV-like antigen has been detected in the glomerular lesions of some patients *(325)*. Furthermore, deposits of IgG, IgM, and the third component of complement have been observed in the kidney of patients with chronic hepatitis *(338)*.

HCV has been suggested, so far with little evidence, as the etiologic agent of several other diseases of unknown origin. These include lichen planus, idiopathic pulmonary fibrosis, Sjögren's syndrome, autoimmune thyroiditis, and Mooren's corneal ulcers *(206,325,335)*. Initial suggestions that HCV might be the cause of aplastic anemias and other afflictions have not been substantiated. Recently, a high prevalence of HCV infection in patient with B-cell nonHodgkin lymphoma has been noted *(339,340)*.

5. Epidemiology

As shown by serosurveys, HCV is present worldwide. Its prevalence, however, varies considerably depending on the geographical areas and the groups of individuals examined. In the general population, prevalence rates range around 1% in Europe and the United States, where the incidence of new cases has declined in recent years, between 1 and 3% in the Middle East and Asia, and between 1 and 19% in Africa. Usually, in the population at large, prevalence of HCV infection is not influenced by gender, is inversely related to socioeconomic level, and increases with age *(341,342)*. In chil-

dren under 16 yr old, HCV infection is rare in developed countries but is already frequent in many African and Asian regions *(343)*.

Much higher prevalence rates are found in subjects who have received blood transfusions or organ transplants before sensitive methods for testing donors were used. It is common knowledge that before donors could be screened for anti-HCV antibody a significant proportion of blood-transfused patients developed NANB hepatitis. In more recent years, even prior to screening, the use of surrogate markers (ALT levels and anti-HBV antibody), intensive questioning of donors, and other preventive measures had somewhat reduced its incidence. The risk of contracting HCV infection was even higher in persons treated with commercial blood derivatives obtained by processing large pools of plasma obtained from numerous, often mercenary donors, when these products were not yet virus-inactivated. Clotting factor concentrates were an especially frequent source of infection. As a consequence, currently 60% or more of hemophiliacs are seropositive for HCV, with positivity rates directly related to the total amount of clotting factor received *(344–346)*. Intravenous but not intramuscular immune globulins have also transmitted the infection *(347–349)*.

Because of the sharing of syringes and other blood-contaminated equipment, HCV is also extremely widespread among parenteral drug consumers. In these subjects, seroprevalence usually ranges between 50 and 90%, with higher rates in individuals using drugs for longer periods. HCV prevalence is also high in patients with chronic renal failure requiring dialysis (10–40% seropositive in different countries and dialysis units), while descriptions of nosocomial infections outside this subset of patients are few *(350)*. Other risk groups include professionals occupationally exposed to blood, such as surgeons, dentists, pathologists, laboratory technicians, and barbers *(351–354)*. For example, studies have shown that approx 10% of infected needle stick accidents result in virus transmission *(355,356)*.

Cases of HCV infection occurring outside the risk groups mentioned above are called sporadic, cryptogenetic, or community-acquired. In many such cases there is no history of overt parenteral exposure and the routes of transmission remain obscure *(357)*, despite some evidence that HCV may be shed with genital secretions, breast milk and saliva, possibly from blood contamination *(358–361)*. Nonsexual spread to household contacts is so infrequent that its occurrence is still uncertain. Sexual transmission does occur, as suggested by higher infection rates in sexually promiscuous individuals *(362)* and demonstrated by sequence analysis of the viruses found in sexual partners *(363,364)*, but its efficiency is markedly lower than for HBV *(365)*. Mother-to-baby transmission is also known to occur but again its efficiency is low. Thus, sexual and vertical routes are believed to account

only for a small percentage of community-acquired forms, even though their importance may increase when HCV viremia levels are especially elevated caused by HIV coinfection or other reasons *(366,367)*.

The fact that in most sporadic infections the modes of HCV spread remain mysterious has led to questioning whether this is because the existence of routes of transmission are still unrecognized or because of insufficient characterization of infected individuals as members of risk groups *(168)*. Undoubtedly, the possible occasions of inapparent percutaneous exposure are numerous: incorrectly performed invasive therapeutic procedures, tattooing, piercing, acupuncture and other types of folk medications, violent body-contact sport activities, the blood-involving ritual ceremonials still in use in many developing countries, among others. A role for hematophagous arthropod vectors has also been suspected but never substantiated.

Additional studies are needed to clarify the means of HCV transmission occurring outside the parenteral risk groups. In any case, there is little doubt that, at a difference with percutaneous transmission, nonparenteral transmission occurs at low efficiency. Clearly, this would be a strong limitation for HCV survival in nature but is apparently compensated by the virus' ability to produce lifelong infections.

Studies have also investigated the relative prevalences of different HCV genotypes and their geographical distribution. The results have evidenced that certain genotypes, such 1a, 1b, 2a, and 2b, are more prevalent than others, and that considerable geographical differences exist in genotype distribution (Fig. 6). Predominant genotypes are 1a, 1b, 2a, and 2b in North America, South America and Europe; 1b, 2a, and 2b in Japan, Korea, Taiwan and parts of China, type 3 in Thailand, Singapore and other parts of Southeast Asia; type 4 (numerous subtypes) in North Africa and the Middle East; and type 5 in South Africa. Other genotypes appear to be less common and restricted to smaller regions, although further studies are necessary to obtain a more comprehensive picture (references in Table 2; refs. *145,147*). Recently, genotype 2c was recognized as the second most common genotype in Italian patients with community-acquired hepatitis *(368,369)*. As already mentioned in Subheading 4.4., genotype distributions in Europe vary with patients' age, as a likely consequence of temporally distinct epidemic waves. Thus, subtype 1b has been detected in over 80% of patients aged 60 yr or more, in over 60% of patients aged 40–60 yr and only in approx 30% of patients less than 30 yr old. In addition, genotype 1b was detected in 87% of patients known to have acquired infection 20 or more years earlier and in 41% of patients infected since less than 10 yr *(305)*. Differences in genotype distribution have also been noted depending on the mode of infection. For example, in Italy community-acquired infections are mainly caused by genotype 1b, whereas the

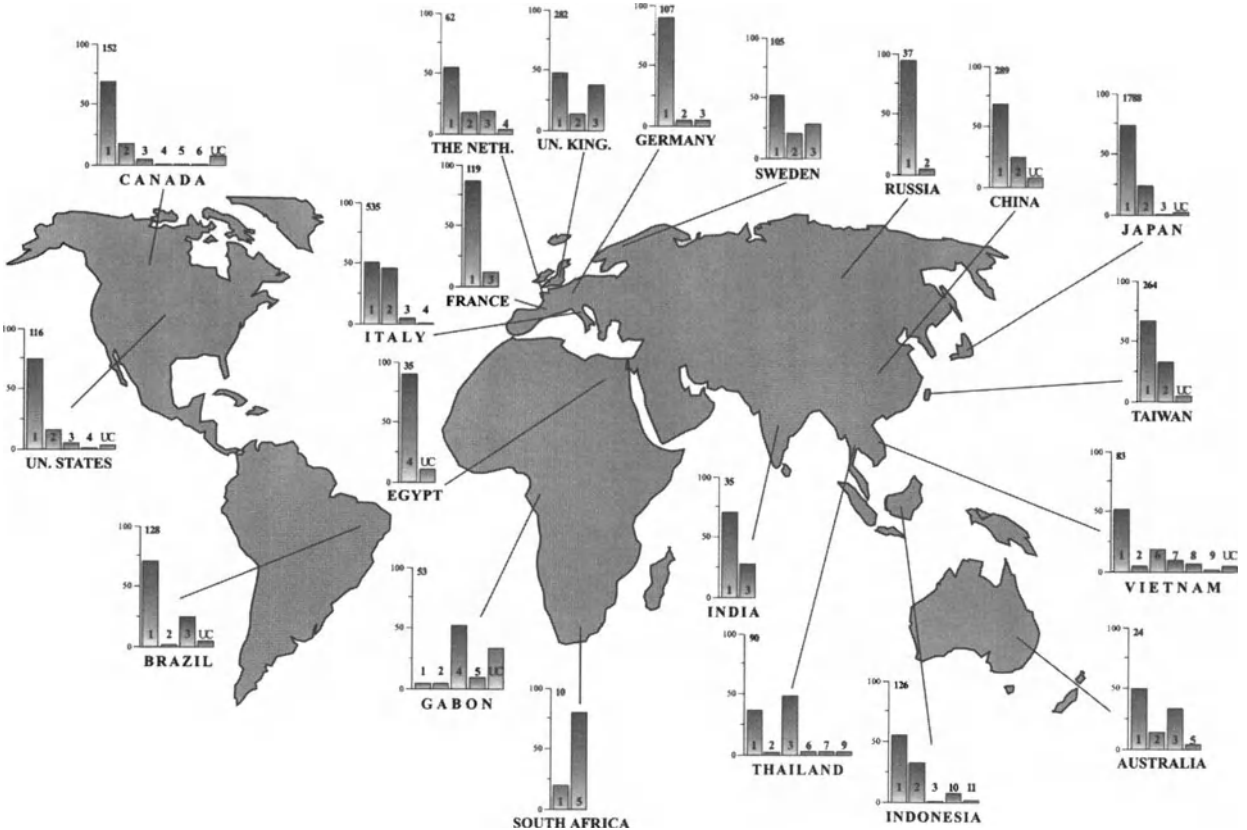


Fig.6. Worldwide distribution of HCV genotypes. Countries where molecular epidemiology surveys were carried out are indicated below each histogram. Columns represent percentages of the genotype indicated above or within each column; UC indicates the unclassified strains.

majority of hemophiliacs are infected with genotype 1a, most likely because the clotting factors used were largely prepared from blood purchased abroad *(370)*. Similarly, in Japan, genotype 1a is confined to hemophiliacs who have received commercial clotting factor concentrates *(371)*.

6. Prevention and Control

Currently, prevention of HCV infection relies mainly on measures directed at interrupting transmission through iatrogenic or accidental exposure to infected blood. As a result of accurate routine anti-HCV screening of blood and organ donors, new posttransfusion and posttransplant infections have now been largely eliminated *(372,373)*. The occasional cases still observed are from the failure of anti-HCV antibody tests to identify infections during the early serologically negative window period. In one study performed in 1992, one of 345 blood-transfused patients became HCV infected and the corresponding blood unit was retrospectively found HCV-positive by PCR *(374)*. Although the increased sensitivity of more recent serological assays has considerably shortened the window period, there is now some debate on whether donors should also be tested by HCV-specific PCR. In recent years, blood derivatives have also become almost 100% safe because of better screening of the blood supply and to the virucidal treatments introduced in the manufacturing processes to combat HIV in the mid-1980s *(349)*. As a result, new hemophilia-associated infections have also almost disappeared *(344,357)*.

As for other bloodborne infections, prevention in professionals exposed to blood and in their clients should be based on the presumption that any person might carry HCV. Hence, precautions to prevent exposure to blood should be meticulous and universally used, and personnel at risk should be trained to use aseptic techniques and disposable or properly sterilized equipment, to prevent needle-sticks and other injuries, to properly dispose of blood, body fluids, and any material potentially contaminated, and to employ safe procedures in the cleaning up of blood spills.

Changes in behavior of illicit intravenous drug users should also be pursued by educational campaigns and other means in the attempt to reduce the risk of bloodborne infections in general. To curb needle sharing, free distribution of disposable syringes has been tried in certain countries with some success.

Until the uncertainties still existing with regard to the nonparenteral routes of HCV spread are resolved, it will be difficult to fashion appropriate guidelines for the prevention of nonpercutaneous transmission. However, HCV-negative sexual partners of known HCV carriers should be advised to use condoms and family members should be educated to avoid contact with

the carrier's blood and body fluids and not to use utensils that might be contaminated with the carrier's blood. Since there are indications that the likelihood of sexual and perinatal transmission correlates with viral load, it is also possible that viremia-reducing therapies may cut down HCV transmission. The observation that in developed countries infection is rare under the age of 16 indicates that improvement of socioeconomic conditions and, possibly, generalized use of disposable medical instruments can considerably cut down transmission rates.

Despite a number of trials *(375,376)*, the efficacy of postexposure prophylaxis with immune globulin after transfusion or needle-stick accidents remains uncertain. In recent studies, passive administration of anti-HCV antibodies to chimpanzees shortly after experimental HCV exposure did not prevent or delay infection but prolonged the incubation period of hepatitis *(377)*.

No doubt, the most important advance in controlling HCV would be the development of efficacious vaccines, but the great genetic diversity of the virus and the poor resistance to reexposure to heterologous and homologous strains of HCV shown by infected humans and chimpanzees *(10,178,179,269)* herald that the route to the development of an effective vaccine will not be an easy one. Because of the high degree of amino acid divergence in the surface glycoproteins of HCV, there is little doubt that a vaccine will have to be multivalent. Additional obstacles are the lack of efficient and reliable in vitro culture systems and the scarcity of experimental hosts. Nevertheless, attempts to develop vaccines against HCV are underway. Choo et al. *(189)* vaccinated seven chimpanzees with E1 and E2/NS1 glycoproteins produced by HeLa cells infected with a recombinant vaccinia virus expressing vector and purified under undenaturating conditions. A strong antibody response was obtained in all vaccines. Following intravenous challenge with approx ten 50% chimpanzee infectious doses of the homologous virus, the five highest responders showed complete protection and the remaining two appeared to have developed a milder infection as compared with four unvaccinated controls. Despite its limits (low challenge dose, challenge performed at peak antibody response, homologous challenge only), this experiment has indicated that at least under certain circumstances, vaccine-induced protection against HCV is achievable. However, many aspects remain to be clarified, including the feasibility of using the above approach to vaccinate against multiple genotypes and the duration of protection. Previous studies in the same laboratory using different immunizations (live recombinant vaccinia virus and glycoproteins produced in yeast or insect cells) had given less encouraging results *(189)*. Other approaches under investigation include the use of DNA vaccines targeting the nucleo-

capsid protein *(378,379)*. This protein would have the advantage of a low sequence variability, but its ability to confer protective immunity remains to be proved. Thus, at present a vaccine against HCV still seems a long way off. Unfortunately, the characteristics of the virus and the infection make it likely that difficulties will be encountered similar to those that are being met in the development of anti-HIV vaccines.

As discussed in the previous sections, the course of HCV infection is highly variable and unpredictable in different patients. When viral and host factors contributing to such variability are better understood, it will also be possible to conceive measures capable of slowing progression to severe disease. In any case, infected individuals should already be advised to refrain from drinking alcohol and to vaccinate against HBV.

Therapeutic options presently available for HCV infection are limited. Chemotherapy alone has proved essentially ineffective and is little used. IFN therapy has instead acquired an important place in the treatment of chronic infections and, though clinical trials are fewer, has also been advantageously used in the treatment of acute infections. However, IFN treatment leads to a successful resolution of infection only in a subset of chronically infected patients. Prolonged IFN-alpha treatment has been shown to reduce biochemical abnormalities in approx 40–50% of chronic infections and to ameliorate liver lesions in a large proportion of responder patients, but clinical benefits are often transient and in about one-half, the initial responders symptoms relapse after cessation of treatment. Complete virus eradication is even rarer.

Because IFN therapy may have considerable side effects and is expensive, studies have focused on the identification of conditions that might predict sustained responsiveness. Existing data indicate that variables affecting the outcome of treatment are several, although none of them is 100% predictive. Also the therapeutic problems posed by nonresponder and relapsing patients are currently the subject of intensive clinical investigation.

Currently, HCV-associated cirrhosis and HCC are the indication for liver transplantation in at least one fourth of liver transplant recipients. Nearly all patients have recurrent, IFN-resistant viremia after transplantation as the result of invasion of the grafted organ and immunosuppression, though not all appear to develop histologic signs of hepatitis. The consequences of HCV recurrence on long-term graft survival have yet to be assessed *(302,380,381)*.

7. Future Directions

One major lesson we have learned from research on HCV is that important progress can come from the interface of the clinic, the basic laboratory,

and biotechnological companies. There is no doubt that HCV research would not be where it stands today had it not been for the molecular cloning of the viral genome. This developed based on previous pioneering investigations in chimpanzees and has since rapidly translated into enormous progress in our understanding of the molecular and immunological properties of the virus as well as in better preventive measures and disease management.

In practical terms, the area that has profited most from the virus cloning breakthrough has been diagnosis. Thanks to the sensitive diagnostic tools developed, the great majority of inapparent infections are now readily recognized. This, in turn, has led to the almost complete disappearance of new transfusion-transmitted infections. It is expected that diagnostic tools and protocols will be further improved in the next few years to eliminate also the few remaining cases.

Another field that has benefited considerably is our perception of the dynamics of HCV infection and of how this is modified by therapies. Methods are now available that permit precise and direct monitoring of the infection by measuring viremia level, which is much more informative than the biochemical alterations on which clinicians previously had to rely entirely. It is easy to foresee that this area of research will expand rapidly in the next few years and will lead to an improved understanding of HCV basic pathobiology as well as to better use of antiviral therapies. Drugs presently available to treat HCV infection are few and only marginally effective. It is expected that the speed of development of new and efficacious treatments will be accelerated in the near future as a result of improved knowledge of HCV enzymes and precursor molecules, the application of newer technologies, and modern concepts in the design of antiviral strategies that are currently emerging from management of HIV infection.

It is anticipated that availability of better drugs will have beneficial effects also on HCV epidemiology by reducing the risks of nonparenteral virus transmission. Especially in certain geographical regions and risk groups, effective prevention will, however, be possible only when anti-HCV vaccines become available. Present skepticism as to whether it will be possible to develop preventive vaccines focuses around the lack of known correlates of immune protection and reliable methods for culturing HCV in vitro, the scarcity of the only animal species in which candidate vaccines can currently be tested, and the uncertainties surrounding the implications of the great genetic diversity of HCV. To surmount these obstacles will undoubtedly require further breakthroughs, but we are confident that our biotechnological era will soon provide the appropriate answers.

HCV infection is an important health problem, as it affects 1% or more of the world's population. Thanks to recent impressive advances, it is now

possible to believe that it will be subdued if not completely conquered in the not too distant future.

References

1. Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244,** 359–362.
2. Bradley, D. W., Maynard, J. E., Popper, H., Cook, E. H., Ebert, J. W., McCaustland, K. A., Schable, C. A., and Fields, H. A. (1983) Post-transfusion non-A non-B hepatitis: physicochemical properties of two distinct agents. *J. Infect. Dis.* **148,** 254–265.
3. van Doorn, L. J. (1994) Review: molecular biology of the hepatitis C virus. *J. Med. Virol.* **43,** 345–356.
4. Houghton, M., Weiner, A. J., Han, J., Kuo, G., and Choo, Q. L. (1991) Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. *Hepatology* **14,** 382–388.
5. Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I., and Okayama, H. (1991) Structure and organization of the hepatitis C virus genome isolated from human carriers. *J. Hepatol.* **65,** 1105–1113.
6. Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G., and Houghton, M. (1991) Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **88,** 2451–2455.
7. Shukla, D. D., Hoyne, P. A., and Ward, C. W. (1995) Evaluation of complete genome sequences and sequences of individual gene products for the classification of hepatitis C viruses. *Arch. Virol.* **140,** 1747–1761.
8. Yoo, B. J., Spaete, R. R., Geballe, A. P., Selby, M., Houghton, M., and Han, J. H. (1992) 5' end-dependent translation initiation of hepatitis C viral RNA and the presence of putative positive and negative control elements within the 5' untranslated region. *Virology* **191,** 889–899.
9. Miller, R. H. and Purcell, R. H. (1990) Hepatitis C virus shares amino acid sequence similarity with pestivirus and flaviviruses as well as members of two plant virus supergroups. *Proc. Natl. Acad. Sci. USA* **87,** 2057–2061.
10. Farci, P., London, W. T., Wong, D. C., Dawson, G. J., Vallari, D. S., Engle, R., and Purcell, R. H. (1992) The natural history of infection with hepatitis C virus (HCV) in chimpanzees: comparison of serologic responses measured with first- and second-generation assays and relationship to HCV viremia. *J. Infect. Dis.* **165,** 1006–1011.
11. Negro, F., Pacchioni, D., Shimizu, Y., Miller, R. H., Bussolati, G., Purcell, R. H., and Bonino, F. (1992) Detection of intrahepatic replication of hepatitis C virus RNA by in situ hybridization and comparison with histopathology. *Proc. Natl. Acad. Sci. USA* **89,** 2247–2251.
12. Shindo, M., Di Bisceglie, A. M., Biswas, R., Mihalik, K., and Feinstone, S. M. (1992) Hepatitis C virus replication during acute infection in the chimpanzee. *J. Infect. Dis.* **166,** 424–427.
13. Bassett, S. E., Brasky, K. M., and Lanford, R. E. (1998) Analysis of hepatitis C virus-inoculated chimpanzees reveals unexpected clinical profiles. *J. Virol.* **72,** 2589–2599.

14. Fong, T. L., Shindo, M., Feinstone, S. M., Hoofnagle, J. H., and Di Bisceglie, A. (1991) Detection of replicative intermediates of hepatitis C viral RNA in liver and serum of patients with chronic hepatitis C. *J. Clin. Invest.* **88,** 1058–1063.
15. Krawczynski, K., Beach, M. J., Bradley, D. W., Kuo, G., Di Bisceglie, A. M., Houghton, M., Reyes, G. R., Kim, J. P., and Alter M. J. (1992) Hepatitis C virus antigen in hepatocytes: immunomorphologic detection and identification. *Gastroenterology* **103,** 622–629.
16. Cho, S. W., Hwang, S. G., Han, D. C., Jin, S. Y., Lee, M. S., Shim, C. S., Lee, D. W., and Lee, H. B. (1996) In situ detection of hepatitis C virus RNA in liver tissue using a digoxigenin-labeled probe created during a polymerase chain reaction. *J. Med. Virol.* **48,** 227–233.
17. Cribier, B., Schmitt, C., Bingen, A., Kirn, A., and Keller, F. (1995) In vitro infection of peripheral blood mononuclear cells by hepatitis C virus. *J. Gen. Virol.* **76,** 2485–2491.
18. Cribier, B., Rey, D., Uhl, G., Schmitt, C., Libbrecht, E., Vetter, D., Lang, J., Kirn, A., and Stoll-Keller, F. (1996) Quantification of hepatitis C virus RNA in peripheral blood mononuclear cells: a comparison between patients chronically infected by HCV and patients coinfected by HIV. *Res. Virol.* **147,** 325–332.
19. Lerat, H., Berby, F., Trabaud, M. A., Vidalin, O., Major, M., Trepo, C., and Inchauspe, G. (1996) Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. *J. Clin. Invest.* **97,** 845–851.
20. Laskus, T., Radkowski, M., Wang, L.-F., Cianciara, J., Vargas, H., and Rakela, J. (1997) Hepatitis C virus negative strand RNA is not detected in peripheral blood mononuclear cells and viral sequences are identical to those in serum: a case against extrahepatic replication. *J. Gen. Virol.* **78,** 2747–2750.
21. Mellor, J., Haydon, G., Blair, C., Livingstone, W., and Simmonds, P. (1998) Low level or absent in vivo replication of hepatitis C virus and hepatitis G virus/GB virus C in peripheral blood mononuclear cells. *J. Gen. Virol.* **79,** 705–714.
22. Iacovacci, S., Sargiacomo, M., Parolini, I., Ponzetto, A., Peschle, C., and Carloni, G. (1993) Replication and multiplication of hepatitis C virus genome in human foetal liver cells. *Res. Virol.* **144,** 275–279.
23. Lanford, R. E., Sureau, C., Jacob, J. R., White, R., and Fuerst, T. R. (1994) Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT-PCR. *Virology* **202,** 607–614.
24. Ito, T., Mukaigawa, J., Zuo, J., Hirabayashi, Y., Mitamura, K., and Yasui, K. (1996) Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titer infectious virus. *J. Gen. Virol.* **77,** 1043–1054.
25. Yoo, B. J., Selby, M. J., Choe, J., Suh, B. S., Choi, S. H., Joh, J. S., Nuovo, G. J., Lee, H. S., Houghton, M., and Han, J. H. (1995) Transfection of a differentiated human hepatoma cell line (Huh7) with in vitro-transcribed hepatitis C virus (HCV) RNA and establishment of a long-term culture persistently infected with HCV. *J. Virol.* **69,** 32–38.
26. Tsuboi, S., Nagamori, S., Miyazaki, M., Mihara, K., Fukaya, K., Teruya, K., Kosaka, T., Tsuji, T., and Namba, M. (1996) Persistence of hepatitis C virus RNA in established human hepatocellular carcinoma cell lines. *J. Med. Virol.* **48,** 133–140.
27. Shimizu, Y. K., Iwamoto, A., Hijikata, M., and Purcell, R. H. (1992) Evidence for in vitro replication of hepatitis C virus genome in a human T-cell line. *Proc. Natl. Acad. Sci. USA* **89,** 5477–5481.

28. Shimizu, Y. K., Purcell, R. H., and Yoshikura, H. (1993) Correlation between the infectivity of hepatitis C virus in vivo and its infectivity in vitro. *Proc. Natl. Acad. Sci. USA* **90,** 6037–6041.
29. Nissen, E., Hohne, M., and Schreier (1994) In vitro replication of hepatitis C virus in a human lymphoid cell line (H9) *J. Hepatol.* **20,** 437.
30. Kato, N., Nakazawa, T., Mizutani, T., and Shimotohno, K. (1995) Susceptibility of human T-lymphotropic virus type I infected cell line MT–2 to hepatitis C virus infection. *Biochem. Biophys. Res. Commun.* **3,** 863–869.
31. Mizutani, T., Kato, N., Saito, S., Ikeda, M., Sugiyama, K., and Shimotohno, K. (1996) 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. Clin. Microbiol.* **70,** 7219–7223.
32. Bertolini, L., Iacovacci, S., Ponzetto, A., Gorini, G., Battaglia, M., and Carloni, G. (1993) The human bone-marrow-derived B-cell line CE, susceptible to hepatitis C virus infection. *Res. Virol.* **144,** 281–285.
33. Valli, M. B., Bertolini, L., Iacovacci, S., Ponzetto, A., and Carloni, G. (1995) Detection of a 5'UTR variation in the HCV genome after long-term in vitro infection. *Res. Virol.* **146,** 285–288.
34. Shimizu, Y. K. and Yoshikura, H. (1994) Multicycle infection of hepatitis C virus in cell culture and inhibition by alpha and beta interferons. *J. Virol.* **68,** 8406–8408.
35. Nakajima, N., Hijikata, M., Yoshikura, H., and Shimizu, Y. K. (1996) Characterization of long-term cultures of hepatitis C virus. *J. Virol.* **70,** 3325–3329.
36. Seipp, S., Muller, H. M., Pfaff, E., Stremmel, W., Theilmann, L., and Goeser, T. (1997) Establishment of persistent hepatitis C virus infection and replication in vitro. *J. Gen. Virol.* **78,** 2467–2476.
37. Morrica , A., Giorgi, M., Maggi, F., Fornai, C., Vatteroni, M. L., Marchi, S., Ricchiuti, A., Antonelli G., Pistello, M., and Bendinelli, M. (1999) Susceptibility of human and non-human cell lines to HCV infection as determined by the centrifugation-facilitated method. *J. Virol. Meth.,* in press.
38. Bradley, D. W., McCaustland, K. A., Cook, E. H., Schable, C. A., Ebert, J. W., and Maynard, J. W. (1985) Posttransfusion non-A, non-B hepatitis in chimpanzees: physicochemical evidence that the tubule-forming agent is a small, enveloped virus. *Gastroenterology* **88,** 773–779.
39. He, L. F., Alling, D., Popkin, T., Shapiro, M., Alter, H. J., and Purcell, R. H. (1987) Determining the size of non-A, non-B hepatitis virus by filtration. *J. Infect. Dis.* **156,** 636–640.
40. Yuasa, T., Ishikawa, G., Manabe, S., Sekiguchi, S., Takeuchi, K., and Miyamura, T. (1991) The particle size of hepatitis C virus estimated by filtration through microporous regenerated cellulose fibre. *J. Gen. Virol.* **72,** 2021–2024.
41. Kaito, M., Watanabe, S., Tsukiyama-Kohara, K., Yamaguchi, K., Kobayashi, Y., Konishi, M., Yokoi, M., Ishida, S., Suzuki, S., and Kohara, M. (1994) Hepatitis C virus particle detected by immunoelectron microscopic study. *J. Gen. Virol.* **75,** 1755–1760.
42. Shimizu, Y., Feinstone, S. M., Kohara, M., Purcell, R. H., and Yoshikura, H. (1996) Hepatitis C virus: detection of intracellular virus particles by electron microscopy. *Hepatology* **23,** 205–209.
43. Takahashi, K., Kishimoto, S., Yoshizawa, H., Okamoto, H., Yoshizawa, A., and Mishiro, S. (1992) p26 protein and 33 nm particle associated with nucleocapsid of hepatitis C virus recovered from the circulation of infected hosts. *Virology* **191,** 431–434.

44. Sato, K., Okamoto, H., Aihara, S., Hoshi, Y., Tanaka, T., and Mishiro, S. (1993) Demonstration of sugar mojety on the surface of hepatitis C virions recovered from the circulation of infected humans. *Virology* **196,** 354–357.
45. Bradley, D., McCaustland, K., Krawczynski, K., Spelbring, J., Humphrey, C., and Cook, E. H. (1991) Hepatitis C virus: buoyant density of the factor VIII-derived isolate in sucrose. *J. Med. Virol.* **34,** 206–208.
46. Hijikata, M., Shimizu, Y. K., Kato, H., Iwamoto, A., Shih, J. W., Alter, H. J., Purcell, R. H., and Yoshikura, H. (1993) Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. *J. Virol.* **67,** 1953–1958.
47. Choo, S. H., So, H. S., Cho, J. M., and Ryu, W. S. (1995) Association of hepatitis C virus particles with immunoglobulin: a mechanism for persistent infection. *J. Gen. Virol.* **76,** 2337–2341.
48. Kanto, T., Hayashi, N., Takehara, T., Hagiwara, H., Mita, E., Naito, M., Kasahara, A., Fusamoto, H., and Kamada, T. (1994) Buoyant density of hepatitis C virus recovered from infected hosts: two different features in sucrose equilibrium density-gradient centrifugation related to degree of liver inflammation. *Hepatology* **19,** 296–302.
49. Durand, J. M., Lefevre, P., Kaplanski, G., and Soubeyrand, J. (1995) Patient to patient transmission of hepatitis C virus. *Lancet* **345,** 1442–1443.
50. Dhillon, A. P. and Dusheiko, G. M. (1995) Pathology of hepatitis C virus infection. *Histopathology* **26,** 297–309.
51. Bradley, D. W. (1985) The agent of non-A, non-B viral hepatitis. *J. Virol. Meth.* **10,** 307–319.
52. Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., and Shimotohno, K. (1990) Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc. Natl. Acad. Sci. USA* **87,** 9524–9528.
53. Ogata, N., Alter, H. J., Miller, R. H., and Purcell, R. H. (1991) Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **88,** 3392–3396.
54. Okamoto, H., Okada, S., Sugiyama, Y., Kurai, K., Iizuka, H., Machida, A., Miyakawa, Y., and Mayumi, M. (1991) Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. *J. Gen. Virol.* **72,** 2697–2704.
55. Okamoto, H., Kojima, M., Sakamoto, M., Iizuka, H., Hadiwandowo, S., Suwignyo, S., Miyakawa, Y., and Mayumi, M. (1994) The entire nucleotide sequence and classification of a hepatitis C virus isolate of a novel genotype from an Indonesian patient with chronic liver disease. *J. Gen. Virol.* **75,** 629–635.
56. Matsuura, Y., and Miyamura, T. (1993) The molecular biology of hepatitis C virus. *Sem. Virol.* **4,** 297–304.
57. Clarke, B. (1997) Molecular virology of hepatitis C virus. *J. Gen. Virol.* **78,** 2397–2410.
58. Major, M. E. and Feinstone, S. M. (1997) The molecular virology of hepatitis C. *Hepatology* **25,** 1527–1538.
59. Takeuchi, K., Kubo, Y., Boonmar, S., Watanabe, Y., Katayama, T., Choo, Q. L., Kuo, G., Houghton, M., Saito, I., and Miyamura, T. (1990) The putative nucleocapsid and envelope protein genes of hepatitis C virus determined by comparison of the nucleotide sequences of two isolates derived from an experimentally infected chimpan-

zee and healthy human carriers. *J. Gen. Virol.* **71,** 3027–3033.
60. Santolini, E., Migliaccio, G., and La Monica, N. (1994) Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J. Virol.* **68,** 3631–3641.
61. Matsumoto, M., Hwang, S. B., Jeng, K. S., Zhu, N., and Lai, M. M. (1996) Homotypic interaction and multimerization of hepatitis C virus core protein. *Virology* **218,** 43–51.
62. Shih, C. M., Chen, C. M., Chen, S. Y., and Lee, Y. H. W. (1995) Modulation of the trans-suppression activity of hepatitis C virus core protein by phosphorylation. *J. Virol.* **69,** 1160–1171.
63. Matsuura, Y., Harada, T., Makimura, M., Sato, M., Aizaki, H., Suzuki, T., and Miyamura, T. (1994) Characterization of HCV structural proteins expressed in various animal cells. *Intervirology* **37,** 114–118.
64. Shih, C. M., Lo, S. J., Miyamura, T., Chen, S. Y., and Lee, Y. H. W. (1993) Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in HuH–7 cells. *J. Virol.* **67,** 5823–5832.
65. Lo, S. Y., Selby, M. J., and Ov, J. M. (1996) Interaction between hepatitis C virus core protein and E1 envelope protein. *J. Virol.* **70,** 5177–5182.
66. Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M. J., Miyamura, T., and Brechot, C. (1997) Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc. Natl. Acad. Sci. USA* **94,** 1200–1205.
67. Zhu, N., Khoshnan, A., Schneider, R., Matsumoto, M., Dennert, G., Ware, C., and Lai, M. M. C (1998) Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. *J. Virol.* **72,** 3691–3697.
68. Miyamura, T., and Matsuura, Y. (1993) Structural proteins of hepatitis C virus. *Trends Microbiol.* **1,** 229–231.
69. Nishihara, T., Nozaki, C., Nakatake, H., Hoshiko, K., Esumi, M., Hayashi, N., Hino, K., Hamada, F., Mizuno, K., and Shikata, T. (1993) Secretion and purification of hepatitis C virus NS1 glycoprotein produced by recombinant baculovirus-infected insect cells. *Gene* **129,** 207–214.
70. Spaete, R. R., Alexander, D., Rugroden, M. E., Choo, Q. L., Berger, K., Crawford, K., Kuo, C., Leng, S., Lee, C., Ralston, R., Thudium, K., Tung, J. W., Kuo, G., and Houghton, M. (1992) Characterization of the hepatitis C virus E2/NS1 gene product expressed in mammalian cells. *Virology* **188,** 819–830.
71. Ralston, R., Thudium, K., Berger, K., Kuo, C., Gervase, B., Hall, J., Selby, M., Kuo, G., Houghton, M., and Choo, Q. L. (1993) Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. *J. Virol.* **67,** 6753–6761.
72. Lanford, R. E., Notvall, L., Chavez, D., White, R., Frenzel, G., Simonsen, C., and Kim, J. (1993) Analysis of hepatitis C virus capsid, E1, and E2/NS1 proteins expressed in insect cells. *Virology* **197,** 225–235.
73. Dubuisson, J., Hsu, H. H., Cheung, R. C., Greenberg, H. B., Russel, D. G., and Rice, C. M. (1994) Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant Vaccinia and Sindbis viruses. *J. Virol.* **68,** 6147–6160.
74. Zibert, A., Schreir, E., and Roggendorf, M. (1995) Antibodies in human sera specific to hypervariable region 1 of hepatitis C virus can block viral attachment. *Virol-*

ogy **208,** 653–661.
75. Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., and Shimotohno, K. (1991) Hypervariable regions in the putative glycoprotein of hepatitis C virus. *Biochem. Biophys. Res. Commun.* **175,** 220–228.
76. Weiner, A. J., Brauer, M. J., Rosenblatt, J., Richman, K. H., Tung, J., Crawford, K., Bonino, F., Saracco, G., Choo, Q. L., Houghton, M., and Han, J. H. (1991) Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. *Virology* **180,** 842–848.
77. Okamoto, H. and Mishiro, S. (1994) Genetic heterogeneity of hepatitis C virus. *Intervirology* **37,** 68–76.
78. Lin, C., Lindenbach, B. D., Pragai, B. M., McCourt, D., and Rice, C. M. (1994) Processing in the hepatitis C virus E2–NS2 region: identification of p7 and two distinct E2–specific products with different C termini. *J. Virol.* **68,** 5063–5073.
79. Mizushima, H., Hijikata, M., Asabe, S. I., Hirota, M., Kimura, K., and Shimotohno, K. (1994) Two hepatitis C virus glycoprotein E2 products with different C termini. *J. Virol.* **68,** 6215–6222.
80. Selby, M. J., Glazer, E., Masiarz, F., and Houghton, M. (1994) Complex processing and protein:protein interactions in the E2:NS2 region of HCV. *Virology* **204,** 114–122.
81. Baumert, T. F., Ito, S., Wong, D. T., and Liang, T. J. (1998) Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J. Virol.* **72,** 3827–3836.
82. Neddermann, P., Tomei, L., Steinkuhler, C., Gallinari, P., Tramontano, A., and De Francesco, R. (1997) The nonstructural proteins of the hepatitis C virus: structure and functions. *Biol. Chem.* **378,** 469–476.
83. Santolini, E., Pacini, L., Fipaldini, C., Migliaccio, G., and La Monica, N. (1995) The NS2 protein of hepatitis C virus is a transmembrane polypeptide. *J. Virol.* **69,** 7461–7471.
84. Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M., and Rice, C. M. (1993) A second hepatitis C virus-encoded proteinase. *Proc. Natl. Acad. Sci. USA* **90,** 10583–10587.
85. Reed, K. E., Grakoui, A., and Rice, C. M. (1995) Hepatitis C virus-encoded NS2–3 protease: cleavage-site mutagenesis and requirements for bimolecular cleavage. *J. Virol.* **69,** 4127–4136.
86. Pieroni, L., Santolini, E., Fipaldini, C., Pacini, L., Migliaccio, G., and La Monica, N. (1997) In vitro study of the NS2–3 protease of hepatitis C virus. *J. Virol.* **71,** 6373–6380.
87. Eckart, M. R., Selby, M., Masiarz, F., Lee, C., Berger, K., Crawford, K., Kuo, C., Kuo, G., Houghton, M., and Choo, Q. L. (1993) The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. *Biochem. Biophys. Res. Commun.* **192,** 399–406.
88. Tomei, L., Failla, C., Santolini, E., De Francesco, R., and La Monica, N. (1993) NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J. Virol.* **67,** 4017–4026.
89. Suzich, J., Tamura, J. K., Palmer-Hill, F., Warrener, P., Grakoui, A., Rice, C. M., Feinstone, S. M., and Collett, M. S. (1993) Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus

and flavivirus enzymes. *J. Virol.* **67,** 6152–6158.
90. Tai, C. L., Chi, W. K., Chen, D. S., and Hwang, L. H. (1996) The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3) *J. Virol.* **70,** 8477–8484.
91. Kim, D. W., Kim, J., Gwack, Y., Han, J. H., and Choe, J. (1997) Mutational analysis of the hepatitis C virus RNA helicase. *J. Virol.* **71,** 9400–9409.
92. Failla, C., Tomei, L., and De Francesco, R. (1994) Both NS3 and NS4a are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J. Virol.* **68,** 3753–3760.
93. Lin, C., Thomson, J. A., and Rice, C. M. (1995) A central region in the hepatitis C virus NS4a protein allows formation of an active NS3–NS4a serine proteinase complex in vivo and in vitro. *J. Virol.* **69,** 4373–4380.
94. Satoh, S., Tanji, Y., Hijikata, M., Kimura, K., and Shimotohno, K. (1995) The N-terminal region of hepatitis C virus nonstructural protein 3 (NS3) is essential for stable complex formation with NS4a. *J. Virol.* **69,** 4255–4260.
95. Shimizu, Y., Yamaji, K., Masuho, Y., Yokota, T., Inoue, H., Sudo, K., Satoh, S., and Shimotohno, K. (1996) Identification of the sequence on NS4A required for enhanced cleavage of the NS5A/5B site by hepatitis C virus NS3 protease. *J. Virol.* **70,** 127–132.
96. Tomei, L., Failla, C., Vitale, R. L., Bianchi, E., and De Francesco, R. (1996) A central hydrophobic domain of the hepatitis C virus NS4A protein is necessary and sufficient for the activation of the NS3 protease. *J. Gen. Virol.* **77,** 1065–1070.
97. Shimotohno, K. (1995) Hepatitis C virus as a causative agent of hepatocellular carcinoma. *Intervirology* **38,** 162–169.
98. Tanji, Y., Hijikata, M., Satoh, S., Kaneko, T., and Shimotohno, K. (1995) Hepatitis C virus-encoded nonstructural protein NS4a has versatile functions in viral protein processing. *J. Virol.* **69,** 1575–1581.
99. Reed, K. E., Xu, J., and Rice, C. M. (1997) Phosphorylation of the hepatitis C virus NS5A protein in vitro and in vivo: properties of the NS5A-associated kinase. *J. Virol.* **71,** 7187–7197.
100. Kaneko, T., Tanij, Y., Satoh, S., Hijikata, M., Shinichi, S., Kimura, K., and Shimotohno, K. (1994) Production of two phosphoproteins from the NS5a region of the hepatitis C viral genome. *Biochem. Biophys. Res. Commun.* **205,** 320–326.
101. Tanji, Y., Kaneko, T., Satoh, S., and Shimotohno, K. (1995) Phosphorylation of hepatitis C virus-encoded nonstructural protein NS5a. *J. Virol.* **69,** 3980–3986.
102. Kato, N., Lan, K.-H., Ono-Nita, S. K., Shiratori, Y., and Omata, M. (1997) Hepatitis C virus nonstructural region 5A protein is a potent transcriptional activator. *J. Virol.* **71,** 8856–8859.
103. Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Izumi, N., Marumo, F., and Sato, C. (1995) Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J. Clin. Invest.* **96,** 224–230.
104. Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Ogura, Y., Izumi, N., Marumo, F., and Sato, C. (1996) Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N. Engl. J. Med.* **334,** 77–81.
105. Gale Jr., M. J., Korth, M. J., Tang, N. M., Tan, S.-L., Hopkins, D. A., Dever, T. E., Polyak, S. J., Gretch, D. R., and Katze, M. G. (1997) Evidence that hepatitis C virus

resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* **230,** 217–227.
106. Hwang, S. B., Park, K.-J., Kim, Y.-S., Sung, Y. C. and Lai, M. M. C. (1997) Hepatitis C virus NS5B protein is a membrane-associated phosphoprotein with a predominantly perinuclear localization. *Virology* **227,** 439–446.
107. Behrens, S. E., Tomei, L., and De Francesco, R. (1996) Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J.* **15,** 12–22.
108. Lohmann, V., Korner, F., Herian, U., and Bartenschlager, R. (1997) Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J. Virol.* **71,** 8416–8428.
109. Chen, P. J., Lin, M. H., Tai, K. F., Liu, P. C., Lin, C. J., and Chen, D. S. (1992) The Taiwanese hepatitis C virus genome: sequence determination and mapping the 5' termini of viral genomic and antigenomic RNA. *Virology* **188,** 102–113.
110. Han, J. H., Shyamala, V., Richman, K. H., Brauer, M. J., Irvine, B., Urdea, M. S., Tekamp-Olson, P., Kuo, G., Choo, Q. L., and Houghton, M. (1991) Characterization of the terminal regions of hepatitis C viral RNA: identification of conserved sequences in the 5' untranslated region and poly(A) tails at the 3' end. *Proc. Natl. Acad. Sci. USA* **88,** 1711–1715.
111. Yen, J. H., Chang, S. C., Hu, C. R., Chu, S. C., Lin, S. S., Hsieh, Y. S., and Chang, M. F. (1995) Cellular proteins specifically bind to the 5'-noncoding region of hepatitis C virus RNA. *Virology* **208,** 723–732.
112. Tsukiyama-Kohara, K., Iiauka, N., Kohara, M., and Nomoto, A. (1992) Internal ribosome entry site within hepatitis C virus RNA. *J. Virol.* **66,** 1476–1483.
113. Wang, C., Sarnow, P., and Siddiqui, A. (1993) Translation of human hepatitis C virus RNA in cultured cells is mediated by internal ribosome-binding mechanism. *J. Virol.* **67,** 3338–3344.
114. Wang, C., Sarnow, P., and Siddiqui, A. (1994) A conserved helical element is essential for internal initiation of translation of hepatitis C virus RNA. *J. Virol.* **68,** 7301–7307.
115. Kolykhalov, A. A., Feinstone, S. M., and Rice, C. M. (1996) Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. *J. Virol.* **70,** 3363–3371.
116. Tanaka, T., Kato, N., Cho, M.-J., Sugiyama, K., and Shimotohno, K. (1996) Structure of the 3' terminus of hepatitis C virus genome. *J. Virol.* **70,** 3307–3312.
117. Umlauft, F., Wong, D. T., Oefner, P. J., Underhill, P. A., Cheung, R. C., Wright, T. L., Kolykhalov, A. A., Gruenewald, K., and Greenberg, M. B. (1996) Hepatitis C virus detection by single-round PCR specific for the terminal 3' noncoding region. *J. Clin. Microbiol.* **34,** 2552–2558.
118. Blight, K. J., and Rice, C. M. (1997) Secondary structure determination of the conserved 98-base sequence at the 3' terminus of hepatitis C virus genome RNA. *J. Virol.* **71,** 7345–7352.
119. Ito, T. and Lai, M. M. C. (1997) Determination of the secondary structure of and cellular protein binding to the 3'-untranslated region of the hepatitis C virus RNA genome. *J. Virol.* **71,** 8698–8706.
120. Tsuchihara, K., Tanaka, T., Hijikata, M., Kuge, S., Toyoda, H., Nomoto, A., Yamamoto, N., and Shimotohno, K. (1997) Specific interaction of polypyrimidine tract-binding protein with the extreme 3'-terminal structure of the hepatitis C virus

genome, the 3'X. *J. Virol.* **71,** 6720–6726.
121. Miyamoto, H., Okamoto, H., Sato, K., Tanaka, T., and Mishiro, S. (1992) Extraordinarily low density of hepatitis C virus estimated by sucrose density gradient centrifugation and polymerase chain reaction. *J. Gen. Virol.* **73,** 715–718.
122. Thomssen, R., Bonk, S., and Thiele, A. (1993) Density heterogeneities of hepatitis C virus in human sera due to the binding of beta-lipoproteins and immunoglobulins. *Med. Microbiol. Immunol.* **182,** 329–334.
123. Blight, K., Trowbridge, R., Rouland, R., and Growans, E. (1992) Detection of hepatitis C virus RNA by in situ hybridization. *Liver* **12,** 286–289.
124. Inchauspe, G., Zevedee, S., Lee, D. H., Sugitani, M., Nasoff, M., and Prince, A. M. (1991) Genome structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. *Proc. Natl. Acad. Sci. USA* **88,** 10292–10296.
125. Okamoto, H., Kurai, K., Okada, S., Yamamoto, K., Lizuka, H., Tanaka, T., Fukuda, S., Tsuda, F., and Mishiro, S. (1992) Full-length sequence of hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. *Virology* **188,** 331–341.
126. Bartenschlager, R., Ahlborn-Laake, L., Mous, J., and Jacobsen, H. (1993) Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. *J. Virol.* **67,** 3835–3844.
127. Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M., and Rice, C. M. (1993) Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* **67,** 2832–2843.
128. Grakoui, A., Wichowski, C., Lin, C., Feinstone, S. M., and Rice, C. M. (1993) Expression and identification of hepatitis C virus polyprotein cleavage products. *J. Virol.* **67,** 1385–1395.
129. Manabe, S., Fuke, I., Tanishita, O., Kaji, C., Gomi, Y., Yoshida, S., Mori, C., Takamizawa, A., Yoshida, I., and Okayama, H. (1994) Production of nonstructural proteins of hepatitis C virus requires a putative viral protease encoded by NS3. *Virology* **198,** 636–644.
130. Hahm, B., Han, D. S., Back, S. H., Song, O. K., Cho, M. J., Kim, C. J., Shimotohno, K., and Jang, S. K. (1995) NS3–4a of hepatitis C virus is a chymotrypsin-like protease. *J. Virol.* **69,** 2534–2539.
131. Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., and Shimotohno, K. (1991) Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc. Natl. Acad. Sci. USA* **88,** 5547–5551.
132. Selby, M. J., Choo, Q. L., Berger, K., Kuo, G., Glazer, E., Eckart, M., Lee, C., Chien, D., Kuo, C., and Houghton, M. (1993) Expression, identification and localization of the proteins encoded by the hepatitis C viral genome. *J. Gen. Virol.* **74,** 1103–1113.
133. Chang, S. C., Yen, J. H., Kang, H. Y., Jang, M. H., and Chang, M. F. (1994) Nuclear localization signals in the core protein of hepatitis C virus. *Biochem. Biophys. Res. Commun.* **205,** 1284–1290.
134. Ravaggi, A., Natoli, G., Primi, D., Albertini, A., Levrero, M., and Cariani, E. (1994) Intracellular localization of full-length and truncated hepatitis C virus core protein expressed in mammalian cells. *J. Hepatol.* **20,** 833–836.
135. Suzuki, R., Matsuura, Y., Suzuki, T., Ando, A., Chiba, J., Harada, S., Saito, I., and Miyamura, T. (1995) Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted. *J. Gen. Virol.* **76,** 53–61.

136. Cuthbert, J. A. (1994) Hepatitis C: progress and problems. *Clin. Microbiol. Rev.* **7,** 505–532.
137. Bukh, J., Purcell, R. H., and Miller, R. H. (1992) Sequences analysis of the 5' noncoding region of hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **89,** 4942–4946.
138. Simmonds, P., Smith, D. B., McOmish, F., Yap, P. L., Kolberg, J., Urdea, M. S., and Holmes, E. C. (1994) Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS5 regions. *J. Gen. Virol.* **75,** 1053–1061.
139. Martell, M., Seteban, J. I., Quer, J., Genescà, J., Weiner, A., Esteban, R., Guardia, J., and Gomez, J. (1992) Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J. Virol.* **66,** 3225–3229.
140. Abe, K., Inchauspe, G., and Fujisawa, K. (1992) Genomic characterization and mutation rate of hepatitis C virus isolated from a patient who contracted hepatitis during an epidemic of non-A, non-B hepatitis in Japan. *J. Gen. Virol.* **73,** 2725–2729.
141. Okamoto, H., Kojima, M., Okada, S. I., Yoshizawa, H., Lizuka, H., Tanaka, T., Muchmore, E. E., Peterson, D. A., Ito, Y., and Mishiro, S. (1992) Genetic drift of hepatitis C virus during an 8. 2–year infection in a chimpanzee: variability and stability. *Virology* **190,** 894–899.
142. Simmonds, P., Holmes, E. C., Cha, T. A., Chan, S. W., McOmish, F., Irvine, B., Beall, E., Yap, P. L., Kolberg, J., and Urdea, M. S. (1993) Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS5 region. *J. Gen. Virol.* **74,** 2391–2399.
143. Simmonds, P., Alberti, A., Alter, H. J., Bonino, F., Bradley, D. W., Brechot, C., Brouwer, J. T., Chan, S. W., Chayama, K., Chen, D. S., Choo, Q. L., Colombo, M., Cuypers, H. T. N., Date, T., Dusheiko, G. M., Esteban, J. I., Fay, O., Hadziyannis, S. J., Han, J., Hatzakis, A., Holmes, E. C., Hotta, H., Houghton, M., Irvine, D., Kohara, M., Kolberg, J. A., Kuo, G., Lau, J. Y. N., Lelie, P. N., Maertens, G., McOmish, F., Miyamura, T., Mizokami, M., Nomoto, A., Prince, A. M., Reesink, H. W., Rice, C., Roggendorf, M., Schalm, S. W., Shikata, T., Shimotohno, K., Stuyver, L., Trèpo, C., Weiner, A., Yap, P. L., and Urdea, M. S. (1994) A proposed system for the nomenclature of hepatitis C viral genotypes. *Hepatology* **19,** 1321–1324.
144. Simmonds, P., Rose, K., Graham, S., Chan, S. W., McOmish, F., Dow, B. C., Follett, E. A. C., Yap, P. L., and Marsden, H. (1993) Mapping of serotype-specific, immunodominant epitopes in the NS4 region of hepatitis C virus: use of type-specific peptides to serologically differentiate infection with HCV types 1, 2 and 3. *J. Clin. Microbiol.* **31,** 1493–1503.
145. Bukh, J., Miller, R. H., and Purcell, R. H. (1995) Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. *Sem. Liver Dis.* **15,** 41–63.
146. Vatteroni, M. L., Maggi, F., Morrica, A., Fornai, C., Giorgi, M., Pistello, M., and Bendinelli, M. (1997) Comparative evaluation of five rapid methods for identifying subtype 1b and 2c of hepatitis C virus isolates. *J. Virol. Meth.* **66,** 187–194.
147. Simmonds, P. (1995) Variability of hepatitis C virus. *Hepatology* **21,** 570–583.
148. Chang, T. T., Young, K. C., Yang, Y. J., Lei, H. Y., and Wu, H. L. (1996) Hepatitis C virus RNA in peripheral blood mononuclear cells: comparing acute and chronic hepatitis C virus infection. *Hepatology* **23,** 977–981.
149. Blight, K., Lesniewski, R. R., LaBrooy, J. T., and Gowans, E. J. (1994) Detection and distribution of hepatitis C-specific antigens in naturally infected liver. *Hepatology* **20,** 553–557.

150. De Vita, S., Sansonno, D., Dolcetti, R., Ferraccioli, G., Carbone, A., Cornacchiulo, V., Santini, G., Crovatto, M., Gloghini, A., Dammacco, F., and Boiocchi, M. (1995) Hepatitis C virus within a malignant lymphoma lesion in the course of type II mixed cryoglobulinemia. *Blood* **86,** 1887–1892.
151. Nouri-Aria, K. T., Sallie, R., Mizokami, M., Portmann, B. C., and Williams, R. (1995) Intrahepatic expression of hepatitis C virus antigens in chronic liver disease. *J. Pathol.* **175,** 77–83.
152. Zignego, A. L., De Carli, M., Monti, M., Careccia, G., La Villa, G., Giannini, C., D'Elios, M. M., Del Prete, G., and Gentilini, P. (1995) Hepatitis C virus infection of mononuclear cells from peripheral blood and liver infiltrates in chronically infected patients. *J. Med. Virol.* **47,** 58–64.
153. Saito, S., Kato, N., Hijikata, M., Gunji, T., Itabashi, M., Kondo, M., Tanaka, K., and Shimotohno, K. (1996) Comparison of hypervariable regions (HVR1 and HVR2) in positive- and negative-stranded hepatitis C virus RNA in cancerous and non-cancerous liver tissue, peripheral blood mononuclear cells and serum from a patient with hepatocellular carcinoma. *Int. J. Cancer* **67,** 199–203.
154. Maggi, F., Fornai, C., Vatteroni, M. L., Giorgi, M., Morrica, A., Pistello, M., Cammarota, G., Marchi, S., Ciccorossi, P., Bionda, A. and Bendinelli, M. (1997) Differences in hepatitis C virus quasispecies composition between the liver, peripheral blood mononuclear cells and plasma. *J. Gen. Virol.* **78,** 1521–1525.
155. Maggi, F., Fornai, C., Morrica, A., Vatteroni, M. L., Giorgi, M., Marchi, S., Ciccorossi, P., Bendinelli, M., and Pistello, M. (1999) Divergent evolution of hepatitis C virus in the liver and peripheral blood mononuclear cells of infected patients. *J. Med. Virol.* in press.
156. Navas, S., Martin, J., Quiroga, J. A., Castillo, I., and Carreno, V. (1998) Genetic diversity and tissue compartmentalization of the hepatitis C virus genome in blood mononuclear cells, liver, and serum from chronic hepatitis C patients. *J. Virol.* **72,** 1640–1646.
157. Shimizu, Y. K., Igarashi, H., Kanematu, T., Fujiwara, K., Wong, D. C., Purcell, R. H., and Yoshikura, H. (1997) Sequences analysis of the hepatitis C virus genome recovered from serum, liver, and peripheral blood mononuclear cells of infected chimpanzees. *J. Virol.* **71,** 5769–5773.
158. Schlauder, G. G., Leverenz, G. J., Amann, C. W., Lesniewski, R. R., and Peterson, D. A. (1991) Detection of the hepatitis C virus genome in acute and chronic experimental infection in chimpanzees. *J. Clin. Microbiol.* **29,** 2175–2179.
159. Abe, K., Inchauspe, G., Shikata, T., and Prince, A. (1992) Three different patterns of hepatitis C virus infection in chimpanzee. *Hepatology* **15,** 690–695.
160. Beach, M. J., Meeks, E. L., Mimms, L. T., Vallari, D., DuCharme, L., Spelbring, J., Taskar, S., Schleicher, J. B., Krawczynski, K., and Bradley, D. W. (1992) Temporal relationships of hepatitis C virus RNA and antibody responses following experimental infection of chimpanzees. *J. Med. Virol.* **36,** 226–237.
161. Farci, P., Alter, H. J., Govindarajan, S., Wong, D. C., Engle, R., Lesniewski, R. R., Mushahwar, I. K., Desai, S. M., Miller, R. H., Ogata, N., and Purcell, R. H. (1992) Lack of protective immunity against reinfection with hepatitis C virus. *Science* **258,** 135–140.
162. Garson, J. A., Tuke, P. W., Makris, M., Briggs, M., Machin, S. J., Preston, F. E., and Tedder, R. S. (1990) Demonstration of viraemia patterns in haemophiliacs treated with hepatitis-C-virus-contaminated factor VIII concentrates. *Lancet* **336,** 1022–1025.
163. Farci, P., Alter, H. J., Wong, D., Miller, R. H., Shih, J. W., Jett, B., and Purcell, R. H.

(1991) A long term study of hepatitis C virus replication in non-A, non-B hepatitis. *N. Engl. J. Med.* **325,** 98–104.
164. Vallari, D. S., Jett, B. W., Alter, H. J., Mimms, L. T., Holzman, R., and Shih, J. W.-K. (1992) Serological markers of posttransfusion hepatitis C viral infection. *J. Clin. Microbiol.* **30,** 552–556.
165. Peters, T., Mohr, L., Scheiffle, F., Schlayer, H. J., Preisler, S., Berthold, H., Gerok, W., and Rasenack J. (1994) Antibodies and viremia in acute post-transfusion hepatitis C: a prospective study. *J. Med. Virol.* **42,** 420–427.
166. Iwarson, S., Norkrans, G., and Wejstal, R. (1995) Hepatitis C: natural history of a unique infection. *Clin. Infect. Dis.* **20,** 1361–1370.
167. Lau, J. N. Y., Davis G. L., Kniffen, J., Qian, K. P., Urdea, M.-S., Chan, C. S., Mizokami, M., Neuwald, P. D., and Wilber J. C. (1993) Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet* **341,** 1501–1504.
168. Alter H. J. (1995) To C or not to C: These are the questions. *Blood* **85,** 1681–1695.
169. Chan, C.-Y., Lee, S.-D., Hwang, S.-J., Lu, R-H., Lu, C.-L., and Lo, K.-J. (1995) Quantitative branched DNA assay and genotyping for hepatitis C virus RNA in chinese patients with acute and chronic hepatitis C. *J. Infect. Dis.* **171,** 443–446.
170. Alter, M. J., Margolis, H. S., Krawczynski, K., Judson, F. N., Mares, A., Alexander W. J., Hu, P. Y., Miller, J. K., Gerber, M. A., Sampliner, R. E., Meeks, E. L., and Beach, M. J., for the Sentinel Countries Chronic Non-A, Non-B Hepatitis Study Team. (1992) The natural history of community-acquired hepatitis C in the United States. *N. Engl. J. Med.* **327,** 1899–1905.
171. Yoshioka, K., Kakumu, S., Wakita, T., Ishikawa, T., Itoh, Y., Takayanagi, M., Higashi, Y., Shibata, M., and Morishima, T. (1992) Detection of hepatitis C virus by polymerase chain reaction and response to interferon-alpha therapy: relationship to genotypes of hepatitis C virus. *Hepatology* **16,** 293–299.
172. Hino, K., Sainokami, S., Shimoda, K., Iino, S., Wang, Y., Okamoto, H., Miyakawa, Y., and Mayumi, M. (1994) Genotypes and titers of hepatitis C virus for predicting response to interferon in patients with chronic hepatitis C. *J. Med. Virol.* **42,** 299–305.
173. Gretch, D., Corey, L., Wilson, C., dela Rosa, C., Willson, R., Carithers, R., Bush, M., Hart, J., Sayers, M., and Han, J. (1994) Assessment of hepatitis C virus RNA levels by quantitative competitive RNA polymerase chain reaction: high-titer viremia correlates with advanced stage of disease. *J. Infect. Dis.* **169,** 1219–1225.
174. Zeuzem, S., Franke, A., Lee, J.-H., Herrmann, G., Ruster, B. and Roth, W. K. (1996) Phylogenetic analysis of hepatitis C virus isolates and their correlation to viremia, liver function tests, and histology. *Hepatology* **24,** 1003–1009.
175. Zeuzem, S., Schmidt, J. M., Lee, J.-H., Ruster, B., and Roth, W. K. (1996) Effect of interferon alfa on the dynamics of hepatitis C virus turnover *in vivo*. *Hepatology* **23,** 366–371.
176. Yoshimura, E., Hayashi, J., Tani, Y., Ohmiya, M., Nakashima, K., Ikematsu, H., Kinukawa, N., Maeda, Y., and Kashiwagi, S. (1994) Inverse correlation between the titre of antibody to hepatitis C virus and the degree of hepatitis C viraemia. *J. Infect.* **29,** 147–155.
177. Krawczynski, K., Beach, M., Bradley, D. W., Meeks, E., and Spelbring, J. E. (1992) Immunosuppression and pathogenetic studies of acute hepatitis C virus (HCV) infection in chimpanzees. *Hepatology* **16,** 131.
178. Lai, M. E., Mazzoleni, A. P., Argiolu, F., De Virgilis, S., Balestrieri, A., Purcell, R.

H., Cao, A., and Farci, P. (1994) Hepatitis C virus in multiple episodes of acute hepatitis in polytransfused thalassaemic children. *Lancet* **343,** 388–390.
179. Okamoto, H., Mishiro, S., Tokita, H., Tsuda, F., Miyakawa, Y., and Mayumi, M. (1994) Superinfection of chimpanzees carrying hepatitis C virus genotype II/1b with that of genotype III/2a or I/1a. *Hepatology* **20,** 1133–1136.
180. Farci, P., Orgiana, G., and Purcell, R. H. (1995) Immunity elicited by hepatitis C virus. *Clin. Exp. Rheumatol.* **13**(Suppl. 13), S9–12.
181. Ching, W.-M., Wychowski, C., Beach, M. J., Wang, H., Davies, C. L., Carl, M., Bradley, D. W., Alter H. J., Feinstone, S. M., and Shih, W.-K. (1992) Interaction of immune sera with synthetic peptides corresponding to the structural protein region of hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **89,** 3190–3194.
182. Ishida, C., Matsumoto, K., Fukada, K., Matsushita, K., Shiraki, H., and Maeda, Y. (1993) Detection of antibodies to hepatitis C virus (HCV) structural proteins in anti-HCV-positive sera by an enzyme-linked immunosorbent assay using syntetic peptides as antigens. *J. Clin. Microbiol.* **31,** 936–940.
183. Sällberg, M., Pumpen, P., Zhang, Z.-X., Lundholm, P., Gusars, I., Rudén, U., Wahren, B., and Magnius, L. O. (1994) Locations of antibody binding sites within conserved regions of the hepatitis C virus core protein. *J. Med. Virol.* **43,** 62–68.
184. Khudyakov Yu, E., Khudyakova, N. S., Jue, D. L., Lambert, S. B., Fang, S., and Fields, H. A. (1995) Linear B-cell epitopes of the NS3-NS4-NS5 proteins of the hepatitis C virus as modeled with synthetic peptides. *Virology* **206,** 666–672.
185. Chien, D. Y., Choo, Q. L., Ralston, R., Spaete, R., Tong, M., Houghton, M., and Kuo, G. (1993) Persistence of HCV despite antibodies to both putative envelope glycoprotein. *Lancet* **342,** 933.
186. Ray, R., Khanna, A., Lagging, L. M., Meyer, K., Choo, Q.-L., Ralston, R., Houghton, M., and Becherer, P. R. (1994) Peptide immunogen mimicry of putative E1 glycoprotein-specific epitopes in hepatitis C virus. *J. Virol.* **68,** 4420–4426.
187. Wang, Y.-F., Brotman, B., Andrus, L., and Prince, A. M. (1996) Immune response to epitopes of hepatitis C virus (HCV) structural proteins in HCV-infected humans and chimpanzees. *J. Infect. Dis.* **173,** 808–821.
188. Weiner, A. J., Geysen, H. M., Christopherson, C., Hall, J. E., Mason, T. J., Saracco G., Bonino, F., Crawford, K., Marion, C. D., Crawford, K. A., Brunetto, M., Barr, P. J., Miyamura, T., McHutchinson, J., and Houghton, M. (1992) Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. *Proc. Natl. Acad. Sci. USA* **89,** 3468–3472.
189. Choo, Q.-L., Kuo, G., Ralston, R., Weiner, A., Chien, D., van Nest, G., Han, J., Berger, K., Thidium, K., Kuo, C., Kansopon, J., McFarland, J., Tabrizi, A., Ching, K., Moss, B., Cummins, L. B., Houghton, M., and Muchmore, E. (1994) Vaccination of chimpanzees against infection by the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **91,** 1294–1298.
190. Zhang, Z.-X., Chen, M., Sönnerborg, A., Weiland, O., and Sällberg, M. (1995) Distinguishing acute from symptomatic chronic hepatitis C virus (HCV) infection by site-directed serology of the HCV structural proteins. *J. Infect. Dis.* **171,** 1356–1359.
191. Van Doorn, L. J., van Hoek, K., de Martinoff, G., Bosman, F., Stuyver, L., Kos, T., Frantzen, I., Sillekens, P., Maertens, G., and Quint, W. (1997) Serological and molecular analysis of Hepatitis C virus envelope regions 1 and 2 during acute and chronic infections in chimpanzees. *J. Med. Virol.* **52,** 441–450.
192. Cerny, A., and Chisari, F. V. (1994) Immunological aspects of HCV infection. *Intervirology* **37,** 119–125.

193. Ferrari, C., Valli, A., Galati, L., Penna, A., Scaccaglia, P., Giuberti, T., Schianchi, C., Missale, G., Marin, M. G., and Fiaccadori, F. (1994) T-cell response to structural and nonstructural hepatitis C virus antigens in persistent and self-limited hepatitis C virus infections. *Hepatology* **19,** 286–295.
194. Jung, M. C., Diepolder, H. M., and Pape, G. R. (1994) T cell recognition of hepatitis B and C viral antigens. *Europ. J. Clin. Invest.* **24**, 641–650.
195. Cerny, A., McHutchinson, J. G., Pasquinelli, C., Brown, M. E., Brothers, M. A., Grabscheid, B., Fowler, P., Houghton, M., and Chisari, F. V. (1995) Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2. 1 binding motif. *J. Clin. Invest.* **95,** 521–530.
196. Hoffman, R. M., Diepolder, H. M., Zachoval, R., Zwiebel, F.-M, Jung, M. C., Scholz, S., Nitschko, H., Riethmüller, G., and Pape, G. R. (1995) Mapping of immunodominant CD4+ T lymphocyte epitopes of hepatitis C virus antigens and their relevance during the course of chronic infection. *Hepatology* **21,** 632–638.
197. Kapprell, H. P., Michel, G., Hampl, H., Bonino, F., and Esteban, J. I. (1996) Demonstration of specific detection of anti-HCV IgM core antibodies. *J. Virol. Meth.* **59,** 121–126.
198. Hellström, U. B., Sylvan, S. P. E., Decker, R. H., and Sönnerborg, A. (1993) Immunoglobulin M reactivity towards the immunologically active region sp75 of the core protein of hepatitis C virus (HCV) in chronic HCV infection. *J. Med. Virol.* **39,** 325–332.
199. Nagayama, R., Miyake, K., Tsuda, F., and Okamoto, H. (1994) IgM antibody to a hepatitis C virus core peptide (CP14) for monitoring activity of liver disease in patients with acute or chronic hepatitis C. *J. Med. Virol.* **43,** 311–317.
200. Brillanti, S., Masci, C., Ricci, P., Miglioli, M., and Barbara, L. (1992) Significance of IgM antibody to hepatitis C virus in patients with chronic hepatitis C. *Hepatology* **15,** 998–1001.
201. Chen, M., Sönnerborg, A., and Sallberg, M. (1995) Levels of hepatitis C virus (HCV) RNA in serum and their relationship to levels of immunoglobulin M and G antibodies against HCV core protein. *J. Clin. Microbiol.* **33,** 778–780.
202. Pawlotsky, J. M., Darthuy, F., Rémiré, J., Pellet, C., Udin, L., Stuyver, L., Roudot-Thoraval, F., Duvoux, C., Douvin, C., Mallat, A., Mavier, J. P., Métreau J. M., Duval, J., and Dhumeaux, D. (1995) Significance of anti-hepatitis C virus core IgM antibodies in patients with chronic hepatitis C. *J. Med. Virol.* **47,** 285–291.
203. Quiroga, J. A., Herrero, M., Castillo, I., Navas, S., Pardo, M., and Carreno, V. (1994) Long-term follow-up study of serum IgM antibody to hepatitis C virus (HCV), HCV replication, and liver disease outcome in chronic hepatitis C. *J. Infect. Dis.* **170,** 669–673.
204. Lesniewski R., Okasinski, G., Carrick, R., van Sant, C., Desai, S., Johnson, R., Scheffel, J., Moore, B., and Mushahwar, I. (1995) Antibody to hepatitis C virus second envelope (HCV-E2) glycoprotein: a new marker of HCV infection closely associated with viremia. *J. Med. Virol.* **45,** 415–422.
205. Tanaka, E., Kiyosawa, K., Nakatsuji, Y., Inoue, Y., Miyamura, T., Chiba, J., and Furuta, S. (1993) Clinical significance of antibodies to nonstructural and core proteins of hepatitis C virus in posttransfusion hepatitis patients during long term follow-up. *J. Med. Virol.* **39,** 318–324.
206. Pawlotsky, J. M., Roudot-Thoraval, F., Pellet, C., Aumont, P., Darthuy, F., Rémiré, J., Duval, J., and Dhumeaux, D. (1995) Influence of hepatitis C virus (HCV) genotypes on

HCV recombinant immunoblot assay patterns. *J. Clin. Microbiol.* **33,** 1357–1359.
207. Maggi, F., Vatteroni, M. L., Pistello, M., Avio, C. M., Cecconi, N., Panicucci, F., and Bendinelli, M. (1995) Serological reactivity and viral genotypes in hepatitis C virus infection. *J. Clin. Microbiol.* **33,** 209–211.
208. Dhaliwal, S. K., Prescott, L. E., Dow, B. C., Davidson, F., Brown, H., Yap, P. L., Follett, E. A. C., and Simmonds, P. (1996) Influence of viraemia and genotype upon serological reactivity in screening assays for antibody to hepatitis C virus. *J. Med. Virol.* **48,** 184–190.
209. Dow, B. C., Buchanan, I., Munro, H., Follett, E. A. C., Davidson, F., Prescott, L. E., Yap, P. L., and Simmonds, P. (1996) Relevance of RIBA–3 supplementary test to HCV PCR positivity and genotypes for HCV confirmation of blood donors. *J. Med. Virol.* **49,** 132–136.
210. Fong, T.-L., Lee, S. R., Briggs, W. K., Valinluk, B., Govindarajan, S., Hoffman, A., Jaczko, B., and Redeker, A. (1996) Clinical significance of hepatitis C viral RNA status and its correlation to antibodies to structural HCV antigens in anti-HCV reactive patients with normal liver tests. *J. Med. Virol.* **49,** 253–258.
211. Zibert, A., Kraas, W., Meisel, H., Jung, G., and Roggendorf, M. (1997) Epitope mapping of antibodies directed against hypervariable region 1 in acute self-limiting and chronic infections due to hepatitis C virus. *J. Virol.* **71,** 4123–4127.
212. Shimizu, Y. K., Hijikata, M., Iwamoto, A., Alter, H. J., Purcell, R. H., and Yoshikura, H. (1994) Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. *J. Virol.* **68,** 1494–1500.
212a. Shimizu, Y. K., Igarashi, H., Kiyohara, T., Cabezon, T., Farci, P., Purcell, R. H., and Yoshikura, H. (1996) A hyperimmune serum against a synthetic peptide corresponding to the hypervariable region 1 of hepatitis C virus can prevent viral infection in cell cultures. *Virology* **223,** 409–412.
213. Farci, P., Alter, H. J., Wong, D. C., Miller, R. H., Govindarajan, S., Engle, R., Saphiro, M., and Purcell, R. H. (1994) Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated *in vitro* neutralization. *Proc. Natl. Acad. Sci. USA* **91,** 7792–7796.
214. Lechner, S., Rispeter, K., Meisel, H., Kraas, W., Jung, G., Roggendorf, M., and Zibert, A. (1998) Antibodies directed to envelope proteins of hepatitis C virus outside of hypervariable region 1. *Virology* **243,** 313–321.
215. Rosa, D., Campagnoli, S., Moretto, C., Guenzi, E., Cousens, L., Chin, M., Dong, C., Weiner, A. J., Lau, J. Y. N., Choo, Q.-L., Chien, D., Pileri, P., Houghton, M., and Abrignani, S. (1996) A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. *Proc. Natl. Acad. Sci. USA* **93,** 1759–1763.
216. Botarelli, P., Brunetto, M. R., Minutello, M. A., Calvo, P., Unutmaz, D., Weiner, A. J., Choo, Q. L., Shuster, J. R., Kuo, G., Bonino, F., Houghton, M., and Abrignani, S. (1993) T-lymphocyte response to hepatitis C virus in different clinical courses of infection. *Gastroenterology* **104,** 580–587.
217. Minutello, M. A., Pileri, P., Unutmaz, D., Censini, S., Kuo, G., Houghton, M., Brunetto, M. R., Bonino, F., and Abrignani, S. (1993) Compartmentalization of T lymphocytes to the site of disease: intrahepatic CD4+ T cells specific for the protein NS4 of hepatitis C virus in patients with chronic hepatitis C. *J. Exp. Med.* **178,** 17–25.
218. Mosmann, T. R., and Sad, S. (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today* **17,** 138–146.

219. Bertoletti, A., D'Elios, M. M., Boni, C., De Carli, M., Zignego, A. L., Durazzo, M., Missale, G., Penna, A., Fiaccadori, F., Del Prete, G., and Ferrari, C. (1997) Different cytokine profiles of intrahepatic T cells in chronic hepatitis B and C virus infections. *Gastroenterology* **112,** 193–199.
220. Diepolder, H. M., Zachoval, R., Hoffmann, R. M., Wierenga, E. A., Santantonio, T., Jung, M. C., Eichenlaub, D., and Pape, G. R. (1995) Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* **346,** 1006–1007.
221. Missale, G., Bertoni, R., Lamonaca, V., Valli, A., Massari, M., Mori, C., Rumi, M. G., Houghton, M., Fiaccadori, F., and Ferrari, C. (1996) Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the antiviral cell-mediated immune response. *J. Clin. Invest.* **98,** 706–714.
222. Tsai, S.-L., Liaw, Y.-F., Chen, M.-H., Huang, C.-Y., and Kuo, G. C. (1997) Detection of type 2–like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *Hepatology* **25,** 449–458.
223. Erickson, A. L., Houghton, M., Choo, Q. L., Weiner A., J., Ralston, R., Muchmore, E., and Walker, C. M. (1993) Hepatitis C virus-specific CTL responses in the liver of chimpanzees with acute and chronic hepatitis C. *J. Immunol.* **8,** 4189–4199.
224. Cerny, A., Ferrari, C., and Chisari, F. V. (1994) The class I-restricted cytotoxic T lymphocyte response to predetermined epitopes in hepatitis B and C viruses. *Curr. Top. Microbiol. Immunol.* **189,** 169–186.
225. Kurokohchi, K., Akatsuka, T., Pendleton, C. D., Takamizawa, A., Nishioka, M., Battegay, M., Feinstone, S. M., and Berzofsky, J. A. (1996) Use of recombinant protein to identify a motif-negative human cytotoxic T-cell epitope presented by HLA-A2 in the hepatitis C virus NS3 region. *J. Virol.* **70,** 232–240.
226. Koziel, M. J., and Walker, B. D. (1997) Characteristics of the intrahepatic cytotoxic T lymphocyte response in chronic hepatitis C virus infection. *Springer Semin. Immunopathol.* **19,** 69–83.
227. Battegay, M., Fikes, J., Di Bisceglie, A. M., Wentworth, P. A., Sette, A., Celis, E., Ching, W.-M., Grakoui, A., Rice, C. M., Kurokohchi, K., Berzofsky, J. A., Hoofnagle, J. H., Feinstone, S. M., and Akatsuka, T. (1995) Patients with chronic hepatitis C have circulating cytotoxic T cells which recognize hepatitis C virus-encoded peptides binding to HLA-A2. 1 molecules. *J. Virol.* **69,** 2462–2470.
228. Kaneko, T., Nakamura, I., Kita, H., Hiroishi, K., Moriyama, T., and Imawari, M. (1996) Three new cytotoxic T cell epitopes identified within the hepatitis C virus nucleoprotein. *J. Gen. Virol.* **77,** 1305–1309.
229. Hiroishi, K., Kita, H., Kojima, M., Okamoto, H., Moriyama, T., Kaneko, T., Ishikawa, T., Ohnishi, S., Aikawa, T., Tanaka, N., Yazaki, Y., Mitamura, K., and Imawari, M. (1997) Cytotoxic T lymphocyte response and viral load in hepatitis C virus infection. *Hepatology* **25,** 705–712.
230. Kato, N., Ootsuyama, Y., Ohkoshi, S., Nakazawa T., Sekiya, H., Hijkata, M., and Shimotohno K. (1992) Characterization of hypervariable regions in the putative envelope protein of hepatitis C virus. *Biochem. Biophys. Res. Commun.* **189,** 119–127.
231. Kato, N., Ootsuyama, Y., Sekiya, H., Ohkoshi, S., Nakazawa, T., Hijkata, M., and Shimotohno, K. (1994) Genetic drift in hypervariable region 1 of the viral genome in persistent hepatitis C virus infection. *J. Virol.* **68,** 4776–4784.
232. van Doorn, L. E., Quint, W., Tsiquaye, K., Voermans, J., Paelinck, D., Kos, T., Maertens, G., Schellekens, H., and Murray, K. (1994) Longitudinal analysis of hepa-

titis C virus infection and genetic drift of the hypervariable region. *J. Infect. Dis.* **169,** 1226–1235.

233. Koizumi, K., Enomoto, N., Kurosaki, M., Murakami, T., Izumi, N., Marumo, F., and Sato, C. (1995) Diversity of quasispecies in various disease stages of chronic hepatitis C virus infection and its significance in interferon treatment. *Hepatology* **22,** 30–35.
234. Odeberg, J., Yun, Z., Sönnerborg A., Uhlén M., and Lundeberg, J. (1995) Dynamic analysis of heterogeneous hepatitis C virus populations by direct solid-phase sequencing. *J. Clin. Microbiol.* **33,** 1870–1874.
235. Kurosaki, M., Enomoto, N., Marumo, F., and Sato, C. (1993) Rapid sequence variation of the hypervariable region of hepatitis C virus during the course of chronic infection. *Hepatology* **18,** 1293–1299.
236. Enomoto, N., Sakamoto, N., Kurosaki, M., Marumo, F., and Sato, C. (1993) The hypervariable region of the HCV genome changes sequentially during the progression of acute HCV infection to chronic hepatitis. *J. Hepatol.* **17,** 415–422.
237. Enomoto, N., Kurosaki, M., Tanaka, Y., Marumo, F., and Sato, C. (1994) Fluctuation of hepatitis C virus quasispecies in persistent infection and interferon treatment revealed by single-strand conformation polymorphism analysis. *J. Gen. Virol.* **75,** 1361–1369.
238. Nakazawa, T., Kato, N., Ootsuyama, Y., Sekiya, H., Fujioka, T., Shibuya, A., and Shimotohno, K. (1994) Genetic alteration of the hepatitis C virus hypervariable region obtained from an asymptomatic carrier. *Int. J. Cancer* **56,** 204–207.
239. Sakamoto, N., Enomoto, N., Kurosaki, M. Marumo, F., and Sato C. (1994) Sequential change of the hypervariable region of the hepatitis C virus genome in acute infection. *J. Med. Virol.* **42,** 103–108.
240. Yamaguchi. K., Tanaka, E., Higashi, K., Kiyosawa, K., Matsumoto, A., Furuta, S., Hasegawa, A., Tanaka, S., and Kohara, M. (1994) Adaptation of hepatitis C virus for persistent infection in patients with acute hepatitis. *Gastroenterology* **106,** 1344–1348.
241. Marrone, A., and Sallie, R. (1996) Genetic heterogeneity of hepatitis C virus. The clinical significance of genotypes and quasispecies behavoir. *Clin. Lab. Med.* **16,** 429–449.
242. Wyatt, C. A., Andrus, L., Brotman, B., Huang, F., Lee, D.-H., and Prince, A. M. (1998) Immunity in chimpanzees chronically infected with hepatitis C virus: role of minor quasispecies in reinfection. *J. Virol.* **72,** 1725–1730.
243. McAllister, J., Casino, C., Davidson, F., Power, J., Lawlor, E., Yap., P. L., Simmonds, P., and Smith, D. B. (1998) Long-term evolution of the hypervariable region of hepatitis C virus in a common-source-infected cohort. *J. Virol.* **72,** 4893–4905.
244. Kato, N., Sekiya, H., Ootsuyama, Y., Nakazawa, T., Hijikata, M., Ohkoshi, S., and Shimotohno, K. (1993) Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. *J. Virol.* **67,** 3923–3930.
245. Taniguchi, S., Okamoto, H., Sakamoto, M., Kojima, M., Tsuda, F., Tanaka, T., Munekata, E., Muchmore, E. E., Peterson, D. A., and Mishiro, S. (1993) A structurally flexible and antigenically variable N-terminal domain of the hepatitis E2/NS1 protein: implication for an escape from antibody. *Virology* **195,** 297–301.
246. van Doorn, L. J., Capriles, I., Maertens, G., DeLeys, R., Murray, K., Kos, T., Schellekens, H., and Quint, W. (1995) Sequence evolution of the hypervariable region in the putative envelope region E2/NS1 of hepatitis C virus is correlated with specific immune response. *J. Virol.* **69,** 773–778.

247. Kumar, U., Monjardino, J., and Thomas, H. C. (1994) Hypervariable region of hepatitis C virus envelope glycoprotein (E2/NS1) in an agammaglobulinemic patient. *Gastroenterology* **106,** 1072–1075.
248. Weiner, A. J., Erickson, A. L., Kansopon, J., Crawford, K., Muchmore, E., Hughes, A. L., Houghton, M., and Walker C. (1995) Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. *Proc. Natl. Acad. Sci. USA* **92,** 2755–2759.
249. Kurosaki, M., Enomoto, N., Marumo, F., and Sato, C. (1995) Variations in the core region of hepatitis C virus genomes in patients with chronic hepatitis. *Arch. Virol.* **140,** 1087–1094.
250. Nowak, M. and Bangham, C. R. M. (1996) Population dynamics of immune responses to persistent viruses. *Science* **272,** 74–79.
251. Corell, A., Morales, J. M., Mandroño, A., Muñoz, M. A., Andrés A., Fuertes A., and Arnaiz-Villena, A. (1995) Immunosuppression induced by hepatitis C virus infection reduces acute renal-transplant rejection. *Lancet* **346,** 1497–1498.
252. Di Bisceglie, A., Goodman, Z. D., Ishak, K. G., Hoofnagle, J. H., Melpolder, J. J., and Alter, H. J. (1991) Long-term clinical and histopathological follow-up of chronic posttransfusion hepatitis. *Hepatology* **14,** 969–974.
253. Gordon, S. C., Elloway, R. S., Long, J. C., and Dmuchowski, C. F. (1993) The pathology of hepatitis C as a function of mode of transmission: blood transfusion vs. intravenous drug use. *Hepatology* **18,** 1338–1343.
254. Alter, H. J., Purcell, R. H., Shih, J. W., Melpolder, J. C., Houghton, M., Choo, Q.-L., and Kuo, G. (1989) Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N. Engl. J. Med.* **321,** 1494–1500.
255. Koretz, R. L., Abbey, H., Coleman, E., and Gitnick, G. (1993) Non-A, non-B post-transfusion hepatitis. Looking back in the second decade. *Ann. Int. Med.* **119,** 110–115.
256. Féray, C., Gigou, M., Samuel D., Reyes G., Bernuau, J., Reynés M., Bismuth, H., and Bréchot, C. (1993) Hepatitis C virus RNA and hepatitis B virus DNA in serum and liver of patients with fulminant hepatitis. *Gastroenterology* **104,** 549–555.
257. Vento, S., Cainelli, F., Mirandola, F., Cosco, L., Di Perri, G., Solbiati, M., Ferraro, T., and Concia, E. (1996) Fulminant hepatitis on withdrawal of chemotherapy in carriers of hepatitis C virus. *Lancet* **347,** 92–93.
258. Seef, L. B. (1997) Natural history of hepatitis C. *Hepatology* **26,** 21S–28S.
259. Seeff, L. B., Buskell-Bales Z., Wright, E. C., Durako, S. J., Alter, H. J., Iber, F. L., Hollinger, F. B., Gitnick, G., Knodell, R. G., Perrillo, R. P., Stevens, C. E., Hollingsworth, C. G., and the National Heart, Lung and Blood Institute Study Group. (1992) Long-term mortality after transfusion-associated non-A, non-B hepatitis. *N. Engl. J. Med.* **327,** 1906–1911.
260. Tremolada, F., Casarin, C., Alberti, A., Drago, C., Tagger, A., Ribero, M. L., and Realdi, G. (1992) Long-term follow-up of non-A, non-B (type C) post-transfusion hepatitis. *J. Hepatol.* **16,** 273–281.
261. Kiyosawa, K., Tanaka, E., Sodeyama, T., and Furuta, S. (1994) Natural history of hepatitis C. *Intervirology* **37,** 101–107.
262. Alberti, A., Morsica, G., Chemello, L., Cavalletto, D., Noventa, F., Pontisso, P., and Ruol, A. (1992) Hepatitis C viraemia and liver disease in symptom-free individuals with anti-HCV. *Lancet* **340,** 697–698.
263. Naito, M., Hayashi, N., Hagiwara, H., Hiramatsu, N., Kasahara, A., Fusamoto, H.,

and Kamada, T. (1994) Serum hepatitis C virus RNA quantity and histological features of hepatitis C virus carriers with persistently normal ALT levels. *Hepatology* **19,** 871–875.

264. Shakil, A. O., Corny-Cantilena, C., Alter, H. J., Hayashi, P., Kleiner, D. E., Tedeschi, V., Krawczynski, K., Conjeevaram, H. S., Sallie, R., Di Bisceglie, A. M., and the Hepatitis C Study Group. (1995) Volunteer blood donors with antibody to hepatitis C virus: clinical, biochemical, virologic and histologic features. *Ann. Int. Med.* **123,** 330–337.
265. Morales, T. G., Sampliner, R. E., Bhattacharyya, A., and Alter, M. J. (1995) Liver histology in anti-HCV-positive persons with normal or minimally elevated aminotransferases. *J. Clin. Gastroenterol.* **21,** 301–305.
266. Brillanti, S., Foli, M., Gaiani, S., Masci, C., Miglioli, M., and Barbara, L. (1993) Persistent hepatitis C viraemia without liver disease. *Lancet* **341,** 464–465.
267. Silini, E., Bono, F., Cividini, A., Cerino, A., Bruno, S., Rossi, S., Belloni, G., Brugnetti, B., Civardi, E., Salvaneschi, L., and Mondelli, M. U. (1995) Differential distribution of hepatitis C virus genotypes in patients with and without liver function abnormalities. *Hepatology* **21,** 285–290.
268. Prince, A. M., Brotman, B., Huima, T., Pascual, D., Jaffery, M., and Inchauspé, G. (1992) Immunity in hepatitis C infection. *J. Infect. Dis.* **165,** 438–443.
269. Jarvis, L. M., Watson, H. G., McOmish, F., Peutherer, J. F., Ludlam, C. A., and Simmonds, P. (1994) Frequent reinfection and reactivation of hepatitis C virus genotypes in multitransfused hemophiliacs. *J. Infect. Dis.* **170,** 1018–1022.
270. Resnick, R. H., and Koff, R. (1993) Hepatitis C-related hepatocellular carcinoma. Prevalence and significance. *Arch. Int. Med.* **153,** 1672–1677.
271. De Mitri, M. S., Poussin, K., Baccarini, P., Pontisso, P., D'Errico, A., Simon, N., Grigioni, W., Alberti, A., Beaugrand, M., Pisi, E., Bréchot C., and Paterlini, P. (1995) HCV-associated liver cancer without cirrhosis. *Lancet* **345,** 413–415.
272. Yotsuyanagi, H., Koike, K., Yasuda, K., Moriya, K., Hino, K., Kurokawa, K., and Iino, S. (1995) Hepatitis C virus genotypes and development of hepatocellular carcinoma. *Cancer* **76,** 1352–1355.
273. Lau, J. N. Y., Davis, G. L., Prescott, L. E., Maertens, G., Lindsay, K. L., Qian, K., Mizokami, M., and Simmonds, P. (1996) Distribution of hepatitis C virus genotypes determined by line probe assay in patients with chronic hepatitis C seen at tertiary referral centers in the United States. Hepatitis Interventional Therapy Group. *Ann. Int. Med.* **124,** 868–876.
274. Bruno, S., Silini, E., Crosignani, A., Borzio, F., and Leandro, G. (1997) Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: a prospective study. *Hepatology* **25,** 754–758.
275. Benvegnu, L., Pontisso, P., Cavalletto, D., Noventa, F., Chemello, L., and Alberti, A. (1997) *Hepatology* **25,** 211–215.
276. Perrillo, R. P. (1997) The role of liver biopsy in hepatitis C. *Hepatology* **26,** 57S–61S.
277. Knodell, R. G., Ishak, K. G., Black, W. C., Chen, T. S., Craig, R., Kaplowitz, N., Kiernan, T. W., and Wollman, J. (1981) Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* **1,** 431–435.
278. Desmet, V. J., Gerber, M., Hoofnagle, J. H., Manns, M., and Scheuer, P. J. (1994) Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* **19,** 1513–1520.
279. Bach, N., Thung, S. N., and Schaffner, F. (1992) The histological features of chronic

hepatitis C and autoimmune chronic hepatitis: a comparative analysis. *Hepatology* **15,** 527–577.
280. Scheuer, P. J., Ashrafzadeh, P., Sherlock, S., Brown, D., and Dusheiko, G. M. (1992) The pathology of hepatitis C. *Hepatology* **15,** 567–571.
281. Czaja, A. and Carpenter, H. A. (1993) Sensitivity, specificity, and predictability of biopsy interpretations in chronic hepatitis. *Gastroenterology* **105,** 1824–1832.
282. Lefkowitch, J. H., Schiff, E. R., Davis, G. L., Perrillo, R. P., Lindsay, K., Bodenheimer, H. C., Balart, L. A., Ortego, T. J., Payne, J., Dienstag, J. L., Gibas, A., Jacobson, I. M., Tamburro, C. H., Carey, W., O'Brien, C., Sampliner, R., van Thiel, D. H., Feit, D., Albrecht, J., Meschievitz, C., Sanghvi, B., Vaughan, R. D., and The Hepatitis Interventional Therapy Group. (1993) Pathological diagnosis of chronic hepatitis C: a multicenter comparative study with chronic hepatitis B. *Gastroenterology* **104,** 595–603.
283. Fong, T.-L., Valinluck, B. Govindarajan, S., Charboneau, F., Adkins, R. H., and Redeker, A. G. (1994) Short-term prednisone therapy affects aminotransferase activity and hepatitis C virus RNA levels in chronic hepatitis C. *Gastroenterology* **107,** 196–199.
284. Magrin, S., Craxì, A., Fabiano, C., Simonetti, R. G., Fiorentino G., Marino, L., Diquattro, O., Di Marco, V., Loiacono, O., Volpes R., Almasio, P., Urdea, M. S., Neuwald, P., Sanchez-Pescador, R., Detmer, J., Wilber J. C., and Pagliaro, L. (1994) Hepatitis C viremia in chronic liver disease: relationship to intereferon-α or corticosteroid treatment. *Hepatology* **19,** 273–279.
285. Gruber, A., Lundberg, L. G., and Bjorkholm, M. (1993) Reactivation of chronic hepatitis C after withdrawal of immunosuppressive therapy. *J. Int. Med.* **234,** 223–225.
286. Chazouilleres, O., Kim, M., Combs, C., Ferrell, L., Bacchetti, P., Roberts, J., Ascher, N. L., Neuwald, P., Wilber, J., and Urdea, M. (1994) Quantitation of hepatitis C virus RNA in liver transplant recipients. *Gastroenterology* **106,** 994–999.
287. Mishiro, S., Hoshi, Y., Takeda, K., Yoshikawa, A., Gotanda, T., Tahahashi, K., Akahane, Y., Yoshizawa, H., Okamoto, H., Tsuda, F., Peterson, D. A., and Muchmore, E. (1990) Non-A, non-B hepatitis specific antibodies directed at host-derived epitope: implication for an autoimmune process. *Lancet* **336,** 1400–1403.
288. Quiroga, J. A., Pardo, M., Navas, S., Martin, J., and Carreno, V. (1996) Patterns of immune responses to the host-encoded GOR and hepatitis C virus core-derived epitopes with relation to hepatitis C viremia, genotypes, and liver disease severity. *J. Infect. Dis.* **173,** 300–305.
289. Lunel, F., Abuaf, N. R., Frangeul, L., Grippon, P., Perrin, M., Le Coz, Y., Valla, D., Borotto, E., Yamamoto, A. M., Huraux, J. M., Opolon, P., and Homberg, J. C. (1992) Liver/kidney microsome antibody type 1 and hepatitis C virus infection. *Hepatology* **16,** 630–636.
290. McFarlane, B. M., Bridger, C., Tibbs, C. J., Saleh, M. G., Fuzio, A., Verucchi, G., Attard, L., Boschi, A., Chiodo, F., McFarlane, I. G., and Williams, R. (1994) Virus-induced autoimmunity in hepatitis C virus infections: a rare event. *J. Med. Virol.* **42,** 66–72.
291. Garson, J. A., Lenzi, M., Ring, C., Cassani, F., Ballardini, G., Briggs, M., Tedder, R. S., and Bianchi, F. B. (1991) Hepatitis C viraemia in adults with type 2 autoimmune hepatitis. *J. Med. Virol.* **34,** 223–226.
292. Lenzi, M., Johnson, P. J., McFarlane, I. G., Ballardini, G., Smith, H. M., McFarlane, B. M., Bridger, C., Vergani, D., Bianchi, F. B., and Williams, R. (1991) Antibodies to hepatitis C virus in autoimmune liver disease: evidence for geographical hetero-

geneity. *Lancet* **338,** 277–280.
293. Czaja, A. (1994) Autoimmune hepatitis and viral infection. *Gastroent. Clin. North Amer.* **23,** 547–566.
294. Michitaka, K., Durazzo, M., Tillmann, H. L., Walker, D., Philipp, T., and Manns, M. P. (1994) Analysis of hepatitis C virus genome in patients with autoimmune hepatitis type 2. *Gastroenterology* **106,** 1603–1610.
295. Kato, N., Yokousuka, K., Otho, M., and Omata, M. (1994) Quantification of hepatitis C virus by competitive reverse transcription-polymerase chain reaction: increase of the virus in advanced liver disease. *Hepatology* **18,** 16–20.
296. Mita, E., Hayashi, N., Kanazawa, Y., Hagiwara, H., Ueda, K., Kasahara, A., Fusamoto, H., and Kamada, T. (1994) Hepatitis C virus genotype and RNA titer in the progression of type C chronic liver disease. *J. Hepatol.* **21,** 468–473.
297. Björkander, J., Cunningham-Rundles, C., Lundin, P., Olsson, R., Söderström, R., and Hanson, L. A. (1988) Intravenous immunoglobulin prophylaxis causing liver damage in 16 of 77 patients with hypogammaglobulinemia or IgG subclass deficiency. *Amer. J. Med.* **84,** 107–111.
298. Martin, P., Di Bisceglie, A. M., Kassianides, C., Lisker-Melman, M., and Hoofnagle, J. H. (1989) Rapidly progressive non-A, non-B hepatitis in patients with human immunodeficiency virus infection. *Gastroenterology*, **97,** 1559–1561.
299. Pereira, B. J., Milford, E. L., Kirkman, R. L., and Levey, A. S. (1991) Transmission of hepatitis C virus by organ transplantation. *N. Engl. J. Med.* **325,** 454–460.
300. Dusheiko, G., Schmilovitz-Weiss, H., Brown, D., McOmish, F., Yap, P.-L., Sherlock, S., McIntyre, N., and Simmonds, P. (1994) Hepatitis C virus genotypes: an investigation of type-specific differences in geographic origin and disease. *Hepatology* **19,** 13–18.
301. Pozzato, G., Kaneko, S., Moretti, M., Crocè L. S., Franzin, F., Unoura, M., Bercich, L., Tiribelli, C., Crovatto, M., Santini, G., and Kobayashi, K. (1994) Different genotypes of hepatitis C virus are associated with different severity of chronic liver disease. *J. Med. Virol.* **43,** 291–296.
302. Féray, C., Gigou, M., Samuel D., Paradis, V., Mishiro, S., Maertens G., Reynés M., Okamoto H., Bismuth, H., and Bréchot C. (1995) Influence of the genotypes of hepatitis C virus on the severity of recurrent liver disease after liver transplantation. *Gastroenterology* **108,** 1088–1096.
303. Preston, F. E., Jarvis, L. M., Makris, M., Philip, L., Underwood, J. C. E., Ludlam, C. A., and Simmonds, P. (1995) Heterogeneity of hepatitis C virus genotypes in hemophilia: relationship with chronic liver disease. *Blood* **85,** 1259–1262.
304. Mita, E., Hayashi, H., Hagiwara, K., Ueda, K., Kanazawa, Y., Kasahara, A., Fusamoto, H., and Kamada, T. (1994) Predicting interferon therapy efficacy from hepatitis C virus genotype and RNA titer. *Dig. Dis. Sci.* **39,** 977–982.
305. Nousbaum, J. B., Stanislas, P., Nalpas, B., Landais, P., Berthelot, P., Bréchot., C., and the Collaborative Study Group. (1995) Hepatitis C virus type 1b (II) infection in France and Italy. *Ann. Int. Med.* **122,** 161–168.
306. Smith, D. B., Davidson, F., Yap, P. L., Brown, H., Kolberg, J., Detmer, J., Urdea, M., Simmonds, P., and the International HCV Collaborative Study Group. (1996) Levels of hepatitis C virus in blood donors infected with different viral genotypes. *J. Infect. Dis.* **173,** 727–730.
307. Moribe, T., Hayashi, N., Kanazawa, Y., Mita, E., Fusamoto, H., Negi, M., Kaneshige, T., Igimi, H., Kamada, T., and Uchida, K. (1995) Hepatitis C viral complexity detected

by single-strand conformation polymorphism and response to interferon therapy. *Gastroenterology* **108,** 789–795.
308. Yeh, B.-I., Han, K.-H., Oh, S.-H., Kim, H.-S., Hong, S.-H., Oh, S.-H., and Kim, Y.-S. (1996) Nucleotide sequence variation in the hypervariable region of the hepatitis C virus in the sera of chronic hepatitis C patients undergoing controlled interferon-α therapy. *J. Med. Virol.* **49,** 95–102.
309. Yun, Z. B., Odeberg, J., Lundeberg, J., Weiland, O., Uhlen, M., and Sönnerborg, A. (1996) Restriction of hepatitis C virus heterogeneity during prolonged interferon-α therapy in relation to changes in virus load. *J. Infect. Dis.* **173,** 992–996.
310. Pawlotsky, J.-M., Germanidis, G., Neumann, A. U., Pellerin, M., Frainais, P.-O., and Dhumeaux, D. (1998) Interferon resistance of hepatitis C virus genotype 1b: relationship to nonstructural 5A gene quasispecies mutations. *J. Virol.* **72,** 2795–2805.
311. Polyak, S. J., McArdle, S., Liu, S.-L., Sullivan, D. G., Chung, M., Hofgartner, W. T., Carithers, R. L., McMahon, B. J., Mullins, J. I., Corey, L., and Gretch, D. R. (1998) Evolution of hepatitis C virus quasispecies in hypervariable region 1 and the putative interferon sensitivity-determing region during interferon theraphy and natural infection. *J. Virol.* **72,** 4288–4296.
312. Smith, D. B., McAllister, J., Casino, C., and Simmonds, P. (1997) Virus "quasispecies": making a mountain out of a molehill? *J. Gen. Virol.* **78,** 1511–1519.
313. Horie, C., Iwahana, H., Horie, T., Shimizu, I., Yoshimoto, K., Yogita, S., Tashiro, S., Ito, S., and Itakura, M. (1996) Detection of different quasispecies of hepatitis C virus core region in cancerous and noncancerous lesions. *Biochem. Biophys. Res. Commun.* **218,** 674–681.
314. Ray, R. B., Lagging, L. M., Meyer, K., and Ray, R. (1996) Hepatitis C virus core protein cooperates with *ras* and transforms primary rat embryo fibroblasts to tumorigenic phenotype. *J. Virol.* **70,** 4438–4443.
315. Chang, J., Yang, S.-H., Cho, Y.-G., Hwang, S. B., Hahn, S., and Sung, Y. C. (1998) Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-*ras* oncogene. *J. Virol.* **72,** 3060–3065.
316. Sakamuro, D., Furukawa, T., and Takegami, T. (1995) Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. *J. Virol.* **69,** 3893–3896.
317. Pares, A., Barrera, J. M., Caballeria, J., Ercilla, G., Bruguera, M., Caballeria, L., Castillo, R., and Rodes, J. (1990) Hepatitis C virus antibodies in chronic alcoholic patients: association with severity of liver injury. *Hepatology* **12,** 1295–1299.
318. Vatteroni, M., Pistello, M., Maggi, F., Cecconi, N., Panicucci, F., and Bendinelli, M. (1994) Hepatitis C virus serological and polymerase chain reactions in human immunodeficiency virus-positive and -negative patients. *Clin. Diag. Virol.* **2,** 7–16.
319. Cribier, B., Rey, D., Schmitt, C., Lang, J.-M., Kirn, A., and Stoll-Keller, F. (1995) High hepatitis C viraemia and impaired antibody response in patients coinfected with HIV. *AIDS* **9,** 1131–1136.
320. Sherman, K. E., Andreatta, C., O'Brien, J., Gutierrez, A., and Harris, R. (1996) Hepatitis C in human immunodeficiency virus-coinfected patients: increased variability in the hypervariable envelope coding domain. *Hepatology* **23,** 688–694.
321. Dorrucci, M., Pezzotti, P., Phillips A. N., Cozzi Lepri, A., and Rezza, G. for the Italian Seroconversion Study. (1995) Coinfection of hepatitis C virus with human immunodeficiency virus and progression to AIDS. *J. Infect. Dis.* **172,**

1503–1508.
322. Kurushita, N., Hayashi, N., Moribe, T., Katayama, K., Kanto, T., Kaneshige, T., Tatsumi, T., Ito, A., Mochizuki, K., Sasaki, Y., Kasahara, A., and Hori, M. (1998) Influence of HLA haplotypes on the clinical courses of individuals infected with hepatitis C virus. *Hepatology* **27,** 240–244.
323. Chang, K. M., Rehermann, B., and Chisari, F. V. (1997) Immunopathology of hepatitis C. *Springer Semin. Immunopathol.* **19,** 57–68.
324. Alric, L., Fort, M., Izopet, J., Vinel, J. P., Charlet, J. P., Selves, J., Puel, J., Pascal, J. P., Duffaut, M., and Abbal, M. (1997) Genes of the major histocompatibility complex class II influence the outcome of hepatitis C virus infection. *Gastroenterology* **113,** 1675–1681.
325. Gumber, S. C. and Chopra, S. (1995) Hepatitis C: a multifaceted disease. Review of extrahepatic manifestations. *Ann. Int. Med.* **123,** 615–620.
326. Czaja, A. (1997) Extrahepatic immunologic features of chronic viral hepatitis. *Dig. Dis.* **15,** 125–144.
327. Hoofnagle, J. H. (1997) Hepatitis C: the clinical spectrum of disease. *Hepatology* **26,** 15S–20S.
328. Lunel, F., Musset, L., Cacoub, P., Frangeul, L., Cresta, P., Perrin, M., Grippon, P., Hoang, C., Piette, J. C., Huraux, J. M., and Opolon, P. (1994) Cryoglobulinemia in chronic liver disease: role of hepatitis C virus and liver damage. *Gastroenterology* **106,** 1291–1300.
329. Wong, V. S., Egner, W., Elsey, T., Brown, D., and Alexander, G. J. (1996) Incidence, character and clinical relevance of mixed cryoglobulinaemia in patients with chronic hepatitis C virus infection. *Clin. Exp. Immunol.* **104,** 25–31.
330. Agnello, V. (1997) The etiology and pathophysiology of mixed cryoglobulinemia secondary to hepatitis C virus infection. *Springer Semin. Immunopathol.* **19,** 111–129.
331. Sansonno, D., De Vita, S., Iacobelli, A. R., Cornacchiulo, V., Baiocchi, M., and Dammacco, F. (1998) Clonal analysis of intrahepatic B cells from HCV-infected patients with and without mixed cryoglobulinemia. *J. Immunol.* **160,** 3594–3601.
332. Sansonno, D., Cornacchiulo, V., Iacobelli, A. R., Di Stefano, R., Lospalluti M., and Dammacco, F. (1995) Localization of hepatitis C virus antigens in liver and skin tissues of chronic hepatitis C virus-infected patients with mixed cryoglobulinemia. *Hepatology* **21,** 305–312.
333. Dammacco, F. and Sansonno, D. (1997) Mixed cryoglobulinemia as a model of systemic vasculitis. *Clin. Rev. Allergy Immunol.* **15,** 97–119.
334. Willems, M., Sheng, L., Roskams, T., Ramdani, B., Doutrelepont, J. M., Nevens, F., Durez, P., Treille, S., Adler, M., Desmet, V., Fevery, J., and Yap, S. H. (1994) Hepatitis C virus and its genotypes in patients suffering from chronic hepatitis C with or without a cryoglobulinemia-related syndrome. *J. Med. Virol.* **44,** 266–271.
335. Hadziyannis, S. J. (1996) Nonhepatic manifestations and combined diseases in HCV infection. *Dig. Dis. Sci.* **41,** 63S–74S.
336. Herrero, C., Vicente, A., Bruguera, M., Ercilla, M. G., Barrera, J. M., Vidal, J., Terés, J., Mascaro, J. M., and Rodés, J. (1993) Is hepatitis C virus infection a trigger of porphyria cutanea tarda? *Lancet* **341,** 788–789.
337. Doutrelepont, J.-M., Adler, M., Willems, M., Durez, P., and Yap, S. H. (1993) Hepatitis C infection and membranoproliferative glomerulonephritis. *Lancet* **341,** 317.
338. Johnson, R. J., Gretch, D. R., Yamabe, H., Hart, J., Bacchi, C. E., Hartwell, P.,

Couser, W. G., Corey, L., Wener M. H., Alpers, C. E., and Willson, R. (1993) Membranoproliferative glomerulonephritis associated with hepatitis C virus infection. *N. Engl. J. Med.* **328,** 465–470.

339. De Vita, S., Sacco, C., Sansonno, D., Gloghini, A., Dammacco, F., Crovatto, M., Santini, G., Dolcetti, R., Boiocchi, M., Carbone, A., and Zagonel, V. (1997) Characterization of overt B-cell lymphomas in patients with hepatitis C virus infection. *Blood* **90,** 776–782.
340. Zuckermann, E., Zuckermann, T., Levine, A. M., Douer, D., Gutekunst, K., Mizokami, M., Qian, D. G., Velankar, M., Nathwani, B. N., and Fong, T. L. (1997) Hepatitis C virus infection in patients with B-cell non-Hodgkin lymphoma. *Ann. Int. Med.* **127,** 423–428.
341. Alter, M. J. (1997) Epidemiology of hepatitis C. *Hepatology* **26,** 62S–65S.
342. Heintges, T. and Wands, J. R. (1997) Hepatitis C virus: epidemiology and transmission. *Hepatology* **26,** 521–526.
343. Hibbs, R. G., Corvin, A. L., Hassan, N. F., Kamel, M., Darwish, M., Edelman, R., Constantine, N. T., Rao, M. R., Khalifa, A. S., Moktar, S., Fam, N. S., Ekladious, E. M., and Bassily, S. B. (1993) The epidemiology of antibody to hepatitis C in Egypt. *J. Infect. Dis.* **168,** 789–790.
344. Pistello, M., Ceccherini-Nelli, L., Cecconi, N., Bendinelli M., and Panicucci, F. (1991) Hepatitis C virus seroprevalence in Italian haemophiliacs injected with virus-inactivated concentrates: five-year follow-up and correlation with antibodies to other viruses. *J. Med. Virol.* **33,** 43–46.
345. Laurian, Y., Blanc, A., Dealney, S. R., and Allain, J. P. (1992) All exposed hemophiliacs have markers of HCV. *Vox Sang.* **62,** 55–56.
346. Watson, H. G., Ludlam, C. A., and Rebus, S. (1992) Use of several second generation, serological assays to determine the true prevalence of hepatitis C virus infection in haemophiliacs treated with non-virus inactivated factor VIII and IX concentrates. *Br. J. Haematol.* **80,** 514–518.
347. Centers for Disease Control. (1994) Outbreak of hepatitis C associated with intravenous immunoglobulin administration—United States, October 1993–June 1994. *MMWR* **43,** 507–509.
348. Flora, K., Schiele, M., Benner, K., Montanaro, A., Johnston, W., Whitham, R., and Press, R. (1996) An outbreak of acute hepatitis C among recipients of intravenous immunoglobulin. *Ann. Allergy Asthma Immunol.* **76,** 160–162.
349. Yap, P. L. (1996) The viral safety of intravenous immune globulin. *Clin. Exp. Immunol.* **104,** Suppl. 1, 35–42.
350. Allander, T., Gruber, A., Naghavi, M., Beyene, A., Söderström, T., Björklom, M., Grillner, L., and Persson, M. A. A. (1995) Frequent patient-to patient transmission of hepatitis C virus in a haematology ward. *Lancet* **345,** 603–607.
351. Sakamoto, N., Enomoto, N., Marumo, F., and Sato, C. (1993) Prevalence of hepatitis C virus infection among long-term hemodialysis patients: detection of hepatitis C virus RNA in plasma. *J. Med. Virol.* **39,** 11–15.
352. Mele, A., Sagliocca, L., Manzillo, G., Converti, F., Amoroso, P., Stazi, M. A., Ferrigno, L., Rapicetta, M., Franco, E., and Adamo, B. (1994) Risk factor for acute non-A, non-B hepatitis and their relationship to antibodies for hepatitis C virus: a case control study. *Amer. J. Publ. Health* **84,** 1640–1643.
353. al Meshari, K., Alfurayh, O., Al Ahdal, M., Qunibi, W., Kessie G., and De Vol, E. (1995) Hepatitis C virus infection in hemodialysis patients: comparison of two new

hepatitis C antibody assays with a second-generation assay. *J. Am. Soc. Nephrol.* **6,** 1439–1444.
354. Tumminelli, F., Marcellin, P., Rizzo, S., Barbera, S., Corvino, G., Furia, P., Benhamou, J.-P., and Erlinger, S. (1995) Shaving as potential source of hepatitis C virus infection. *Lancet* **345,** 658.
355. Kiyosawa, K., Sodeyama, T., Tanaka, E., Nakano, Y., Furuta, S., Nishioka, K., Purcell, R. H., and Alter, H. J. (1991) Hepatitis C in hospital employees with needle stick injuries. *Ann. Int. Med.* **115,** 367–369.
356. Mitsui, T., Iwano, K., Masuko, K., Yamazaki, C., Okamoto, A., Tsuda, F., Tanaka, T., and Mishiro, S. (1992) Hepatitis C virus infection in medical personnel after needlestick accident. *Hepatology* **16,** 1109–1114.
357. Alter, H. J. (1994) Transmission patterns in hepatitis C virus infection, in *Viral Hepatitis and Liver Disease* (Nishioka, K., Suzuki, H., Mishiro, S., and Oda, T., eds.), Springer-Verlag, Tokyo, pp. 445–449.
358. Wang, J. T., Wang, T. H., Sheu, J. C., Lin, J. T., and Chen, D. S. (1992) Hepatitis C virus RNA in saliva of patients with posttransfusion hepatitis and low efficiency of transmission among spouses. *J. Med. Virol.* **36,** 28–31.
359. Numata, N., Ohori, H., Hayakawa, Y., Saitoh, Y., Tsunoda, A., and Kanno, A. (1993) Demonstration of hepatitis C virus genome in saliva and urine of patients with type C hepatitis: usefulness of the single round polymerase chain reaction method for the detection of the HCV genome. *J. Med. Virol.* **41,** 120–128.
360. Ogasawara, S., Kage, M., Kosai, K., Shimamatsu, K., and Kojiro, M. (1993) Hepatitis C virus RNA in saliva and breastmilk of hepatitis C carrier mothers. *Lancet* **341,** 561.
361. Young, K. C., Chang, T. T., Liou, T. C., and Wu, H. L. (1993) Detection of hepatitis C virus RNA in peripheral blood mononuclear cells and in saliva. *J. Med. Virol.* **41,** 55–60.
362. Weinstock, H. S., Bolan, G., Reingold, A. L., and Polish, L. B. (1993) Hepatitis C virus infection among patients attending a clinic for sexually transmitted diseases. *JAMA* **269,** 392–394.
363. Kao, J. H., Chen, P. J., Lei, M. Y., Wang, T. H., and Chen, D. S. (1993) Sexual transmission of HCV. *Lancet* **342,** 626.
364. Rice, P. S., Smith, D. B., Simmonds, P., and Holmes, E. (1993) Heterosexual transmission of hepatitis C virus. *Lancet* **337,** 1052–1053.
365. Brettler, D. B., Mannucci, P. M., Gringeri, A., Rasko, J. E., Forsberg, M. G., Rumi, R. J., Garsia, K. A., Richard, K. A., and Colombo, M. (1992) The low risk of hepatitis C virus among sexual partners of hepatitis C-infected hemophiliacs males: an international multicenter study. *Blood* **80,** 540–543.
366. Dienstag, J. L. (1997) Sexual and perinatal transmission of hepatitis C. *Hepatology* **26,** 66S–70S.
367. Thomas, S. L., Newell, M. L., Peckham, C. S., Ades, A. E., and Hall, A. J. (1998) A review of hepatitis C virus (HCV) vertical transmission: risks of transmission to infants born to mothers with and without HCV viraemia or human immunodeficiency virus infection. *Intern. J. Epidemiol.* **27,** 108–117.
368. Cammarota, G., Maggi, F., Vatteroni, M. L., Da Prato, L., Barsanti, L., Bendinelli, M. L., and Pistello, M. (1995) Partial nucleotide sequencing of six subtype 2c hepatitis C viruses detected in Italy. *J. Clin. Microbiol.* **33,** 2781–2784.
369. Maggi, F., Vatteroni, M. L., Fornai, C., Morrica, A., Giorgi, M., Bendinelli, M., and

Pistello, M. (1997) Hepatitis C virus subtype C is highly prevalent in Italy and is heterogeneous in the NS5A region. *J. Clin. Microbiol.* **35,** 161–164.
370. Pistello, M., Maggi, F., Vatteroni, L., Cecconi, N., Panicucci, F., Bresci, G. P., Gambardella, L., Taddei, M., Bionda, A., Tuoni, M., and Bendinelli, M. (1994) Prevalence of hepatitis C virus genotypes in Italy. *J. Clin. Microbiol.* **32,** 232–234.
371. Kinoshita, T., Miyake, K., Okamoto, H., and Mishiro, S. (1993) Imported hepatitis C virus genotype in Japanese hemophiliacs. *J. Infect. Dis.* **168,** 249–250.
372. Alter, H. J. and Seeff, L. B. (1993) Transfusion-associated hepatitis, in *Viral Hepatitis: Scientific Basis and Clinical Management* (Zuckerman, A. J. and Thomas, H. C., eds.), Churchill Livingstone, Edinburgh.
373 Yoshizawa, H. (1994) Prevention of post-transfusion hepatitis in Japan, in *Viral Hepatitis and Liver Disease* (Nishioka, K., Suzuki, H., Mishiro, S., and Oda, T., eds.), Springer-Verlag, Tokyo, pp. 554–557.
374. Nishioka, K. (1994) Epidemiological studies on hepatitis C virus infection: detection, prevalence exposure and prevention. *Intervirology* **37,** 58–67.
375. Al-Khaya, N., Roberts, D. G., Belboul, A., Lopere, V., Bergman, P., Radberg, G., Hermodsson, S., and Olsson, R. G. (1991) Gammaglobulin prophylaxis to reduce post-transfusion non-A, non-B hepatitis after cardiac surgery with cardiopulmonary bypass. *Scand. J. Thorac. Cardiovasc. Surg.* **25,** 7–12.
376. Geberdin, J. L. and Henderson, D. H. (1992) Management of occupational exposures to bloodborne pathogens: hepatitis B virus, hepatitis C virus, and human immunodeficiency virus. *Clin. Infect. Dis.* **14,** 1179–1185.
377. Krawczynski, K., Alter, M. J., Tankersley, D. L., Beach, M., Robertson, B. H., Lambert, S., Kuo, G., Spelbring, J. E., Meeks, E., Sinha, S., and Carson, D. A. (1996) Effect of immune globulin on the prevention of experimental hepatitis C virus infection. *J. Infect. Dis.* **173,** 822–828.
378. Lagging, L. M., Meyer, K., Hoft, D., Houghton, M., Belshe, R. B., and Ray, R. (1995) Immune responses to plasmid DNA encoding the hepatitis C virus core protein. *J. Virol.* **69,** 5859–5963.
379. Major, M. E., Vivitski, L., Mink, M. A., Schleef, M., Whalen, R. G., Trépo, C., and Inchauspé G. (1995) DNA-based immunization with chimeric vectors for the induction of immune responses against hepatitis C virus nucleocapsid. *J. Virol.* **69,** 5798–5805.
380. Wright, T. L., Combs, C., Kim M., Ferrell, L., Bacchetti, P., Acsher, N., Roberts, J., Wilber, J., Sheridan, P., and Urdea, M. (1994) Interferon-α therapy for hepatitic C virus infection after liver transplantation. *Hepatology* **20,** 773–779.
381. Poterucha, J. J. and Gross, J. B. (1995) Hepatitis C after liver transplantation. *Gastroenterology* **108,** 1314–1317.
382. Bukh, J., Purcell, R. H., and Miller, R. H. (1993) At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. *Proc. Natl. Acad. Sci. USA* **90,** 8234–8238.
383. Tokita, H., Okamoto, H., Luengrojanakul, P., Vareesangthip, K., Chainuvati, T., Iizuka, H., Tsuda, F., Miyakawa, Y., and Mayumi, M. (1995) Hepatitis C virus variants from Thailand classifiable into five novel genotypes in the sixth (6b), seventh (7c, 7d) and ninth (9b, 9c) major genetic groups. *J. Gen. Virol.* **76,** 2329–2335.
384. Tokita, H., Okamoto, H., Iizuka, H., Kishimoto, J., Tsuda, F., Lesmana, L. A., Miyakawa, Y., and Mayumi, M. (1996) Hepatitis C virus variants from Jakarta,

Indonesia classifiable into novel genotypes in the second (2e and 2f), tenth (10a) and eleventh (11a) genetic groups. *J. Gen. Virol.* **77,** 293–301.

385. Mori, S., Kato, N., Yagyu, A., Tanaka, T., Ikeda, Y., Petchclai, B., Chiewsilp, P., Kurimura, T., and Shimotohno, K. (1992) A new type of hepatitis C virus in patients in Thailand. *Biochem. Biophys. Res. Commun.* **183,** 334–342.
386. Tokita, H., Shrestha, S. M., Okamoto, H., Sakamoto, M., Horikita, M., Iizuka, H., Shrestha, S., Miyakawa, Y., and Mayumi, M. (1994) Hepatitis C virus variants from Nepal with novel genotypes and their classification into the third major group. *J. Gen. Virol.* **75,** 931–936.
387. Bukh, J., Purcell, R. H., and Miller, R. H. (1994) Sequence analysis of the core gene of 14 hepatitis C virus genotypes. *Proc. Natl. Acad. Sci. USA* **91,** 8239–8243.
388. Tokita, H., Okamoto, H., Tsuda, F., Song, P., Nakata, S., Chosa, T., Iizuka, H., Mishiro, S., Miyakawa, Y., and Mayumi, M. (1994) Hepatitis C virus variants from Vietnam are classifiable into the seventh, eighth, and ninth major genetic groups. *Proc. Natl. Acad. Sci. USA* **91,** 11,022–11,026.
389. Mizokami, M., Gojobori, M., Ohba, K. I., Ikeo, K., Ge, X. M. and Orito, E. (1996) Hepatitis C virus genotypes 7, 8 and 9 should be classified as genotype 6 subtypes. *J. Hepatol.* **24,** 622–624.
390. Simmonds, P., Mellor, J., Sakuldamrongpanich, T., Nuchaprayoon, C., Tanprasert, S., Holmes, E. C. and Smith, D. B. (1996) Evolutionary analysis of variants of hepatitis C virus found in South-East Asia: comparison with classifications based upon sequence. *J. Gen. Virol.* **77,** 3013–3024.
391. de Lamballerie, X., Charrel, R. N., Attoui, H., and De Micco, P. (1997) Classification of hepatitis C virus variants in six major types based on analysis of the envelope 1 and nonstructural 5B genome regions and complete polyprotein sequences. *J. Gen. Virol.* **78,** 45–51.

5

Hepatitis D Virus

Biology, Pathogenesis, Epidemiology, Clinical Description, and Therapy

Antonia Smedile and Giorgio Verme

1. Biology of HDV

1.1. Taxonomy and Genotypes

Hepatitis D virus (HDV), the only member of the genus Deltavirus, is from the Deltaviridae family *(1)*. It is a negative-stranded RNA virus that depends on a DNA virus (HBV) from the family of Hepadnaviridae for propagation but not for RNA replication *(2)*. The helper virus, HBV, does not share either sequence homologies or functional similarities with HDV in their mechanisms of genome replication. Instead, HDV shares some functional and structural similarities with viroids and virusoids, both of which belong to the world of plant viruses *(3)*. However, there is no evidence reflecting evolutionary lineage or similar evolution between HDV and the plant viruses. Thus, HDV is unique among animal viruses. The virion particle of 35–37 nm is an hybrid made of the ribonucleic acid *(HDV–RNA)* and delta antigen (HDAg) enveloped by the surface antigen (HBsAg) of HBV *(4)*. Recent analysis of HDV RNA sequences by polymerase chain reaction (PCR) amplification of a higly conserved region of the RNA genome revealed the presence of three major HDV genotypes with different geographic and demographic distributions *(5)*. HDV genotype I is predominant in North America and Italy; HDV isolates of genotype II have been identified in Japan and Taiwan. By contrast, HDV genotype III isolates are associated exclusively with countries from northern South America (Columbia, Venezuela, Peru). So far, HDV genotype III only have been correlated to disease severity. Genotype F of HBV, the single genotype described as pre-

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

dominant in the Americas has been associated with HDV in this endemic area of HDV infection *(6)*.

1.2. HDV Genome

The HDV genome is a 1.7-kb single-stranded circular RNA. Similar to plant virus RNAs, it is self-complementary and folds into an unbranched rod structure in which about 70% of the nucleotides are base-paired *(7)*. Three major RNA species have been found in infected cells and humans. The first is the 1.7-kb genomic RNA (negative polarity [–]) predominant in the virions. The second is the complementary antigenomic RNA (+) which is present in greater amounts in the liver. The third species is present in lesser amounts in the liver; it is shorter (0.8 kb) than full-length genome RNA and is a polyadenylated mRNA *(8)*. Several isolates of HDV RNA have been fully or partially sequenced; a common feature is the ability to fold into the rod structure. Like subviral agents of plants, HDV RNA has two unique features that are crucial to the replication of the virus, which is based on the rolling circle replication strategy *(9)*.

The first is transcription by a host RNA polymerase, the second property is the autocatalytic capacity (self-cleavage and self-ligation) present in both genomic and antigenomic strands, which is required for processing of linear transcripts to circular replication products.

These autocatalytic RNA segments have been isolated and studied in vitro. Although these elements are funtionally similar to the "hammerhead" ribozymes described in plant viruses, they are different, suggesting that this catalytic domain of HDV RNA represents a new and different ribozyme motif. Mutagenesis and biochemical analysis data give strongest support to a pseudoknot structure for the HDV ribozyme. New hopes for a different approach to therapy may derive form the unique ribozyme activity of the virus as a potential target for antiviral agents *(10)*.

1.3. Functional Domains of HDAg and Transcription

HDV encodes a single protein, HDAg, which is produced from a 0.8 kb-mRNA. Transcription produces two isoforms of the HDV protein (large, L-HDAg; small, S-HDAg); identical in sequences except that the large contains an additional 19 amino acids at its carboxyl terminus *(11)*. Several functional domains that are essential for viral replication, assembly, propagation, and spread of the virus in the liver in the HDAg gene have been described *(12)*. Among them are:

1. a coiled coil domain that mediates formation of HDAg dimers,
2. arginine-rich motifs that are necessary for RNA binding activity, and
3. an isoprenylation signal sequence at the C-terminus.

The two forms of HDAg, HDAg- p24, or HDAg- p27, derived from the same open reading frame, are generated during the replication cycle of HDV because of a specific posttranscriptional RNA editing process that changes the coding capacity of the viral genome. HDV RNA is modified such that the amber stop codon (UAG) for HDAg-S is converted to a tryptophan codon (UGG). The modification occurs at an adenosine at position 1012 in the antigenomic RNA. HDV-RNA editing was documented initially in transfected cells, but it also occurs in infected humans, chimpanzees, and woodchucks. A host enzyme, double-stranded RNA adenosine deaminase (dsRAD) is responsibile for editing that occurs in the antigenomic strand *(13)*. The efficiency of the editing process is variable in different genotypes and isolates as shown by the different levels of virion RNA in serum. Editing at this site (1012), referred to as the amber/W site, requires a highly conserved base-paired structure within the unbranched rod structure of HDV RNA. Mutations that alter this structure, either by disrupting base pairs or by changing the A-C mismatch pair, led to site-mutagenesis studies to test lack of editing efficiency, which results in vitro in the reduction of p27 protein and virion production *(14)*. HDAg-p24 promotes HDV replication and is produced by infectious RNA; HDAg -p27 inhibits replication and is required for virion assembly. The extent of the RNA editing, which is a unidirectional process (edited genomes produce only HDAg-p27) may reflect a fine regulation and balance between replication and assembly of HDV infectious virions. Transcription studies in vitro demonstrated that an 199-nucleotide (nt) genomic sense RNA molecule containing a region that represent one end of the HDV rod, upstream of the HDAg gene, has promoter activity in vitro *(15)*. A computer-generated secondary structure analysis of the HDV promoter revealed a highly ordered RNA secondary structure with an internal and external bulge region and a stem-loop conserved between group I and II HDV isolates *(16)*.

1.4. HDV Replication and Life Cycle

It has been obvious from the first sequencing study that HDV RNA can fold on itself into an unbranched rodlike structure. This rod shape has many features in common with the plant viroids; however, since the viroids are much smaller than HDV and do not code for known proteins, some authors have divided the HDV genome into a "viroid domain," as distinct from the remainder which includes the ORF for the delta antigen. Based on the biological similarities of HDV and viroids it is postulated that replication is likely to occur by the rolling circle model proposed for other plant RNA viruses *(17)*. More recent experiments, however, support the idea of alternative structures that also have importance in the cycle of genome replication

in addition to the rod-like conformation and ribozyme activity. The replication cycle starts with the attachement of the virus to the hepatocytes membrane and ends with its release from the infected cells. Since the HDV virion is coated with the envelope proteins of the helper virus (HBV), it is likely that HBV and HDV share a common receptor on hepatocyte membranes recognized by the preS1 domain of the HBsAg-L. Inside the cytoplasm, HDV is moved to the nucleus were replication occurs. The nuclear localization signal domain identified in the HDAg gene seems to mediate this initial step, then the viral genome is replicated by a host DNA-dependent RNA polymerase II (Pol II), which will interact with the specific region (199-nt RNA) of the rod-structure, where the RNA promoter has been identified, allowing the transcription of the only ORF of HDAg. In this model, circular genomic RNA is transcribed by Pol II to yield multimeric linear transcripts of antigenomic sense that undergo autocatalytic cleavage and ligation to produce circular monomeric antigenomic RNA. Then the antigenomic RNA serves as a template for replication of circular genomic RNA by similar transcription and processing. Meanwhile, the extent of the editing process regulates the production of the L-HDAg. The large form acts as a dominant negative inhibitor of genome replication and is also needed for packaging of HDV RNA into the virus particles *(18)*. Both the HBsAg-L and the small HBsAg protein present in Dane particles (infectious HBV) are required for the encapsidation of HDV; this takes place by budding into the endoplasmic reticulum of the Golgi.

1.5. Propagation of HDV in Cell Cultures

The propagation of HDV in cell cultures has not been successful *(19,20)*. Primary hepatocyte cultures from woodchucks and chimpanzees were infected with HDV, yielding a single cycle of replication in a small proportion of cells. Partial- and full-length HDV cDNA or cRNA clones have been introduced in transfected cells; however, the infectious cycle appears to be limited to primary hepatocytes, and the complete replication of the virus is limited to hepatocytes that are transfected with an hepadnavirus. In vitro synthesized virions released in the medium are fully infectious *(21)*. By contrast, in vitro studies of cDNA clones of specific RNA elements have dramatically improved our understanding of the genome structure and virus replication. Site-mutagenesis analysis in the antigenomic RNA have shed light to the efficiency and fine regulation of the cellular editing process essential for the replication and packaging of HDV virions. Mutants generated by making point mutations, small insertions, or deletions at the top of the rod structure have shown in vitro how this part of the genome may affect HDV replication by dropping replication efficiency between 100–1000-fold compared with the wild type

(22). In vitro transcriptional studies strongly support the presence of an HDV promoter in the top of the rod, upstream of the HDAg gene, which may help us understand how the HDV promoter operates.

1.6. Infection in Experimental Animals

The host range of HDV infection includes man and experimental animals (chimpanzee, woodchuck, and duck) carrying the HBV-related hepadnavirus (HBV, WHV, DHV) *(23,24)*. Experimental infections have reproduced the modes of infection, coinfection, and superinfection that mediate HDV transmission in humans. A common feature of experimental HDV infection is the inhibition of the HBV-helper virus, shown by the decrease of HBsAg and of HBV DNA in serum and the liver. Serial passage of HDV in chimpanzees and woodchucks produced a shortening of the period of incubation and an increased severity of acute hepatitis D *(25)*. Infection has been initiated also by direct injection in the liver of cDNA clones capable of generating infectious virions *(26)*. Transgenic mice, not susceptible to HBV infection were used to study the effect of HDV by itself in the absence of the helper virus. Replicative intermediates of HDV and HDAg in the liver were demonstrated in the injected rodents *(27)*.

1.7. Reinfection in Liver Transplants

Liver transplantion provides a valid treatment option in patients with liver failure caused by HDV. There is enough evidence to suggest that orthotopic liver transplantation will cure all the HDV recipients if standard anti-HBIg prophylaxis is provided after surgery. The recipients demonstrate a much lower (almost none) risk of graft reinfection than in ordinary HBV infection, and long-term survival is good *(28)*. Initial suggestions that the transplant setting may be an additional mode of HDV infection (latent infection) with different virologic properties have not been confirmed. At that time, using hybridization assays for HDV RNA and HBV DNA, the virological profiles of HDV reinfection following liver transplantation demonstrated that in over 50%, HDV appeared in the graft early on but was not accompained by HBV *(29)*. This observation, together with the detection of HDV viremia before the appearance of HBV markers had led to the suggestion that HDV may not always require HBV, and that it could exist in a latent form that could have been rescued by subsequent HBV infection. Recent analysis, using highly sensitive nested-PCR assays for both viruses (HDV and HBV) indicates that both were simultaneously present in the serum, and that the particle appearing after the transplant had similar cesium chloride (CsCl) density properties of the virion that was present before transplantation *(30)*.

2. Transmission

The parenteral route is the most efficient modality of HDV transmission, explaining the elevated rates of infection reported in groups such as drug addicts, hemophiliacs, and recipients of blood derivates. In recent years, the screening of blood for viral hepatitis and the use of HBV vaccine have led to the virtual elimination of the risk of posttransfusion hepatitis D; the infection has also declined in drug-addict communities *(31)*.

Transmission of HDV in endemic areas occurs through inapparent parenteral routes. Sexual transmission has been well documented in Taiwanese prostitutes and sexual partners of HDV-infected carriers *(32)*. Poor hygienic conditions and overcrowding favor the spread of the virus; in Southern Italy, coexistence with an HDV carrier within the household was identified as a major risk for HDV transmission *(33)*. The increased practice of body piercing and tattooing may add a new way of transmission among modern youth.

An unusual mode of infection with HDV was noted in liver grafts after transplantation. In this mode, early replication of HDV accompanied by minimal HBV replication (detectable only by sensitive PCR assays) has remained clinically silent with no biochemical or histologic evidence of graft damage until HBV infection has also reactivated to the full expression of its antigenic and genetic markers; as a rule, this event enhanced HDV replication, transforming a latent HDV infection into florid hepatitis D of the graft.

3. Epidemiology

HDV infection has been reported worldwide. Prevalence rates have varied from very low in temperate and cold climates to intermediate and high in many tropical and subtropical countries *(34)*. Clearly, a major determinant of the prevalence of hepatitis D in a given population is the prevalence of HBV infection; nevertheless, the rate of HDV infections is not a simple function of the rate of HBV infection, as in many countries the prevalence of HDV infection is negligible despite elevated rates of HBV endemicity. This would suggest various degrees of penetration of HDV in "susceptible areas". The improved control of HBV achieved in Europe in recent years has led to a marked decline of HDV infection as well; public health measures to control the AIDS epidemic have also had an impact on transmission of HBV/HDV, a virus transmitted through the same modalities as the HIV. Data from the SEIEVA (Surveillance System for Acute Viral Hepatitis) indicate that in 1992 the HBV/HDV infection rate has dropped from 3.1 to 1.2 per million inhabitants *(35)*. Throughout the Mediterranean

basin, prevalence rates have also significantly diminished among chronic HBsAg carriers.

Nevertheless, despite the general reduction of new cases of acute infection reported, other countries in the world are only just now reporting the prevalence of HDV infection. Recently, new foci of HDV infection were identified in St. Petersburg, Russia, the island of Okinawa in Japan, and isolated villages in China, Northern India, and Albania *(36–38)*. By contrast, the regions of northern South America (Peru, Columbia, Venezuela), in particular the subtropical area close to the jungle used for military personnel stations, continue to be identified as a reservoir of epidemic outbreaks of HDV infection *(39)*.

What determines the different spreading and clinical types of HDV infection is unclear. Casey et al. reported that HBV genotype F was predominant in HDV-infected cases isolated from an outbreak of hepatitis D among military personnel in Peru *(6)*. Thus, one factor may be HBV, whereas other factors may be involved in the spread of HDV, including host susceptibility to HDV, possibly different infections, and pathogenic potential of HBV and HDV genotypes.

Molecular epidemiology of HDV has become an area of extreme interest as it might provide an explanation to the variable features of HDV infection based on the viral genetics of HDV. Recent analysis of HDV RNA sequences by PCR amplification of a semiconservative region of virion RNA has shown that there are three major HDV genotypes (a fourth genotype is still uncertain) with different geographic and demographic distributions *(5)*. Recent evidence supports the correlations between different genotypes and disease severity *(6)*. HDV genotype I is found worldwide and is predominant in North America and Europe. Within a semiconserved region of the HDV genome, genotype I isolates are at least 85% identical to each other. Some clustering of sequences has suggested the possible existence of two subgroups (IA and IB) of this genotype. Interestingly, the distribution of the subgroups is sometimes geographically distinct: the 20 isolates found in the Unites States are group IB, and the six published East Asian isolates are group IA. Mixed patterns are found in areas of past endemic infection, such as Italy and Greece, where both subgroups are represented in the population. Such data suggest that HDV genotype I was introduced to the Mediterranean area earlier and more often than into the U.S.A and East Asia population. Preliminary data also suggest an association of genotype IB with intravenous drug use in Italy. HDV genotype II have been exclusively found in isolates from Japan and Taiwan. In Taiwan, based on large screening of HDV isolates with RFPL analysis (restriction fragment polymorphism), a new genotype (genotype IV or a subtype of genotype II) has been identified *(40)*.

HDV genotype III is exclusively associated with HDV infection occurring in the northern part of South America. This genetic association is correlated to severe forms of acute and fulminant HDV hepatitis. More intriguing, is that the type of HBV isolated in these epidemics is genotype F. The meaning of this association is unknown. Since HBV genotype F is predominant in this part of the world, it is necessary to clarify whether other HBV genotypes are associated with particular genotypes of HDV and disease outcome. The strong geographic component of the distribution of HDV genotypes I, II and III, raises questions about the possibility of whether new genotypes or subtypes may exist in regions were HDV infection has not yet been detected in association with its helper virus, HBV, despite extensive testing. In the antigen-coding region, most of the genotype-specific sequence divergence is found in the C terminus of HDAg-p27. The C-terminus of the large form HDAg is involved in virion formation and inhibition of replication. There are also substantial variations between the structures of the RNA editing sites of the different genotypes suggesting possibile effects on the efficiency or mechanisms of editing. Altogether, these observations suggest the possibility of subtle variations in the strategy of replication of the virus to accomodate different adaptations of HDV to the local strains of HBV that predominate in different populations.

4. Pathogenesis

The pattern of HDV disease associated with chronic HDV infection vary in different epidemiological settings. Relatively benign forms of liver disease and asymptomatic carriage of HDV have been described in areas were HDV is endemic, such as in the Greek Island of Rhodes, the islands of America Samoa, Okinawa, and Southern Italy. In areas were HDV is not endemic (northern Europe and North America), the outcome of hepatitis D is more severe; most of the patients are intravenous drug abusers. In South America, where HDV infection is endemic in the Northern part of the continent (Venezuela, Columbia, Peru), the course of HDV disease is often more severe with a subfulminant or fulminant course described in some communities *(41,42)*. To explain these different patterns of HDV infection, many factors have been advocated; particular interest has led to the discovery of geographical genetic diversities and virus interactions.

The analysis of HDV genotypes as a means to explain the different degree of liver disease severity associated with HDV infection has only recently been pursued. To demonstrate a direct role of HDV and its genetic variability in inducing liver damage, it is necessary to demonstrate a close relationships between given HDV genotypes and disease. In HDV hepatitis,

only genotype III seems to correlate with cases of severe or fulminant acute delta hepatitis such as those occurring in communites in the northern part of South America. A recent virological analysis of HDV and HBV strains carried out in one of these outbreaks, which occurred in 1992–1993 among troops stationed at four jungle outposts in Peru, has demonstrated that the HDV isolate was exclusuvely of genotype III and the HBV isolate was of genotype F *(6)*. These findings would support a direct contribution of the HDV to the particular severity of liver disease and specific alterations at liver pathology (microvesicular steatosis without necroinflammatory disease) through a cytotoxic mechanism of liver damage *(43–44)*.

Genotype II HDV, isolated only in Japan, seems to cause only a mild form of HDV infection, as demonstrated by recent data in the Irabu islands, Okinawa, Japan. In this area of the Far East, in which HBV and HDV infections are endemic, a recent large study in the general population found that among 2207 subjects tested, 210 (9.5%) were HBsAg positive. Anti-HD antibody was detected in 47 out of 210 (22.4%) and 21 out of 43 anti-HD positive were HDV RNA positive by PCR assay. More interesting, those subjects with anti-HD antibody referred to hospital for clinical evaluation rarely showed biochemical alterations or histological lesions of liver diseases *(45)*. These intriguing findings await analysis by genetic variability to determine the relationship of genotype and severity.

Genetic analysis was used to characterize the biology of the particular HDV isolate from sera of patients collected in the outbreak of HDV fulminant hepatitis that occurred in Bangui (Central Africa). The cloning and sequencing of that particular isolate amplified after inoculation in the woodchuck animal model showed a specific point mutation in the gene of HDAg that was responsible for the abnormal size of the HDAg identified using immunoblotting *(46)*. An analogous virologic approach was used to explain the benign form of HDV infection noted in patients living in the Greek island of Rhodes. After cloning and sequencing of some sera from this region, a point mutation was found to be prevalent. By contrast, this mutation was not found in sera taken from patients with progressive chronic hepatitis D. HDV genotype I, prevalent in Italy and North America, seems to be more heterogenous. Phylogenetic analysis of 46 HDV samples from Italian chronic carriers have been shown to segregate into two major subgroups (IA and IB), but no clear conclusions were derived regarding this association with HDV disease severity *(47)*. This type of phylogenetic analysis offers hope for tracking HDV diffusion among drug addicts and in families in which an HDV-infected member has been identified *(47)*. The same method of analysis was used to demonstrate the spread of HDV infection and how isolates were identical in spouses of infected HDV carriers *(48)*.

The contribution of HBV infection to HDV disease remains the most intriguing question. HDV inhibits HBV replication as shown by the low rate of HBV DNA replication in chronic HDV carriers *(49)*. However, with PCR assays for HBV-DNA, it has been demonstrated that HBV persists and thrives in a patient with active HDV hepatitis when HDV reaches full replication. The model of HDV reinfection in liver transplantation using sensitive PCR assays to measure both HDV and HBV, has finally made clear the serum and liver interference between these two agents, as well as the sequential serological events taking place before acute overt HDV hepatitis occurs in the graft *(30)*.

Multiple viral infections (HBV, HDV, HCV, and HIV) occur more frequently in drug abusers *(50)*. In contrast, sexual transmission of HDV and HCV is relatively inefficient, so that coinfection with HBV and these other two viruses is seen predominantly in patients with injection drug use as their risk factor for transmission.

In multiple viral infections, interference is a frequent phenomenon, HDV/HBV coinfection represents the first example of viral suppression. In this case, HBsAg is not detected in serum, and there is a decrease in HBV DNA levels. In patients coinfected with HBV/HCV or HDV/HCV, a clear suppression of HCV viremia occurs *(51)*. The inhibition of HCV replication may explain the less severe course of HCV posttransplantation hepatitis in patients coinfected with HDV/HBV/HCV prior to OLT compared with those infected by HCV only (personal observation). Injection drug users are also at high risk for HIV infection. In a study of 88 HBV infected drug users in New York, HDV and HIV infection were present in 67% and 58%, respectively. Although, HDV was associated with more severe liver disease, this was not further aggravated by concomitant HIV infection *(52)*. This was not the experience recently reported from Caredda and coworkers in a study conducted in Milan, where an epidemic of HDV infection occurred in the 1980s in drug users of this metropolitan area *(53)*. Most of those drug abusers infected by HDV were found to be infected with HIV. Comparison between HIV/HDV-positive patients and HIV patients alone showed that the former had high levels of viremia and a greater risk for developing chronic severe hepatitis than those without HIV infection *(54)*.

5. Clinical Features and Outcome

5.1. Acute Infection

No specific clinical feature differentiates acute hepatitis D from other types of acute viral hepatitis. The SEIEVA report for cases of acute hepatitis D that occurred in Italy in recent years has indicated that the majority required

hospitalization. This would suggest that the overall course is more severe than for hepatitis B alone. Acute coinfections resolve in over 95% of cases; in contrast, superinfection is most often severe and advances to chronicity in over 70% of cases *(55)*. Overall HDV infections remain the second most common cause of fulminant viral hepatitis worldwide *(56)*. Severe outbreaks of hepatitis D have been reported in the Yupca Indians of South America. This part of the world continues to be highly endemic for HDV infection, with new outbreaks recently reported in local populations and military personnel living in the Peruvian jungle. HDV infection persists in the area despite efforts to implement HBV vaccination in the general population.

5.2. Chronic Infections

No specific clinical features mark the progression of hepatitis D from acute to chronic. This is recognized from seroconversion to IgM anti-HD, the increase of IgM anti-HD, and the persistence of HDV-RNA in serial blood samples monitored after the acute episode. Chronic HDV infection is rarely observed in healthy carriers of HBsAg (less than 4–8% of cases). In areas where HDV is endemic, it prevails in young cirrhotic patients and in patients with active forms of chronic hepatitis *(57)*. Chronic hepatitis D evolves to cirrhosis more rapidly than HBV infection alone, thus explaining the younger cumulative age (by a decade) of patients with HBV/HDV cirrhosis compared with those with HBV cirrhosis alone *(58)*. Although the advance to cirrhosis is usually rapid, when cirrhosis is established the disease course does not appear to differ from HBV cirrhosis. The estimated 5- and 10-yr probability of survival free of virus after OLT was 98% and 95% for patients with chronic active hepatitis, 93% and 64% for patients with histological cirrhosis, and 49% and 29% for patients with clinical cirrhosis *(59)*. The estimated 93% 5-yr survival is comparable with the 84% 5-yr survival in HBsAg-positive histological cirrhosis and with the 87% 5-yr survival probability reported by Fattovich *(60)*.

Three studies in Rotterdam, North Europe, and Italy and Greece reevaluated the natural history of HDV infection. By analyzing a cohort of infected HDV patients and the final outcome of liver disease (death, liver transplantation), it was found that the course of HDV liver disease can be slowly progressive, indolent for decades *(61,62,59)*. It has been suggested that there are three clinical phases of HDV liver disease: an early stage with active HDV replication and suppression of HBV, a second stage characterized by moderate active disease with decreasing HDV and reactivating HBV, and a third late stage characterized either by the development of cirrhosis and hepatocellular carcinoma (HCC) caused by replication of either virus or by remission resulting from the reduction of replication of both viruses.

These data derived from years of monitoring chronic delta-infected carriers also suggest the importance of HBV infection; in the long run, this may influence the final outcome of HDV disease.

A rapid course to liver failure was reported in Italy in the 1980s in 10–15% of patients with HDV infection; most of them exhibited active infection with both HBV and HDV. This course, typical in drug addicts, was probably caused by coinfection with HCV; when testing for anti-HCV became available in the 1990s, most of these patients were recognized also to have HCV markers.

Hepatocellular carcinoma develops in HDV chronic carriers at approximately the same rate as for patients with HBV cirrhosis alone. In a recent study, HCC was the cause of death for 7/19 (37%) patients who were followed for a period of 5–10 yr *(59)*. In Greece, a recent study reported that within 12 yr of follow-up, 40% of HDV chronic carriers developed HCC *(62)*. The same screening program for HCC is recommended for patients with cirrhosis type D, using tests such as alpha-fetoprotein and ultrasound control.

HDV replication has not been detected in animal tissues and extrahepatic human organs; the liver is the only target for HDV. Transgenic mice have been used as a model to study HDV replication in the presence and absence of HBV. The tissue from muscle fibers of infected animals seems to actively replicate the virus in vitro *(63)*. HDV as well as other hepatitis viruses are able to trigger an autoimmune reaction as shown by the detection of autoantibodies. Those with active disease may develop autoantibodies against nuclear lamin C *(64)*, thymic cells, and autoantibodies reactive against the microsomal membranes of the liver and kidney (LKM antibodies) *(65–67)*. LKM3 is used to distinguish viral-induced autoantibody from idiopathic LKM 1 and LKM 2 elicited in hepatitis induced by tyenilic acid. LMK3 is directed against a 55-kd microsomal band containing an antigen of the UDP glucoronyl transferase 1 gene family (UGT1) *(68)*.

6. Pathology of HDV Hepatitis

The liver histology in acute coinfections and superinfections is not different from histology in other cases of viral hepatitis *(69)*. Acute fulminant hepatitis D in the Western world is characterized by extensive stromal collapse with disappearance of hepatocytes except those regenerating in a pseudoductular arrangement; the portal tracts are only moderately infiltrated with lymphocytes *(70)*. In the outbreaks in Northern South America and in Yupca Indians of Western Venezuela, HDV superinfection caused severe disease in populations with a high rate of HBsAg carriers, producing peculiar histological features. The liver specimens showed extensive small droplet steatosis with some necrosis as well as portal lymphocytic infiltration; the

intralobular inflammatory cells were mainly macrophages containing PAS-positive nonglycogenic granules. The cells named "morula cells" were first described during the outbreak of Labrea fever in the Amazon River; the cells contained HD antigen *(71)*. The HDV-associated microsteatosis has also been described in other cases of hepatitis D in Brazil, Venezuela, and Colombia, and was recently observed in experimentally infected woodchucks that died after acute HDV hepatitis (Callea and Ponzetto, personal observation). The mechanism underlying this cell metamorphosis is not understood; possibly the overload of cellular organelles with virus gene products (about 300,000 copies of HDV RNA per cell) might cause the storage of fat or phospholipids typical for this type of cell damage. Clusters of shrunken hepatocytes with deeply acidophilic cytoplasm, in which the nucleous is absent or undergoing pyknosis are often seen in hepatitis D; these cells have irregular oulines and sometimes assume angular or rhomboid shapes. They represent an example of "apoptosis," a form of degeneration seen in all types of viral hepatitis, considered an expression of immune-mediated cell necrosis *(72)*.

7. Natural History of HDV Infection

As in vivo HDV infection requires the presence of HBV infection, two modalities of viral acquisition were diagnosed for this infection. Coinfection is the result of a simultaneous acquisition of both HBV/HDV by an individual who has not been previously infected with HBV. This typically results in an acute hepatitis characterized by the presence of viremia (HDV RNA and HBV DNA lasting a few days) and of serological markers of primary infection (antibody of class IgM to the core antigen of HBV, anti-HBc IgM, and IgM antibody to delta antigen, anti-HD IgM). In the liver, HDAg and core antigen can be expressed simultaneously or sequentially. In most cases, acute coinfection is self-limiting, but it can evolve to fulminant hepatitis. The serological profile of asymptomatic cases of coinfection is recognized only from the delayed increase of IgM and IgG anti-HD *(73)*. In severe disease, the serological profile is more sequential, with viremia preceding the early finding of HDAg and HBcAg in the liver, followed shortly by the seroconversion first to IgM and then to IgG anti-HD; the former wanes in a few weeks, the latter after a few months, leaving no serological evidence of past HDV infection *(74)*.

Acute hepatitis D acquired through superinfection is usually a severe disease accompained by jaundice and liver dysfunction. The outcome of acute hepatitis can be fulminant or subacute with the underlying liver disease caused by HBV modulating its severity. In superinfection early viremia is a frequent finding followed by a quick IgM and IgG antibody reaction. In

superinfection that resolves, the IgM antibody wanes in a few weeks and the IgG antibody persists for 1–2 yr; both antibodies rise to high titers and persist indefinitely in superinfections that progress to chronicity *(75)*.

Currently, new HDV infections continue to be acquired via these two mechanisms; however, superinfection seems to prevail over coinfection as evidenced by the number of chronic HBV carriers at risk of HDV infection worldwide. In a recent study in North India, coinfections were significantly higher in acute hepatitis (80%), whereas superinfections predominated (66.7%) in chronic liver disease *(76)*.

8. Prevention of HDV Infection

Two main strategies exist for the prevention of HDV infection: behavior modification to prevent disease transmission, and active immunization against HBV. Changes in sexual practices in respose to HIV infection have probably contributed to the declining incidence of HBV and HDV infection in Italy and abroad. Improved screening measures of blood products in blood banks have undoubtedly reduced the risk of transfusion-associated hepatitis. Other primary preventive measures, such as needle exchange programs for injection drug users, are more difficult to implement. Other routes of transmission, such as tattooing and body piercing, are emerging as risk factors of transmission. Behavior modification is unlikely to be beneficial in developing countries where HDV is endemic and where neonates and young adults are at greatest risk of acquiring infection. Prophylaxis for HDV infection is possible by HBV vaccination, and this mode of prevention is possible for coinfections among HBV-susceptible individuals. However, prophylaxis of HBV carriers from HDV superinfection remains unresolved. Active immunization with synthethic peptides derived from HDAg have been used in experimental studies in the woodchuck animal model by several groups *(77,78)*; but the vaccine failed to prevent HDV infection, although the amount of circulating HDV virions was considerably reduced. New strategies based on the biological properties of HDV, such as its ribozyme activities, seems to work much better in cell cultures, but clearly, more research is need to confirm and extend these alternative approaches.

Universal immunization against HBV will be the most effective measure for preventing HDV infection.

9. Therapy for HDV Disease

Alpha-Interferon (IFN) is the only drug available for treatment of chronic hepatitis D. However, in the majority of responders, the disease reoccurred after therapy was stopped; there was no sustained response of IFN

against HDV, as in most patients the level of viremia was not affected by therapy *(79)*. By contrast, when high doses of IFN are administered for a prolonged period of time, some patients have biochemical and histological responses to treatment. In one study, ALT become normal in 10 of 14 (71%) patients treated with 9 MU IFN 3X wk for 48 wk. In comparison, ALT normalized only in 4 of 14 patients who were treated with 3 MU 3X wk for the same period of time and in 1 of 13 untreated controls. In those treated with the high regimen (9 MU), the normalization of ALT was associated with a marked improvement in histological findings, and no HDV RNA was detected during the course of therapy *(80)*. Nevertheless, HDV viremia usually persists despite the high dosage of drug; sustained responses are achieved only in a minority of treated patients, and clearance of HBsAg has been observed in patients with short duration of hepatitis D *(81)*. In addition, side effects are common with this high regimen of IFN.

In the past, other immunomodulators and antiviral agents were proposed to treat HDV infection. However, therapy with streroids, thymosin, levamisole, and ribavarin were disappointing and have not provided benefit *(82–84)*. Thymosin alpha-1, a polypeptide with immunomodulatory effects, can induce disease remission in patients with chronic hepatitis B. This finding induced a pilot study in patients with chronic hepatitis D. Twelve patients were randomly assigned to either thymosin alpha-1 at a dose of 900 μg/m 2X wk for 6 mo or no treatment. None of the controls had normal ALT levels at the end of follow-up. In the thymosin alpha-1 group, ALT values became normal in two patients and remained within the normal range throughout the subsequent follow-up. Both patients had a short history of chronic liver disease *(85)*. The use of antivirals, nucleoside analog such as lamivudine, ganciclovir, famciclovir, or lobucovir appear to be the most promising new agents currently in clinical trials for HBV treatment. Considering the persistence of HBV-DNA as measured by PCR assays in patients with chronic hepatitis D, it may suggest the use of antiviral agents to completely abate levels of HBV replication, especially in infected carriers with continuing HBV replication *(86)*. Recent studies of antisense oligonucleotides directed against functional domains of HDV have shown that they are effective inhibitors of HDV replication in vitro *(87)*. HBV antisense oligonucleotides have been considered in the prevention of HDV replication by altering those HBV functions that are considered essential for HDV replication *(88)*.

10. Transplantation for Chronic Delta Hepatitis

Liver transplantation is a valid therapeutic measure for the therapy of chronic liver failure in cirrhotic HDV patients. Delta hepatitis is an uncom-

mon cause for liver transplantation in the United States *(89)*, but represents a large proportion of transplant candidates in Italy and France *(90,91)*. Hepatitis delta infection can recur after liver transplantation is performed, generally shortly after the surgery. Of 27 HDV transplants in Italy and Belgium who received short HBIg prophylaxis against HBV, or no prophylaxis at all, 22 *(81%)* became reinfected with HDV, but only 11 (14%) were also reinfected with HBV and had a HDV relapse *(92)*. Treatment of recurrent delta hepatitis has been unsatisfactory. Various therapies, most often targeted to HBV, have been tried without great evidence of success *(93)*. However, recurrence of hepatitis after transplantation for chronic delta hepatitis is less frequent than that for chronic hepatitis B, the reasons being that the delta hepatitis cannot recur unless hepatitis B also recurs. As a consequence, prevention of recurrence of HBV using passive immunoprophylaxis has also worked satisfactorily for the prevention of HDV infection. In Italy between 1985 and 1995, there were 154 transplants for HBV correlated cirrhosis, 59% of those who were HBsAg positive and anti-HBe positive were also anti-HD positive. The reinfection rate, after the introduction of standard HBIg prophylaxis against HBV, has dropped to 9–12% in two large series studied in Italy and France. Thus, long-term prophylaxis with immunoglobulins against HBsAg is highly effective. The 5-yr and 10-yr survival rate of 76 HDV transplants in Paris was 88%, and in Italy (Torino) results were excellent; 45 HDV transplants are alive and none was lost during the 8–10 yr of follow-up (Smedile, personal observation).

Liver transplantation also represents a valid model to study HDV infection of the graft. HDV by itself can infect the new liver but that, without the helper function of hepatitis B (probably HBsAg), is not disease-producing. The reinfection is limited in scope, persisting at low levels and causing only hepatic damage. If hepatitis B does then recur, HDV replication increases exponentially and can cause significant clinical disease *(94)*.

11. Summary

Viral hepatitis D, despite its decreasing prevalence in the world, continues to be an important health problem with significant morbidity and mortality. HDV is still the principal cause of severe or fulminant hepatitis in undeveloped countries and the cause of rapidly progressive chronic hepatitis in industrialized countries. New foci of endemicity and new clinical aspects continue to emerge in different epidemiological settings. In recent years, important advances in molecular biology of the virus have contributed to the identification of its three major genotypes and the understanding of its life cycle. Its RNA editing properties may explain the pathogenesis and degree

of severity of liver damage. Studies on epidemiology and the natural course of HDV hepatitis have clarified modes of transmission and clinical patterns of HDV infection. Potential prognostic factors of benign or severe outcome for chronic delta hepatitis may be identified. Liver transplantation provides a good cure for HDV-associated liver failure.

References

1. Gerin, J. L. (1994) The taxonomy of hepatitis delta virus, in *Viral Hepatitis and Liver Disease*. Nishioka K., Suzuki H., Mishiro S., Oda T. eds, Springer-Verlag, Tokyo, pp. 63–64.
2. Taylor, J. M. (1990) Structure and replication of hepatitis delta virus. *Semin. Virol.* **1,** 135–141.
3. Tanner, N. K., Schaff, S., Thill, G., Petit-Koskas, E., Crain-Denoyelle, A. M., and Westhof, E. (1994) A three-dimensional model of hepatitis delta virus ribozyme based on biochemical and mutational analyses. *Curr. Biol.* **4,** 488–498.
4. Smedile, A., Rizzetto, M., and Gerin, J. L. (1994) Advances in hepatitis D virus biology and disease, in *Progress in Liver Disease* (Boyer, J. L. and Okner R. K., eds.), W. B. Saunders, pp. 157–175.
5. Casey, J. L., Brown, T. L., Colan, E. J., et al. (1993) A genotype of hepatitis D virus that occurs in northern South America. *Proc. Natl. Acad. Sci. USA* **90,** pp. 9016–9020.
6. Casey, J. L., Niro, G. A., Engle, R., Vega, A., Gomez, H., McCarthy, M., Watts, D. M., et al. (1996) Hepatitis B virus/hepatitis D virus co-infection in outbreaks of acute hepatitis in the Peruvian Amazon basin: the role of HDV genotype III and HBV genotype F. *J. Infect. Dis.* **174,** 920–926.
7. Chen, P. J., Kalpana, G., Goldberg, J., et al. (1986) Structure and replication of the genome of the hepatitis delta virus. *Proc. Natl. Acad. Sci. USA* **83,** 8774–8778.
8. Taylor, J. M. (1990), Hepatitis delta virus: cis and trans functions required for replication. *Cell,* **61,** 371–373.
9. Ryu, W.-S., Bayer, M., and Taylor, J. (1992) Assembly of hepatitis delta virus particles. *J. Virol.* **66,** 2310–2315.
10. Cech, T. R. (1988) Ribozymes and their medical implications. *JAMA* **260,** 3030–3034.
11. Lin, J. H., Chang, M. F., Baker, S. C., Govindarajan, S., and Lai, M. M. C. (1990) Characterization of hepatitis delta antigen: specific binding to hepatitis delta virus RNA. *J. Virol.* **64,** 4051–4058.
12. Kuo, M. Y. P., Chao, M., and Taylor, J. (1989) Initiation of replication of the human hepatitis delta virus genome from cloned DNA: role of delta antigen. *J. Virol.* **63,** 1945–1950.
13. Casey, J. L., Bergman, K. F., Brown, T. L., and Gerin, J. L. (1992) Structural requirements for RNA editing in hepatitis delta virus: evidence for a uridine-to-cytidine editing mechanism. *Proc. Natl. Acad. Sci. USA* **89,** 1749–1753.
14. Polson, A., Bass, B., and Casey, J. (1996) RNA editing of hepatitis delta virus antigenome by dsRNA-adenosine deaminase. *Nature* **380,** 454–456.
15. Macnaughton, T., Beard, M., Chao, M., Gowans, E., and Lai, M. (1993) Endogenous promoters can direct the transcription of hepatitis delta virus RNA from a recircularized cDNA template. *Virology* **196,** 629–636.

16. Beard, M. R., Gowans, E. J., and Macnaughton, T. (1997) A transcriptional promoter for antigenomic-sense HDV RNA, in *Viral Hepatitis and Liver Disease* (Rizzetto, M., Purcell, R. H., Gerin, J. L., and Verme, G., eds.), Minerva Medica, Torino, Italy, pp. 298–301.
17. Branch, A. D. and Robertson, H. D. (1984) A replication cycle for viroids and other small infectious RNAs. *Science* **223,** 450–455.
18. Lazinski, D. W. and Taylor, J. M. (1994) Expression of hepatitis delta virus RNA deletions: cis and trans requirements for self-cleavage, ligation and RNA packaging. *J. Virol.* **68,** 2879–2888.
19. Taylor, J. M., Mason, J., Summers J., Goldenberg, C., Aldrich, L., Coates, J., Gerin, J. L., and Gowans, E. (1987) Replication of human hepatitis delta virus in primary cultures of woodchuck hepatocytes. *J. Virol.* **61,** 2891–2895.
20. Wu, J. C., Chen, M. Y. P., Kuo, S. D., Lee, Chen, D. S., and Ting, L. P. (1991) Production of hepatitis delta virus and suppression of helper hepatitis B virus in a human hepatoma cell line. *J. Virol.* **65,** 1099–1104.
21. Sureau, C., Moriarty, A. M., Thorton, G. B., and Landford R. (1992) Production of infectious hepatitis delta virus in vitro and neutralization with antibodies directed against hepatitis B virus pre-s antigens. *J. Virol.* **66,** 1241–1245.
22. Casey, J. L. and Gerin, J. L. (1995) Hepatitis delta virus RNA editing and genotype variations, in *The Unique Hepatitis Delta Virus* (Dinter-Gottlieb, G., ed.), Springer-Verlag, New York, pp. 111–124.
23. Rizzetto, M., Canese, M. G., Gerin, J. L., London, W. T., Sly, D. L., and Purcell, R. H. (1980) Transmission of the hepatitis B virus-associated delta antigen to chimpanzees. *J. Infect. Dis.* **141,** 590–602.
24. Ponzetto, A., Cote, P. J., Popper, H., Hoyer, B. H., London, W. T., Ford, E. C., Bonino, F., Purcell, R. H., and Gerin, J. L. (1984) Transmission of the hepatitis B-virus associated delta agent to the eastern woodchuck. *Proc. Natl. Acad. Sci. USA* **81,** 2208–2212.
25. Ponzetto, A., Negro, F., Popper, H., et al. (1988) Serial passage of hepatitis delta virus in chronic hepatitis B virus carrier chimpanzees. *Hepatology* **8,** 1655–1661.
26. Tai, F., Chen, P., Chang, F., Chen, D. (1993) Hepatitis delta virus cDNA monomer can be used in transfection experiments to initiate viral RNA replication. *Virology* **197,** 137–142.
27. Netter, H. J., Kajino, K., and Taylor, J. M. (1993) Experimental transmission of human hepatitis delta virus to the laboratory mouse. *J. Virol.* **67,** 3357–3362.
28. Smedile, A. and Rizzetto, M. (1992) Orthotopic liver transplantation for chronic viral hepatitis: an overview. *Int. J. Clin. Lab. Res.* **22,** 211–215.
29. Ottobrelli, A., Marzano, A., Smedile, A., et al. (1991) Patterns of hepatitis delta virus reinfection and disease in liver transplantation. *Gastroenterology* **101,** 1649–1655.
30. Smedile, A., Casey, J. L., Cote, P., Rizzetto, M., and Gerin, J. L. (1998) HDV viremia following orthotopic liver transplantation involves a typical HDV virion with an hepatitis B surface antigen envelope. *Hepatology* **27,** 1723–1729.
31. Tassoupoulous, N. C., Kanzelou, M. G., Papatheodoridis, G. V., Kalantzakis, Y. S., and Hatzakis, A. E. (1993) Acute delta hepatitis in Greek parenteral drug abusers. *Proc. Clin. Biol. Res.* **324,** 221–227.
32. Liaw, V. F., Chin, K. W., Chen, C. M., Sheen, I. S., and Huang, M. (1990) Heterosexual transmission of hepatitis delta virus in the general population of an area endemic for hepatitis B virus infection: a prospective study. *J. Infect. Dis.* **162,** 1170–1172.

33. Bonino, F., Caporaso, N., Dentico, P., et al. (1985) Familiar clustering and spreading of delta infection. *J. Hepatol.* **1,** 221–226.
34. Rizzetto, M., Hadziyannis, S., Hansson, B. G., Toukan, A., and Gust, I. (1992) Hepatitis delta virus infection in the world, epidemiological patterns and clinical expression. *Gastroenterol. Intern.* **5,** 18–32.
35. Stroffolini, T., Fenigno, L., Cialdea, L., Catapano, R., Palumbo, F., Noveco, F., Moiraghi, A., Galanti, C., Bernacchia, R., Mele, A., and SIEVA Collaborating Group. (1994) Incidence and risk factors of acute delta hepatitis in Italy, results from a national surveillance system. *J. Hepatol.* **21,** 1123–1126.
36. Navascues, C. A., Rodriguez, M., Sotorrio, N. G., Sala, P., Linares, A., Suarez, A., and Rodrigo, L. (1995) Epidemiology of hepatitis D virus infection. Changes in the last 14 years. *Amer. J. Gastroenterol.* **90,** 198–1984.
37. Sakugawa, H., Nakasone, H., Shokita, H., et al. (1995) Seroepidemiological study of hepatitis delta virus infection in Okinawa, Japan. *J. Med. Virol.* **45,** 312–315.
38. Singh, V., Goenka, M. K., Bhasin, D. K., Kochahar, R., and Singh, K. (1995) A study of delta virus infection in patients with acute and chronic liver disease from Northern India. *J. Virol.* **2,** 151–154.
39. Sjiogren, M. H., Colichon, A., Vildosola, H., Cantella, R., and Romero, J. (1997) Initial and 8 years serologic follow-up of HBV and HDV in resident of a hyperendemic area in the amazon basin. *IX Trien. Intern. Symp. Viral Hep. Liver Dis.*, Rome, April 22–26, 1996 (abstract A289), p. 119.
40. Shakil, A. O., Casy, J. L., Niro, G. A., Hadziyannis, S., Di Bisceglie A. M., Hoofnagle, J. H., and Gerin, J. L., Sequence variability of hepatitis delta virus genome. Analysis of global distribtution of genotypes and subtypes. *Fifth Int. Symp. Hep. Delta Virus Liver Dis.* (abstract), Gold Coast, Australia, August 2–September 3, D1.
41. Hadler, S. C., de Monzon, M., Ponzetto, A., et al. (1984) An epidemic of severe hepatitis due to delta virus infection in Yupca Indians of Venezuela. *Ann. Intern. Med.* **100,** 339–344
42. Hadler, S. C., de Monzon, M. A., Bensabath, G., et al. (1991) Epidemiology of hepatitis delta virus infection in less developed countries. *Proc. Clin. Biol. Res.* **364,** 21–31.
43. Ljunggren, K., Patarroyo, M. E., Engle, R., Purcell, R. H., and Gerin, J. L. (1985) Viral hepatitis in Colombia: a study of the "Hepatitis de Sierra Nevada de Santa Marta." *Hepatology* **5,** 299–304.
44. Cole, S., Gowans, E. J., Macnaughton, T. B., et al. (1991) Direct evidence for cytotoxicity associated with expression of hepatitis delta virus antigen. *Hepatology* **13,** 845–851.
45. Sakugawa, H., Nakasane, H., Kawakami, Y., et al. (1998) *Determination of hepatitis delta virus (HDV)-RNA in asymptomatic cases of HDV infection*, in press.
46. Tang, J. R., Hantz, O., Vitvitski, J. P., Lamelin, R., Parana, R., Cova, L., Lesbordes, J. L., and Trepo, C. (1993) Discovery of a novel point mutation changing the HDAg expression of a delta virus isolate from Central African Republic. *J. Virol.* **74,** 1827–1835.
47. Niro, G. A., Smedile, A., Andriulli, A., Rizzetto, M., Gerin, J. L., and Casey, J. L. (1997) The predominance of hepatitis delta virus genotype I among chronically infected Italian patients. *Hepatology* **25,** 728–734.
48. Niro, G. A., Garubba, M., Casey, J. L., Durazzo, M., Conoscitore, P., Rizzetto, M., and Andriulli, A. (1997) Intrafamilial transmission of HDV is implicated by sequence

analysis of HDV isolated from siblings. *IX Trien. Intern. Symp. Viral Hep. Liver Dis.*, Rome, April 22–26, 1996 (abstract 292) pp. 119.
49. Chen, P. S., Chen, D. C., Chen, C. R., et al. (1988) Delta infection in asymptomatic carriers of hepatitis B surface antigen: low prevalence of delta activity and effective suppression of hepatitis B virus infection. *Hepatology* **8,** 1121–1124.
50. Liaw, Y. F. (1995) Role of hepatitis C virus in dual and triple hepatitis virus infection. *Hepatology* **22,** 1101–1108.
51. Pontisso, P., Ruvoletto, M. G., Fattovich, G., Chemello, L., Gallorini, A., Ruol, A., and Alberti, A. (1993) Clinical and virological profiles in patients with multiple hepatitis virus infections. *Gastroenterology* **105,** 1529–1533.
52. Castillo, I., Bartolome, J., Madejon, A., Melero, M., Porres, J. C., and Carreno, V. (1991) Hepatitis delta virus RNA detection in chronic HBsAg carriers with and without HIV infection. *Digestion* **48,** 149–156.
53. Caredda, F., Antinori, S., Coppin, P., et al. (1991) The influence of human immunodeficiency virus infection on acute and chronic HBsAg-positive hepatitis. *Prog. Clin. Biol. Res.* **364,** 365–375.
54. Caredda, F., Vag, L., Mainini, F., di Marco, A., Nebuloni, M., and Moroni, M., Strong association between hepatitis D virus (HDV) infection and liver cirrhosis in chronic carriers of HBsAg with AIDS. *IX Trien. Intern. Symp. Viral Hep. Liver Dis.*, Rome, April 22–26, 1996 (abstract 285) pp. 118.
55. Caredda, F., Antinori, S., Pastecchia, C., Coppin, P., Ponzetto, A., Rizzetto, M., and Moroni, M. (1989) Presence and incidence of hepatitis delta virus infection in acute HBsAg-negative hepatitis. *J. Infect. Dis.* **159,** 977–979.
56. Saracco, G., Maccagno, S., Caredda, F., Antinori, S., and Rizzetto, M. (1988) Serologic markers with fulminant hepatitis in persons positive for hepatitis B surface antigen. A worldwide epidemiologic and clinical survey. *Ann. Int. Med.* **106,** 380–384.
57. Negro, F. and Rizzetto, M. (1993) Pathobiology of hepatitis delta virus. *J. Hepatol.* **17,** S149–S153.
58. Smedile, A., Niro, G. A., and Rizzetto, M. (1997) Hepatitis D virus, in *Clinical Virology* (Richman, D. D., Whitley, R. J., and Hayden, F. G., eds.), Churchill Livingstone, New York, pp. 1273–1284.
59. Rosina, F., Rocca, G., Giuliani, A., et al. (1995) Natural history of chronic hepatitis D in a cohort of 148 Italian patients. *Fifth Intern. Symp. Hep. Delta Virus Liver Dis.* Gold Coast, Australia, August 28–29, D21.
60. Fattovich, G., Giustina, G., Realdi, G., Schalm, S. W. and the EUROHEP Study Group on Cirrhosis. (1997) Effectiveness of interferon alpha in caucasian patients with viral compensated cirrhosis, in *Viral Hepatitis and Liver Disease* (Rizzetto, M., Purcell, R. H., Gerin, J. L., and Verme, G., eds.), Minerva Medica, Torino, Italy, pp. 787–790.
61. de Man, R. A., Sprey, P. R., Niesters, H. G. M., Heijtink, R. A., Zondervan, P. E., Hop, W., and Schalm, S. W. (1995) Survival and complications in a cohort of patients with anti-delta positive liver disease presenting in a tertiary referral clinic. *J. Hepatol.* **23,** 662–667.
62. Gioustozi, A., Manesis, E. K., and Hadzyiannis, S. (1995) Long-term follow-up of patients with chronic hepatitis D in the community of Archangelos compared to other areas of Greece: survival and development of HCC. *Fifth Intern. Symp. Hep. Delta Virus Liver Dis.*, Gold Coast, Australia, August 28–29, D21.

63. Polo, J. M., Lim, B., Govindarajan, S., and Lai, M. M. C. (1995) Replication of hepatitis delta virus RNA in mice after intramuscular injection of plasmid DNA. *J. Virol.* **69,** 5203–5207.
64. Wesierka-Gadek, J., Penner, E., Hitchaman, E. R., et al. (1990) Antibodies to nuclear lamin C in chronic hepatitis delta virus infection. *Hepatology* **12,** 1129–1133.
65. Amengual, M. J., Catalfamo, M., Pujol, et al. (1989) Autoantibodies in chronic delta virus infection recognize a common protein of 46 kd in rat forestomach basal cell layer and stellate thymic epithelial cells. *Clin. Exp. Immunol.* **78,** 80–84.
66. Zauli, D., Crespi, C., Bianchi, F. B., et al. (1986) Autoimmunity in chronic liver disease caused by hepatitis delta virus. *J. Clin. Pathol.* **39,** 897–899.
67. Crivelli, O., Lavarini, C., Chiaberge, E., et al. (1983) Microsomal autoantibodies in chronic infection with the HBsAg associated delta (d) agent. *Clin. Exp. Immunol.* **54,** 232–238.
68. Philipp, T., Durazzo, M., Trautwein, C., et al. (1994) Recognition of uridine diphosphate glucuronosyl transferases by LKM-3 autoantibodies in chronic hepatitis D. *Lancet* **344,** 578–581.
69. Verme, G., Amoroso, P., Lettieri, G., et al. (1986) A histologic study of hepatitis delta virus liver disease. *Hepatology* **6,** 1303–1307.
70. Saracco, G., Maccagno, S., Caredda, F., Antinori, S., and Rizzetto, M. (1988) Serologic markers with fulminant hepatitis in persons positive for hepatitis B surface antigen. A worldwide epidemiologic and clinical survey. *Ann. Int. Med.* **106,** 380–384.
71. Popper, H., Thung, S. N., Gerber, M. A., et al. (1983) Histologic studies of severe delta agent infection in Venezuelan Indians. *Hepatology* **3,** 906–912.
72. Xia, L., Wang, L., Sun, Z., Harris, C. C. (1997) Interactive effects of p53 tumor suppressor gene and hepatitis B virus in hepatocellular carcinogenesi, in *Viral Hepatitis and Liver Disease* (Rizzetto, M., Purcell, R. H., Gerin, J. L., and Verme, G., eds.), Minerva Medica, Torino, Italy, pp. 484–489.
73. Smedile, A., Lavarini, C., Crivelli, O., et al. (1982) Radioimmunoassay detection of IgM antibodies to the HBV-associated delta (d) antigen; clinical significance in d infection. *J. Med. Virol.* **9,** 131–138.
74. Aragona, M., Macagno, S., Caredda, F., et al. (1987) Serological response to the hepatitis delta virus in hepatitis D. *Lancet* **1,** 478–480.
75. Farci, P., Aragona, M., Crivelli, et al. (1986) Diagnostic and prognostic significance of the IgM antibody to the hepatitis delta virus. *JAMA*, **225,** 1443–1446.
76. Dalekos, G. N., Zervou, E., Karabini, F., and Tsianos, E. V. (1995) Prevalence of viral markers among refugees fron southern Albania: increased incidence of infection with hepatitis A, B and D viruses. *Eur. J. Gastroenterol.* **7,** 553–558.
77. Ponzetto, A., Forzani, B., Forzani, I., D'Urso, N., Avanzini, L., Smedile, A., and Verme, G. (1989) Immunization with hepatitis delta antigen does not prevent superinfection with hepatitis delta virus in the woodchuck. *Gastroenterology* **96,** A646.
78. Karayiannis, P., Saldanha, J., Monjardino Jackson A., Luther, S., and Thomas, H. C. (1993) Immunisation of woodchucks with hepatitis delta antigen expressed by recombinant vaccinia and baculoviruses controls HDV superinfection. *Prog. Clin. Biol. Res.* **382,** 193–199.
79. Rosina, F., Pintus, C., Meschievitz, C., et al. (1991) A randomized controlled trial of a 12-mo course of recombinant Human Interferon-a in chronic delta (Type D) hepatitis: a multicenter study. *Hepatology* **13,** 1052–1056.
80. Farci, P., Mandas, A., Coiana, A., et al. (1994) Treatment of chronic hepatitis D with interferon alfa-2a. *N. Engl. J. Med.* **330,** 88–94.

81. Marzano, A., Ottobrelli, A., Spezia, C., et al. (1992) Treatment of early chronic delta hepatitis with lymphoblastoid alpha interferon: a pilot study. *Ital. J. Gastroenterol.* **24**, 119–121.
82. Rizzetto, M., Verme, G., Recchia, S., et al. (1983) Chronic HBsAg hepatitis with intrahepatic expression of delta antigen: an active and progressive disease unresponsive to immunosuppressive treatment. *Ann. Int. Med.* **98,** 437–441.
83. Arrigoni, A., Ponzetto, A., Actis, G., et al. (1983) Levamisole and chronic delta hepatitis. *Ann. Int. Med.* **98,** 1024.
84. Garripoli, A., Di Marco, V., Cozzolongo, R., et al. (1994) Ribavirin treatment for chronic hepatitis D: a pilot study. *Liver* **14,** 154–157.
85. Zavaglia, C., Bottelli, R., Smedile, A., Iamoni, G., Mondazzi, L., Airoldi, A., Lanzani, F., and Ideo, G. (1996) A pilot study of thymosin-alpha1 therapy for chronic hepatitis D. *J. Clin. Gastroenterol.* **2,** 162–163.
86. Marzano, A., Smedile, A., Rizzetto, M., et al. (1998) Antiviral therapy for the treatment of HBV recurrence in liver transplantation. *J. Hepatol.*, submitted for publication.
87. Korba, B. E., Casey, J. L., and Gerin, J. L. (1995) Antisense oligonucleotides are effective inhbitors of hepatitis delta virus replication in vivo. *Fifth Intern. Symp. Hep. Delta Virus Liver Dis.* (abstract), Gold Coast, Australia, August 2–September 3, D48.
88. Korba, B., Casey, G., and Gerin, J. L. (1997) In vitro models for the antiviral activities of deoxyoligonucleotides against HBV and HDV replication, in *Viral Hepatitis and Liver Disease*, pp. 754–757.
89. Wright, T. L. and Pereira, B. (1995) Liver transplantation for chronic viral hepatitis. *Liver Transplant Surg.* **1,** 30–42.
90. Fagiouli, S., Leandro, G., Bellati, A., Gasbarrini, A., Rapaccini, G. L., Pompili, M., et al. (1996) Liver transplantation in Italy: preliminary 10-year report. *Ital J. Gastroenterol.* **28,** 343–350.
91. Samuel, D., Zignego, A. L., Feray, C., et al. (1995) Long term clinical and virologic outcome after liver transplantation for delta cirrhosis. *Hepatology* **21,** 333–339.
92. Ottobrelli, A., Marzano, A., Smedile, A., et al. (1991) Patterns of hepatitis delta virus reinfection and disease in liver transplantation. *Gastroenterology* **101,** 649–1655.
93. Smedile, A. (1998) Transplantation for chronic delta hepatitis. *J. Hepatol.*, submitted for publication.
94. Smedile, A., Casey, J. L., Cote, P., Durazzo, M., Lavezzo, B., Purcell, R. H., Rizzetto, M., and Gerin, J. L. (1998) Hepatitis D viremia following orthotopic liver transplantation involves a typical HDV virion with an hepatitis B surface antigen envelope. *Hepatology* **27,** 1723–1729.

6

Hepatitis E Virus

Biology, Pathogenesis, Epidemiology, Clinical Description, Diagnosis, and Prevention

Patrice O. Yarbough and Albert W. Tam

1. Hepatitis E Virus

Hepatitis E virus (HEV) is the etiologic agent responsible for the large majority of acute enterically-transmitted non-A hepatitis. While hepatitis E has been documented many times worldwide as major epidemic outbreaks involving thousands of infected individuals with overt disease *(1,2)*, it also accounts for a significant proportion of sporadic hepatitis cases in endemic regions (*1,2*, *see* Table 1). The molecular virology and biology of the recently identified HEV, even with its genome completely cloned and sequenced, have not been fully characterized. Current understanding of HEV virus biology, disease manifestations, mode of pathogenesis, its sero-epidemiology, and prospects for vaccine intervention will be reviewed in these ensuing sections.

2. Biology

2.1. Experimental Models

Retrospective studies in 1980 of epidemic water-borne hepatitis cases in India, using then newly available specific antibody test for detection of acute hepatitis A virus (HAV) infection and finding no serologic proof, rendered convincing evidence for the existence of a novel viral agent responsible for an enterically transmitted form of non-A, non-B hepatitis (ET-NANBH, refs. *3,4*). Experimental transmission of this agent, which has been named hepatitis E virus (HEV), since its molecular identification, to nonhuman primates was first reported in 1983 *(5)*. Cynomolgus macaques

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

Table 1
Epidemiologic Features of Hepatitis E Virus

Enterically transmitted
Subclinical anicteric disease
Self-limited icteric disease, no chronicity observed
Fulminant cases of hepatic failure
High mortality during pregnancy
Epidemic cases in developing countries
Sporadic cases in adults and children in endemic regions
Sporadic cases among travelers in Western countries

were inoculated with fecal suspensions obtained from patients with ET-NANBH in an outbreak in Tashkent of the former Soviet Union. These cynomolgus monkeys developed hepatitis with the recovery of viruslike particles (VLPs), during the course of infection, similar to those observed in the human inoculum. Further, the VLPs were found to be immunoreactive with antibodies from the source patients as well as with sera from the experimentally infected animals. These observations satisfied but one of Koch's postulates for the association of a previously unidentified etiologic agent with a pathological condition or disease. HEV was shown to be disease-associated, being present only in infected sources, and immunogenic, being responsible for the elicitation of an antibody response in exposed individuals capable of specific immune recognition of the agent itself. The last postulate of the ability to isolate in vitro VLPs or infectious genome was not fulfilled until much later (*see* discussion below).

Other nonhuman primates shown to be susceptible to HEV infection include chimpanzees, rhesus monkeys, African green monkeys, tamarins, and owl monkeys (for a review, *see* ref. *2*). The most reliable animal model to date remains the cynomolgus monkey. A 3–8-wk incubation period following virus inoculation has typically been observed prior to onset of liver enzyme elevations *(6–9)*. Unimodal as well as bimodal enzyme changes have been documented. Histopathologic changes include general liver cell degeneration and focal hepatic necrosis with accumulations of blood-derived mononuclear macrophages and activated Kupffer cells *(6,10)*. Peak viremia and virus shedding usually occur during the incubation period and early acute disease phase, just before the peak level of enzyme elevation *(5,10,11)*. Multiple passages of the same virus in the cynomolgus monkeys has resulted in the shortening of the incubation period *(12,13)*, suggesting adaptation of the virus to the host or heightened response to an increased virus load.

Anti-HEV has been detected in wild-caught monkeys and rodents *(14–16)*, and natural infection of domestic pigs and chickens has been reported *(17)*, indicating the zoonotic nature of the virus. These observations could serve to explain the endemicity of certain geographic areas where water sanitation measures are substandard, and humans and animals might at times share a common pool of water supply. Additionally, experimental infection of pigs has been shown *(18)*, and most recently HEV infection of laboratory rats was also reported *(19)*. However, the relative susceptibility of these species to HEV infection has not been systematically determined.

2.2. *Physical Chemical Properties*

The use of immune electron microscopy (IEM) has been the only means allowing for direct visualization of HEV particles. When coated and aggregated by antibodies obtained from sera of human patients during epidemic outbreaks or experimentally inoculated animals during the course of infection, the particles appeared as small, spherical, nonenveloped structures with spikes and indentations on their surfaces. These surface features are similar, though less prominent, to those found on caliciviruses and the Norwalk agents *(20)*, and can be contrasted from the relatively smooth surface of the virions of picornaviruses, including HAV. HEV particles found in feces and bile fluid have been described variously as 27 nm, 27–30 nm, 27–34 nm, and 32–34 nm in diameters *(2,15)*. The variability in the reported particle sizes reflects, not only an actual potential size range of the VLPs, but also the differential degree of antibody-coating and nonidentical measurement techniques being employed in different reporting laboratories. Proteolytic degradation of the virus as it traverses the digestive tract may also be important in determining the eventual size of the particles found in the fecal materials. Single experiments comparing the virion sizes of HEV sourced from geographically diverse locations yielded an average diameter of 30–32 nm *(2,12)*, compared to a size of 28 nm in average diameter for HAV. Rotational enhancement of the antibody-coated virion images suggested that the HEV particles have an icosahedral symmetry *(15)*.

Several lines of evidence have indicated that HEV may be extremely labile and has not survived well in a number of routine laboratory conditions *(6)*. The virus will not tolerate exposure to high concentration of salts, including CsCl, and no VLPs have been recovered from cesium gradients. HEV appeared to degrade also following high-speed pelleting in sucrose gradients or from fecal suspensions. Although appearing to be stable when stored in the vapor phase of liquid nitrogen, it has been reported that HEV is unstable when stored in suspensions at temperatures between –70 and +8°C and disintegrates when subjected to freeze/thaw cycles. The observed labile

nature of HEV is rather ambivalent to its natural existence as a water-transmissible etiologic agent. However, rate-zonal banding of the virus in linear, preformed sucrose gradients yielded sufficiently purified intact 32–34-nm-diameter particles suitable for IEM and animal transmission studies *(21)*. The partially purified intact HEV particles have a calculated sedimentation coefficient of 183S and a buoyant density of 1.29 g/cm^3 in a potassium tartrate/glycerol gradient *(6,12)*. A second peak presumably corresponding to defective particles devoid of a complete RNA genome was found by rate zonal banding sedimenting at 165S.

2.3. *Molecular Cloning*

Initial cloning of HEV was accomplished by two altogether different approaches, namely "plus-minus" differential hybridization screening and immunoscreening based on specific antibody-antigen interaction. While selected stool samples of clinical and experimental specimens were found to be suitable for immune aggregation and IEM analysis, the low virus titer present rendered molecular cloning by conventional techniques impractical. With the advent of polymerase chain amplification (PCR) technology, a method for the nonspecific expansion of heretofore uncharacterized cDNA called Sequence-Independent Single Primer Amplification (SISPA) was developed *(22)*. Such heterogeneous amplification of cDNA molecules irrespective of their sequences, thus overcame the scarcity issue of source materials for molecular biology analysis. The cloning of the virus genome was also facilitated by the finding of intact particles in infectious bile of experimentally infected cynomolgus monkeys *(9,23)*. With a much lower nucleic acid complexity in bile fluid, a differential "plus-minus" hybridization screening strategy using labeled infected and uninfected source probes afforded sufficient sensitivity for initial clone identification from a cDNA library constructed from HEV-Burma strain-infected cynomolgus bile *(23)*. ET1.1 was the first among candidate clones firmly established as virus-specific in origin. Further, strand-specific probes generated from ET1.1 confirmed the single-stranded, positive-sense nature of the virus *(24)*.

The use of immunoscreening for clone identification similarly relied on SISPA-amplified infected source RNA. Two epitope clones, 406.3-2 and 406.4-2, derived from an HEV-Mexico strain cDNA expression library, were determined to be immunoreactive with anti-HEV antibodies contained in convalescent phase ET-NANBH human sera *(25)*. Paneling of the expressed proteins against paired acute- and convalescent-phase documented hepatitis E human sera from geographically diverse localities, as well as preinoculation, acute- and convalescent-phase sera from infected nonhuman primates demonstrated their immunogenicity and thus their viral derivation *(25)*.

With ET1.1 as a molecular probe, a set of overlapping cDNA clones encompassing the entire virus genome of the Burma strain were identified in a series of manipulations analogous to genomic walking *(26)*. The 3' end of the cloned full-length genome was marked by the presence of a polyadenylated tract, while the 5' end viral sequence was first delineated by primer extension analysis and then successfully cloned with the use of an anchored PCR amplification technique *(26)*. Empirically determined genomic sequence totaled approx 7.2 kb with a 3' poly-(A) tail of at least 200–300 residues long *(26)*, the sum of which is in good agreement with a 7.5-kb polyadenylated species detected in Northern blot analysis of HEV-infected liver RNA *(23,25)*. A search of the current Genbank database reveals several other complete or partial HEV sequences that have been reported to date. These isolates include virus characterized from Algeria *(27)*, Chad *(27)*, China *(28–30)*, India *(31,32)*, Mexico *(33)*, Pakistan *(34)*, and the former Soviet Union *(34,35)*.

2.4. Genome Organization

Open reading frame (ORF) analysis of the Burma strain genomic nucleotide sequence reveals the presence of two large ORFs. The first open reading frame (ORF1) extends approx 5.1 kb in length from the 5' end and encodes the nonstructural proteins of the virus *(26)*. Initial alignment of the deduced ORF1 amino acid sequence identified conserved motifs belonging to the viral helicase and RNA-dependent RNA polymerase (RDRP) gene domains *(26,35)*. A subsequent detailed computer comparison with other positive-strand RNA plant and animal viruses uncovered additional conserved functional protein domains *(36)*. The current proposed order of the ORF1 viral nonstructural proteins, beginning at the 5' end, is as follows: a methyltransferase, Y domain, a papain-like cysteine protease, a proline-rich hinge domain, X-domain, RNA helicase, and RNA-dependent RNA polymerase (RDRP) (*see* Table 2). The assignment of these nonstructural genes in ORF1 helps elucidate a 5'-nonstructural, 3'-structural genomic organization for HEV. Initiation of synthesis of the nonstructural polyprotein has yet to be carefully examined. The first potential AUG initiation codon is found at nucleotide 28, giving a relatively short untranslated segment of 27 nucleotides. A second in-frame methionine codon is located another 147 nucleotides downstream. Based on Kozak's rules, it appears the first AUG may be the favored codon for ORF1 transcription initiation.

The second large open reading frame (ORF2) starts at nucleotide 5114 in the plus-1 coding frame, just four nucleotides downstream from the termination codon of ORF1. ORF2 itself extends 1980 nucleotides from the first in-frame methionine at nucleotide 5147 and terminates at nucleotide 7127,

Table 2
Genomic Organization and Functional Gene Domains Oof Hepatitis E Virus

Reading frame and viral protein domain	Putative gene function
ORF1 (nts.[a] 28–5106)	
Methyltransferase	Capping of genomic RNA
Y domain	Unknown biologic role
Papain-like cysteine protease	Proteolytic processing of nonstructural viral proteins
Proline-rich hinge	Flexibility of polyprotein molecule
X domain	Unknown biologic role
Helicase	Winding and unwinding of RNA molecule
RNA-dependent RNA polymerase	Transcription of genomic RNA (and synthesis of subgenomic RNA species?)
ORF2 (plus-1 coding frame, nts. 5114–7126)	
Capsid	Structural protein
ORF3 (plus-2 coding frame, nts. 5106–5474)	
Unassigned	Potential RNA binding activity

[a]nts., nucleotide number of virus genome based on HEV-Burma strain.

yielding a 3' untranslated region of 65 nucleotides immediately upstream of the poly-(A) tail *(26)*. Analysis of the primary sequence of the ORF2-encoded gene product reveals a novel protein similar in its basic amino acid content (clusters of arginine residues near the N-terminal) and electropositive charges to other known capsid proteins of alphaviruses and rubella *(26)*. It has been demonstrated that this hydrophilic, electropositive domain in alphaviruses served to encapsulate the electronegatively charged virion RNA *(37,38)*. Potential N-linked glycosylation sites have also been identified, suggesting the putative capsid is likely a glycoprotein. Epitope 406.3-2, identified by immunoscreening procedure, was mapped to the carboxyl terminus of this protein *(25)*. The antigenicity and diagnostic utilities of the 406.3-2 peptide, reflecting its role as an immunodominant epitope of the viral structural protein, have been repeatedly demonstrated *(25,39)*.

A third open reading frame (ORF3) in the plus-2 coding frame was detected by the mapping of the other immunodominant epitope clone, 406.4-2, to the 3' end of this small ORF of 369 bp in length *(25)*. The ORF3 initiating methionine codon overlaps with ORF1 by a single nucleotide, but the reading frame shares significant overlapping sequences with ORF2, which initiates 41 bp downstream from the start of ORF3 *(26)*. The biologic func-

tion of this 123 amino acid ORF3-encoded protein has yet to be clearly elucidated, even though an RNA binding activity has been reported for a recombinant form of the protein *(40)*.

In addition to the utilization of all three forward reading frames for the expression of its proteins, two subgenomic HEV RNA messages of 3.7 and 2.0 kb have also been detected *(25,26)*. Both of these subgenomic messages were found to be 3' coterminal with the full-length genomic RNA at the poly-(A) tract *(26)*. It has been speculated that the 2.0-kb species might be involved in the transcription and translation of the ORF2-encoded putative capsid protein *(26)*. However, the exact mechanism of the synthesis and functional role of either subgenomic transcript has not been determined.

2.5. *Virus Diversity*

Genetic heterogeneity has been observed among the different strains of HEV cloned thus far. Phylogenetic analysis through sequence comparison indicates the existence of at least two genetic groups for the virus. The HEV-Burma and HEV-Mexico strains appear to represent the most genetically diverse isolates identified to date. Sequence homology between the two strains was calculated to be 74% identity at the nucleotide level for the ORF1 coding region, 81% for ORF2, and 90% for ORF3. Amino acid sequence is most conserved at 93% identical for the ORF2-encoded capsid, while percent identities are 83 and 87 for ORF1 and ORF3 proteins, respectively *(33)*. Of the other strains with known full-length genomic sequences, all are derived from a region in Asia in close proximity to Burma and are found to be very well conserved. The Burma, China, India, and Pakistan strains differ little more than 6% in nucleotide sequence and 2% in amino acid sequence throughout the virus genome. However, even though the genetic distances are not large, these four isolates can be divided into two subgroups of Southeast Asia (Burma and India) and Northern and Central Asia (China and Pakistan) *(41,42)*. A region of sequence hypervaribility has also been detected within ORF1 in the region of the proline-rich hinge domain *(33,34)*. This hypervariable region extends from nucleotide 2002 to 2424, with sequence homology between Mexico and the Asian isolates falling to about 60% nucleotide identity and below 50% amino acid identity *(33,34)*. While the divergence is not as notable among the Asian strains, this segment of the virus genome does appear to be less conserved by comparative analysis of other ORF1 regions. No biologic significance has yet been suggested for the presence of this sequence hypervaribility.

Partial HEV sequences have been reported recently for isolates obtained from outbreaks in Chad and Algeria *(27)*. A region corresponding to the 3' third of ORF2 was cloned by RT-PCR amplification. Limited

sequence comparison of these two African strains indicated they are more closely related to the Asian strains (87–90% nucleotide identity), but nevertheless, they appear phylogenetically to occupy the continuum between the two diverse ends as represented by the Burma and Mexico isolates. Of further interest is the report of another partial cloning of an U.S. isolate from a single case of nonimported hepatitis E *(43)*. PCR-generated sequences from the 5' end of ORF1 and the overlapping region of ORF2/3 suggest they are as distantly related to the Mexico strain as the latter is to the Asian isolates. Much as this appears contrary to the anticipated geographic distribution of HEV diversity; full phylogenetic analysis of the virus has to await the complete cloning and characterization of the United States as well as additional isolates from South America, South Africa, and the Middle Eastern Gulf region where hepatitis E has been documented.

2.6. Serologic Crossreactivity

Whereas genetic heterogeneity is present between different strains of HEV as defined by their country of origin, there presently appears to be only one serotype of the virus identified. Serologic assays available using key antigens from the ORF2-encoded structural protein indicate the ability of these tests to detect acute infection as well as past exposure to HEV by the most divergent strains such as Burma and Mexico. The early use of the immunodominant epitope antigen 406.3-2 in enzyme immunoassays demonstrated its utility in diagnosing hepatitis E cases worldwide *(25)*. Subsequent incorporation of larger versions or the complete recombinant capsid protein yielded several improved, more-specific and more-sensitive assays *(8*,44). Because of high degree of amino acid sequence homology in the viral structural protein among known strains, it is not surprising that the use of a single key ORF2 antigen is capable of detecting them all. The ORF3-encoded 406.4-2 epitope antigen has also been found useful in the diagnosis of hepatitis E infection, especially among experimentally inoculated nonhuman primates, where host and species adaptation may play a role *(25)*. However, development of improved diagnostic assays with the incorporation of large recombinant ORF2 proteins may have lessened the requirement of the ORF3 antigen.

In the past few years, several reports have pointed to the possibility of the existence of either an atypical strain of HEV or a yet-to-be identified enteric agent. One atypical strain was determined to be similar morphologically (particle size and shape) to the Burma strain and caused disease in animal transmission studies but was found to be serologically unreactive to antisera known to recognize the prototype HEV strains *(45)*. A separate report presented evidence for hepatitis cases from at least one water-borne epidemic in India that were not detectable by currently configured HEV

diagnostics *(46)*. It is not clear if further improved HEV serodiagnosis would be capable of detecting a minor but cross-reactive epitope, but these findings suggest conceivably the presence of separate serotype(s) of HEV.

2.7. Viral Replication

The study of the biology of HEV has been hampered by a lack of a cell culture system capable of propagating the virus proficiently in vitro. Nothing is known about the cellular receptor to which HEV binds to gain entry into susceptible cells. The mechanisms by which uncoating, packaging, and transport of the virus occur are unknown. Questions also remain as to the strategies used in infected cells for virus genome replication and protein processing. In vitro transcription and translation of ORF1 nonstructural proteins has not yielded much useful information (A. W. Tam, unpublished data). Recent reports of expression of ORF2-encoded capsid in the baculovirus system indicated posttranslational processing of the protein, but it is not clear if the proteolytic modifications are biologically relevant or only specific to the insect cell expression system used *(47,48)*.

Growing of two different strains of HEV in cultivated cells have been reported. Fetal rhesus monkey kidney (FRhK-4) cells, cocultured with primary cynomolgus kidney cells isolated from animals experimentally infected with the Osh, Russia HEV strain, were found to harbor HEV even after multiple passages *(49)*. A Chinese strain (87A) was also reported to be propagated in human embryo lung diploid cells *(50,51)*. Recovered virus was found to be reactive with anti-HEV sera obtained from HEV-infected human patients and nonhuman primates. Large numbers of virus particles in the form of a crystalline array in the cytoplasm of infected cells was observed under electron microscopy. Cytopathic effects on the infected cells were also described. As both of these observations have not been attributed previously to HEV infected cells, this finding of HEV replication awaits independent confirmation. More recently, HEV was shown to be propagated and produced in vitro from infected primary cynomolgus hepatocytes maintained in long-term culture using a serum-free medium formulation *(52)*. This hepatocyte culture system has previously been used to successfully replicate hepatitis C virus (HCV) and hepatitis D virus (HDV) *(53–57)*. Replicative negative-strand viral RNA was directly demonstrated in the infected cells by a highly strand-specific reverse-transcription PCR *(52,54)*. Presence of virus particles shed into the culture medium was illustrated both biochemically with the detection of viral genomic RNA and physically with the aggregation of HEV VLPs (*see* Fig. 1). Consistent in vitro infection of primary hepatocytes and apparent passaging of infectious culture medium have also been reported (A. W. Tam, et al., manuscript in preparation), hence fulfilling the

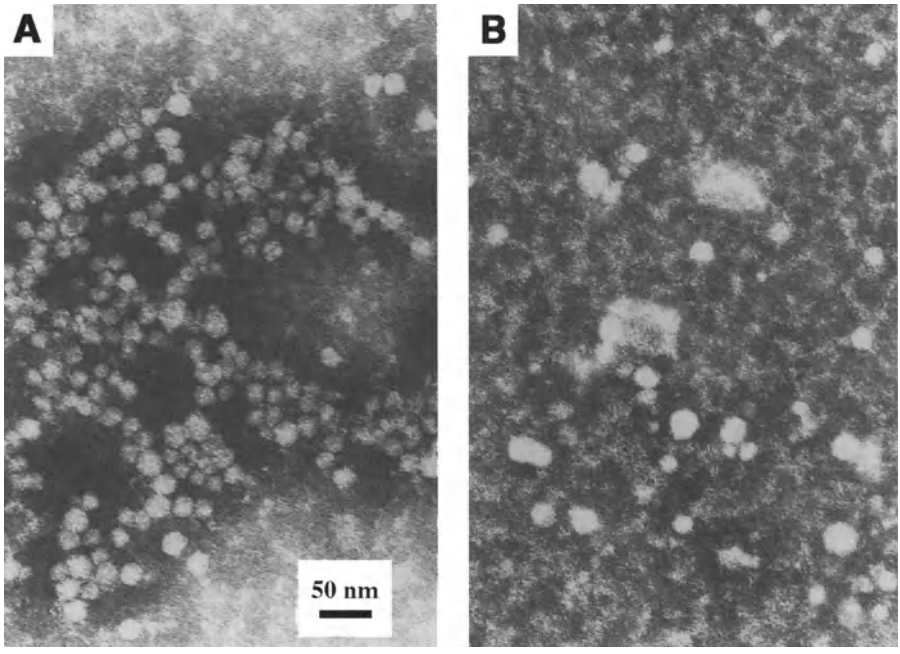


Fig. 1. IEM aggregation of HEV VLPs produced in vitro. Immune aggregation of HEV VLPs secreted into tissue culture medium by in vivo-infected primary cynomolgus hepatocytes that had been isolated and placed in long-term culture. **(A)** Convalescent phase anti-HEV antibodies from a cynomolgus macague experimentally infected with HEV were used to aggregate viruslike particles produced in vitro by cultured primary hepatocytes isolated from an in vivo infected cyno with clinical signs of disease. The culture media were concentrated and treated with formalin to inactivate the virus and to preserve virus particle structure. Clusters of HEV VLPs with an antibody-coating appearing as more densely staining materials could be observed, whereas similar aggregates of viruslike particles were not detected with the use of preimmune serum antibodies from the same animal **(B)**. (From ref. *52*. Used with permission from *Virology*.)

last of the Koch's postulates. Further development of the hepatocyte culture system to grow HEV should provide an useful tool to study the replicative process of the virus.

3. Epidemiology

3.1. Epidemics in Developing Countries

HEV infection is a significant cause of morbidity and mortality because of the large number of cases involved in endemic regions and the high mor-

tality rates among pregnant women. Hepatitis E was first documented in New Delhi, India, in 1955. Over 29,000 cases of icteric hepatitis were reported following fecal contamination of the public drinking water *(58)*. Epidemics in Asia and Africa have since been reported in Nepal, Pakistan, Burma, the former Soviet Union, India, Borneo, Somalia, Sudan, and China *(12)*. Examples of the large number of cases are: 20,000 icteric cases in Mandalay, Burma in 1976; 10,000 cases in Kathmandu Valley, Nepal in 1973; and 79,000 jaundiced people in Kanpur, India in 1991. The only documented outbreak of hepatitis E reported in North America occurred in Mexico in 1986 *(59)*. Over 200 cases, all icteric with additional signs of acute viral hepatitis, were recognized in two neighboring towns south of Mexico City. Virus-like particles recovered from stool samples from these Asian, African, and Mexican cases suggest that one virus was the responsible agent for these cases of entericallly transmitted hepatitis. In the outbreaks listed here, hepatitis E was associated with the ingestion of fecally contaminated water. Control measures that focus on making the water supply safe are the first line of defense.

3.2. *Sporadic Cases in Endemic Regions*

Cyclic outbreaks of hepatitis E have been observed in tropical areas of Asia and Africa, usually occurring after seasonal flooding. In these HEV-endemic regions there are cases of hepatitis E that occur between the epidemic cycles at a low level, in the form of sporadic hepatitis. HEV was found to be the cause of acute sporadic hepatitis in Sudanese children *(60)* and in children living in Cairo, Egypt *(61)*. In sporadic hepatitis E cases, the reservoir of HEV between cycles is often not known nor is it clear how circulating virus may lead to ensuing epidemics that favor the adult population. In the earlier mentioned cases, there was no evidence of person-to-person transmission, nor was the primary source of virus identified. In spite of the limited understanding of the cause of sporadic hepatitis E in endemic regions, the virus is implicated in causing more than 50% of the sporadic acute viral hepatitis cases worldwide *(12)*.

3.3. *Cases Among Travelers in Developed Countries*

All confirmed cases of hepatitis E in North America and Western Europe have been traced to immigrants and tourists from countries where HEV is endemic. HEV was judged the cause of acute hepatitis in two English patients that had traveled abroad to India *(62)*. The two patients had biochemical evidence of hepatitis and were found to be antibody positive for the IgM class of antibody. In the Netherlands, there were several cases of hepatitis E among patients who became acutely ill after traveling to

Bangledesh, the Middle East, and Somalia *(63)*. Patient serum was positive for IgM antibody to HEV. Following jaundice that was reported as severe, the patients resolved disease. In 1992, a case of hepatitis E in a U.S. traveler to Pakistan was confirmed by serologic assay *(64)*. One month after returning from Pakistan, the jaundiced male, with no evidence of acute hepatitis A or B, tested IgM antibody positive for acute phase antibody to HEV. Follow-up sera taken 3 yr and 4 yr after acute disease still contained high titer IgG antibodies to HEV.

These "imported cases" confirm the global distribution of the virus and demonstrates that hepatitis E is a worldwide public health problem. Even though travelers to HEV-endemic regions are at low risk of infection, HEV infection should be considered in all persons that travel abroad and develop symptoms of acute hepatitis in the absence of serologic markers for hepatitis A, B, or C.

4. Clinical Description

4.1. Clinical Manifestation

The clinical profile for HEV infection is similar to that of other forms of acute viral hepatitis. In a comparison of the clinical manifestations in patients with hepatitis E and hepatitis A *(65)*, laboratory tests for serum ALT, AST, and bilirubin were typical of acute hepatitis and indistinguishable from each other. Only serologic assays proved specific diagnosis. Hepatitis E usually becomes apparent after an incubation period of 2–9 wk. The average period is 6 wk, longer than the duration for hepatitis A *(4)*. Hepatitis E patients typically complain of fatigue, pain, and gastrointestinal upsets. The symptoms of infection are chills, fever, nausea, vomiting, diarrhea, joint pain, headache, and abdominal enlargement *(4*,65). These preicteric phase symptoms usually occur up to 1 wk to 10 d before the onset of jaundice. The icteric phase follows for 15–40 d and occurs with jaundice, dark urine and clay-colored stools. Jaundice and elevated serum ALTs last on average for 1–6 wk. During this time necro-inflammatory changes in the liver are consistent with acute virus hepatitis. Common histologic changes include focal necrosis, cellular and lobular cholestasis, portal inflammation, and hepatocyte ballooning *(58)*. Upon recovery, biopsy returns to normal and liver enzyme tests return to baseline. The time of convalescence varies from 3 to 14 wk with a mean of 8 wk *(65)*.

4.2. Subclinical and Clinical Cases

The extent of hepatitis E infection can be subclinical, acute, or fulminant stage hepatitis. Some persons infected with HEV are anicteric and free

of classical symptoms of hepatitis. In these cases, HEV infection can be confirmed by the presence of viral RNA in fecal specimens, with or without seroconversion, in the absence of jaundice. In sero-epidemiologic studies in Kathmandu and Abbottabad, the subclinical infection rate reported was higher than that observed for clinically overt disease *(66)*. The data from the Abbottabad study suggest that the presence of measurable antibody directed to HEV conferred protection from disease and reinfection. The range of HEV infection was defined in a patient population of 2–76 yr of age in Kashmir, India *(67)*. Acute hepatitis E, as defined by antibody response, was most prevalent in patients 20–39 yr old. None of the children 10 yr old or younger displayed any signs of infection. In a study in Egypt *(68)*, children of ages 2 mo to 15 yr that were hospitalized with acute hepatitis (accompanied by jaundice and elevated serum ALTs) were tested for antibody to HEV. Sera from 15 of 36 *(42%)* patients were antibody positive for HEV IgG. Diagnosis of acute hepatitis E was confirmed in 40% of the IgG positive sera by acute-phase IgM antibody. Hepatitis E, although more likely to be subclinical, anicteric, and symptom free in infants and young children, indeed causes acute illness in children. During epidemics of hepatitis E, the clinical attack rate averages 5%, with the most severe disease being observed in young adults *(10)*. Usually, hepatitis E is self-limiting with full recovery and no progression to chronic liver disease. One of the striking differences between the course of disease in HAV infections and HEV infections is the higher case fatality rate in hepatitis E, primarily caused by the progression to fulminant hepatic failure. Among 562 cases of hepatitis E in Hetian City, China, 9% progressed to fulminant hepatitis *(69)*. Of these 52 cases: 48% were pregnant women, 19% nonpregnant women, and 33% were men. The mortality rate was between 16 and 20%. Fulminant hepatitis E occurs in men and women but is mostly associated with high mortality rates in third-trimester pregnancy. The timing of fulminant hepatitis during pregnancy will be discussed in a later section.

5. Pathogenesis

5.1. Temporal Patterns of Viral RNA, Antigens, and Antibodies

In studies with experimentally infected monkeys, inoculation with HEV lead to necro-inflammatory changes in the liver tissue coexistent with elevation of serum aminotransferases. HEV enters the body through the intestine, enters the blood from the enteric tract and via the portal vein infects the hepatocytes. The virus replicates in the liver cells, is released from the hepatocytes into the bile and subsequently excreted in the feces *(15)*. The

course of disease is marked by virus excretion in the feces, viremia, liver enzyme elevation (ALTs), and seroconversion, followed by the clearing of the virus and resolution of disease.

For infections in humans, there is not enough data to thoroughly characterize the pattern of virus excretion. The limited data comes from two human self-inoculation studies. A stool suspension from an HEV-infected patient in India was taken orally by a volunteer in 1990, and the clinical course of infection was reported *(70)*. HEV was detected in the serum and feces 22 d and 34 d respectively after self-inoculation. Anicteric symptoms of hepatitis began 30 d postinoculation (p.i.). Icterus began 8 d later and lasted for more than 80 d. IgM and IgG were detectable at the time of onset of illness. IgG antibody was detected 41 d after infection and ALT elevations peaked 5 d later and persisted for 10 wk. Although the hepatitis was quite severe as ascertained by the prolonged jaundice, the volunteer did completely recover with no indication of chronic liver disease. The earliest human volunteer report dates back to 1983 in a clinical case of hepatitis from ingestion of what is now know as the Asian strain HEV *(5,71)*. The source of virus was acute phase stool suspensions from jaundiced persons infected during an epidemic of hepatitis E in Central Asia. In this volunteer study, virus-like particles of 27–30 nm were recovered in the feces as early as 28 d p.i.; this was prior to the elevation of serum ALTs at 36 d p.i. The virus collected on day 42 p.i. was subsequently transmitted to two cynomolgus macaques. The pathogenecity observed in these monkeys marked the beginning of the use of these animals as a model system to study hepatitis E. Although the pattern of hepatitis in monkeys is the same as that observed in humans, in people the incubation time is longer and the diseased state varies from moderate to severe.

5.2. Mortality and Morbidity in Adults

Hepatitis E has often been referred to as a disease of young adults. Overt disease has been observed in children *(68)* although illness predominantly occurs in infected young adults. A study among school children, with enterically transmitted non-A, non-B hepatitis in Pune, India, suggested that the ratio of icteric and nonicteric illness in children is 1:4 *(72)*. It is presumed that most of the hepatitis E in children manifests itself as subclinical disease. During epidemics of hepatitis E, the highest reported attack rate has typically been in adults aged 15–40 yr *(58)*. This has been a consistent observation among epidemics of hepatitis E in various geographical locations. In the New Delhi, India epidemic of 1955, the incubation period averaged 40 d. There were more than 29,000 cases of icteric hepatitis, representing 2.3% of the susceptible population. Most of the clinically ill persons were young

adults aged 15–39 yr. Approximately 1–2% of hospitalized patients from the general population died. In the South Xinjian epidemic of 1986–1988, hepatitis E primarily caused disease in young adults with 90% of the patients showing acute symptoms of disease followed by jaundice *(73)*. Hepatitis was severe in 10% of the cases. In the well-characterized outbreak of hepatitis E in Huitzilla and Telixtac, Mexico in 1986 *(59)*, the overall attack rate was 5–6%. However, more than 80% of the icteric cases were in persons older than 15 yr of age. The highest attack rate was 11–13% in the group aged 15–24 yr. In the hepatitis E epidemic in Hetian City in the fall of 1987 *(69)*, 75% of the patients seen were aged 15–40 yr. Of these, 87% developed icteric hepatitis. The overall case fatality rate was 1.4%. Fulminant hepatitis occurred in 10.7% of the patients; of these 33% were males, 19% were nonpregnant females, and 48% were pregnant women.

5.3. Mortality in Pregnant Women

While the overall mortality for hepatitis E in the general population is estimated at 0.5–1.0%, the observed mortality in pregnant women has been reported to rise as high as 20% *(12)*. This significant increase in mortality is primarily influenced by the high rate of fulminant hepatitis in pregnant women during the third trimester *(58)*. Although there is no proven explanation for progression to fulminant hepatitis in pregnancy, this observation is a characteristic feature of the non-A, non-B enterically transmitted viral hepatitis that we now know is attributed to HEV infection. In the 1981 outbreak of hepatitis E in Kashmir, India *(74)*, it was pregnant women that suffered the most severe illness. The attack rate was 2–3% for men and nonpregnant women; the attack rate and morbidity rate was 17.3% for pregnant women. Fulminant hepatic failure occurred in 22% of the pregnant women and developed only in the third trimester. In the Xinjian, China epidemic of 1986–1988, the mortality rate was 10–20% in pregnant women *(73)*. Mortality was proportional to the length of the pregnancy. In the first trimester, mortality was reported at 1.5%, in the second trimester it was 8.5%, and in the third trimester, 39 of 186 (21%) pregnant women died. During the hepatitis E epidemic in Hetian City, China *(69)*, the case fatality rate was highest among pregnant women at 5.2%. There were 58 pregnant women that developed acute hepatitis; more than one half were in the final trimester. Of these pregnancies, there were a total of 14 deaths by abortion, stillbirth, or premature delivery. There were a total of 25 pregnant women who developed fulminant hepatitis; 19 of these were in their third trimester. Three (15.7%) of the women in the final stage of pregnancy had hepatic failure and died.

The mechanism for fulminant hepatic failure during pregnancy is unknown. Poor nutrition has often been suggested as a basis for the severe

liver dysfunction observed in pregnant women in developing countries. However, there are no conclusive scientific data to substantiate the claims for HEV infections. Endotoxin-mediated cytotoxicity has also been proposed as a cause for the fulminating disease. This hypothesis, reviewed by Purcell and Ticehurst *(58)*, is not proven but provides one plausible explanation for the characteristic mortality in pregnant women. Hypothetically, hepatitis E damages the sinusoidal cells, which in turn fail to protect the hepatocytes from injury by endotoxins released by bacteria in the intestinal tract. The sensitivity of pregnant women to an endotoxin-mediated effect may explain increased severity of disease. Currently there is no animal model to study fulminant hepatitis E. The course of disease in experimentally infected pregnant and nonpregnant rhesus macaques were very similar *(75)*. There was no histopathologic, serologic, or biochemical evidence of more acute disease in the pregnant female monkeys. Nor was there any evidence of HEV infection in the newborn offspring.

5.4. Modes of Transmission

HEV, like HAV, is spread through the fecal-oral route, usually through contaminated water sources. Unlike hepatitis A, person-to-person transmission does not appear to routinely occur *(3,76)*. Since there is no evidence that HEV leads to a chronic carrier state, blood transfusions are not thought to be an important medium of transmission. There is, however, a remote possibility that the virus could be transmitted by blood donated during subclinical infections or during a viremic stage that occurs prior to the appearance of clinical symptoms. The studies from multitransfused hemophiliacs *(77)* show that the risk of HEV being transmitted through the blood supply is minimal.

Although pregnant women and their unborn babies are at the highest risk of mortality following HEV infection, very little is currently known about transmission of HEV from infected mothers to their babies. Vertical transmission has recently been demonstrated in infants born to mothers infected with HEV in their third trimester during an outbreak in South Kashmir, India *(78)*.

Blood specimens were taken from eight mothers during illness and at the time of delivery; cord blood and blood specimens were taken from their babies at birth and at 6 mo later. All specimens were assayed for serum ALTs, bilirubin, IgM and IgG antibodies to HEV, and HEV RNA by RT-PCR. HEV RNA was detected in acute phase sera from five of the mothers; four of the mothers were diagnosed with fulminant hepatitis, one mother died during delivery. HEV RNA was detected in cord or birth blood of five infants; four of these infants were born of mothers with fulminant hepatic

failure and two died within 1 d of birth. There was serologic and clinical evidence of HEV infection in all five of the infants suspected of HEV infection by vertical transmission. Research to define the risk factors associated with the mechanism for fulminant hepatic failure during pregnancy is desperately needed.

6. Prototype Vaccine for Hepatitis E

6.1. Prospects for Prevention

A vaccine for HEV could prevent large outbreaks in developing countries, reduce the morbidity and mortality in pregnant women, and provide protection to persons that travel to HEV-endemic regions. The cynomolgus macaque is susceptible to hepatitis E virus infection and is the best animal model for the overall course of disease after viral infection *(79)*. It is well established that within 2 wk following experimental infection, HEV particles are found in the bile and feces. Pathologic changes in the liver and rises of serum ALTs, the secondary signs of active infection, are observed within 4 wk of experimental infection. The succeeding antibody response to HEV marks the clearing of the virus and resolution of hepatitis. The overall course of disease in infected monkeys is very similar to that of naturally infected humans. The cynomolgus macaque has thus been a suitable model to investigate the efficacy of various candidate recombinant vaccines.

6.2. Experimental Vaccination in Nonhuman Primates

Analysis of the primary sequence of ORF2 of HEV revealed a novel protein with characteristics similar to that of other viral capsid proteins *(26)*. Antigenicity studies showed that the ORF2 contains immunodominant epitopes that are recognized by human sera from naturally infected persons *(25)*. The development of a subunit vaccine for hepatitis E has been based on the 660 amino acid ORF2 structural protein of HEV believed to encode the capsid. The earliest report described the immunization of cynomolgus macaques with C2, a truncated portion of the HEV Burma strain ORF2 expressed in *E. coli (80)*. This protein provided full protection against homologous wild-type challenge with the HEV Burma isolate; however, the monkey challenged with the heterologous HEV Mexico isolate was protected from hepatitis but still showed evidence of virus replication. A later study utilized a larger portion of the ORF2 expressed by recombinant baculoviruses *(81)*. The protein product known as 55K contains amino acids 112–609 of ORF2 from the HEV Pakistan isolate. After one- and two-dose vaccination, the monkeys were challenged with 1000–10,000 cynomolgus 50% infectious doses (CID_{50}) of the homologous Pakistan isolate of HEV.

All the vaccinated monkeys were protected from hepatitis. Animals that receive a single dose of vaccine excreted virus to the feces. Animals that received a double dose of vaccine were completely free of any clinical signs of virus infection. A titration study employing rhesus monkeys defined the minimum dose of 55K required as a vaccine to protect against homologous and heterologus HEV challenge *(47)*. Animals given as little as 0.4 μg of recombinant 55K protein were protected against hepatitis, although the presence of virus in feces verified HEV infection and limited replication after homologous challenge and the heterologous Mexico HEV challenge dose.

In an independent study, we used r62K as an immunogen in the experimental vaccination of cynomolgus monkeys *(82)*. This protein from ORF2 of the HEV Burma isolate was purified from recombinant baculoviruses that were reengineered to contain amino acids 112–660 of the ORF2 *(83)*. The antigenic properties exhibited by the protein are conceivably caused by a conformational structure retained by this larger protein product expressed in baculoviruses. Cynomolgus monkeys were immunized with r62K and challenged with wild-type virus of the divergent HEV Mexico strain. Postchallenge, there was no sign of HEV infection by antigen in the liver in any of the three vaccinated animals. There was delayed and transient presence of viral RNA in the feces of only one of the three animals indicating limited "breakthrough" viral replication. There was no biochemical or histopathologic evidence for hepatocellular damage in any of the immunized animals. Vaccination conferred protection against hepatitis in all animals. The effectiveness of the r62K protein to protect against disease and infection in cynomolgus monkeys is attributed to the retaining of the amino acid sequences at the 3' end of the ORF2 previously postulated to encode a conformation epitope. In vitro neutralization studies are in progress to test this hypothesis (A. W. Tam et al., unpublished observations).

References

1. Bradley, D. W. (1992) Hepatitis E: epidemiology, aetiology, and molecular biology. *Med. Virol.* **2,** 19–28.
2. Krawczynski, K. (1993) Hepatitis E. *Hepatology* **17,** 932–941.
3. Khuroo, S. M. (1980) Study of an epidemic of non-A, non-B hepatitis. Possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. *Amer. J. Med.* **68,** 818–824.
4. Wong, D. C., Purcell, R. H., Sreenivasan, M. A., Prasad, S. R., and Pavri, K. M. (1980) Epidemic and endemic hepatitis in India: evidence for non-A/non-B hepatitis virus etiology. *Lancet* **2,** 876–878.
5. Balayan, M. S., Andjaparidze, A. G., Savinskaya, S. S., Ketiladze, E. S., Braginsky, D. M., Savinov, A. P., and Poleschuk, V. F. (1983) Evidence for a virus in non-A/non-B hepatitis transmitted via the fecal-oral route. *Intervirology* **20,** 23–31.

6. Bradley, D. W., Krawczynski, K., Cook, E. H., Jr., McCaustland, K. A., Humphrey, C. D., Spelbring, J. E., Myint, H., and Maynard, J. E. (1987) Enterically transmitted non-A, non-B hepatitis: Serial passage of disease in cynomolgus macaques and tamarins and recovery of disease-associated 27 to 34-nm viruslike particles. *Proc. Natl. Acad. Sci. USA* **84,** 6277–6281.
7. Ticehurst, J., Rhodes, L. L., Krawczynski, K., Asher, L. V. S., Engler, W. F., Mensing, T. L., Caudill, J. D., Sjogren, T. L., Hoke, C. H., LeDuc, J. W., Bradley, D. W., and Binn, L. N. (1992) Infection of owl monkeys *(Aotus trivirgatus)* and cynomolgus monkeys *(Macaca fascicularis)* with hepatitis E virus from Mexico. *J. Infect. Dis.* **165,** 835–845.
8. Tsarev, S. A., Tsareva, T. S., Emerson, S. U., Kapikian, A. Z., Ticehurst, J., London, W., and Purcell, R. H. (1993) ELISA for antibody to hepatitis E virus (HEV) based on complete open-reading frame-2 protein expressed in insect cells: Identification of HEV infection in primates. *J. Infect. Dis.* **168,** 369–378.
9. Uchida, T., Win, K. M., Suzuki, K., et al. (1990) Serial transmission of a putative causative virus of enterically transmitted non-A, non-B hepatitis to Macaca fascicularia and Macaca mulatta. *Japan J. Exp. Med.* **60,** 13–21.
10. Bradley, D. W., Krawczynski, K., Cook, E. H., Jr., McCaustland, K. A., Robertson, B. H., Humphrey, C. D., Kane, M. A., Spelbring, J., Weisfuse, I., Andjaparidze, A., Balayan, M., Stetler, H., and Velazquez, O. (1988) Enterically transmitted non-A, non-B hepatitis: Etiology of disease and laboratory studies in nonhuman primates, in *Viral Hepatitis and Liver Disease* (Zuckerman, A. J., ed.), Alan R. Liss, New York, pp. 138–147.
11. Tsarev, S. A., Emerson, S. U., Tsareva, T. S., Yarbough, P. O., Lewis, M., Govindarajan, S., Reyes, G. R., Shapiro, M., and Purcell, R. H. (1993) Variation in course of hepatitis E in experimentally infected cynomolgus monkeys. *J. Infect. Dis.* **167,** 1302–1306.
12. Bradley, D. W. (1990) Enterically-transmitted non-A, non-B hepatitis. *Brit. Med. Bull.* **46,** 442–461.
13. Bradley, D. W. (1990) Hepatitis non-A, non-B viruses become identified as hepatitis C and E viruses, in *Progress in Medical Virology*, vol. 37 (Melnick, J. L., ed.), Karger, Basel, pp. 101–135.
14. Balayan, M. S. (1991) HEV infection: Historical perspectives, global epidemiology, and clinical features, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), Williams and Wilkins, Baltimore, pp. 498–501.
15. Ticehurst, J. (1991) Identification and characterization of hepatitis E virus, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), Williams and Wilkins, Baltimore, pp. 501–513.
16. Karetnyi, Y. V., Dzhumalieva, D. I., Usmanov, R. K., Titova, I. P., Litvak, Y. I., and Balayan, M. S. (1993) Probable involvement of rodents in the spread of viral hepatitis. *Zh. Mikrobiol. Epidemiol. Immunobiol.* **4,** 52–56.
17. Clayson, E. T., Snithan, R., Ngarmpochana, M., Vaughn, D. W., and Shrestha, M. P. (1996) Evidence that the hepatitis E virus (HEV) is a zoonotic virus: detection of natural infections among swine, rats, and chickens in an area endemic for human disease, in *Enterically-Transmitted Hepatitis Viruses* (Buisson, Y., Coursaget, P., and Kane, M., eds.), La Simarre, Tours, pp. 329–335.
18. Balayan, M. S., Usmanov, R. K., Zamyatina, N. A., Djumalieva, D. I., and Karas (1990) Brief report: experimental hepatitis E infection in domestic pigs. *J. Med. Virol.* **32,** 58–59.

19. Maneerat, Y., Clayson, E. T., Khin, S. A., Myint, G., Young, D., and Innis, B. L. (1996) Experimental infection of the laboratory rat with the hepatitis E virus. *J. Med. Virol.* **48,** 121–128.
20. Cubitt, W. D., Blacklow, N. R., Hermann, J. E., Nowak, N. A., Nakata, S., and Chiba, S. (1987) Antigenic relationships between human caliciviruses and Norwalk virus. *J. Infect. Dis.* **56,** 806–814.
21. Bradley, D. W., Andjaparidze, A., Cook, E. H., Jr., McCaustland, K. A., Balayan, M., Stetler, H., Velazquez, O., Robertson, B., Humphrey, C., Kane, M., and Weisfuse, I. (1988) Etiologic agent of enterically-transmitted non-A, non-B hepatitis. *J. Gen. Virol.* **69,** 731–738.
22. Reyes, G. R. and Kim, J. P. (1991) Sequence-independent, single-primer amplification (SISPA) of complex DNA populations. *Mol. Cell Probes* **5,** 473–481.
23. Reyes, G. R., Purdy, M. A., Kim, J. P., Luk, K. C., Young, L. M., Fry, K. E., and Bradley, D. W. (1990) Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science* **247,** 1335–1339.
24. Reyes, G. R., Huang, C. C., Yarbough, P. O., Young, L. M., Tam, A. W., Moeckli, R. A., Yun, K. Y., Nguyen, D., Smith, M. M., Fernandez, J., Guerra, M. E., Kim, J. P., Luk, K. A., Krawczynski, K., Purdy, M. A., Fry, K. E., and Bradley, D. W. (1991) Hepatitis E virus (HEV): epitope mapping and detection of strain variation, in *Viral Hepatitis C, D., and E* (Shikata, T., Purcell, R. H., and Uchida, T., eds.), Elsevier, Amsterdam, pp. 237–245.
25. Yarbough, P. O., Tam, A. W., Fry, K. E., Krawczynski, K., McCaustland, K. A., Bradley, D. W., and Reyes, G. R. (1991) Hepatitis E virus: identification of type-common epitopes. *J. Virol.* **65,** 5790–5797.
26. Tam, A. W., Smith, M. M., Guerra, M. E., Huang, C. C., Bradley, D. W., Fry, K. E., and Reyes, G. R. (1991) Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* **185,** 120–131.
27. van Cuyck-Gandre, H., Zhang, H. Y., Clements, N. J., Cohen, S. G., Caudill, J. D., Coursaget, P., Buisson, Y., Warren, R. L., and Longer, C. F. (1996) Partial sequence of HEV isolates from North Africa and Pakistan: comparison with known HEV sequences, in *Enterically-Transmitted Hepatitis Viruses* (Buisson, Y., Coursaget, P., and Kane, M., eds.), La Simarre, Tours, pp. 301–310.
28. Aye, T. T., Uchida, T., Ma, X. Z., Iida, F., Shikata, T., Zhuang, H., and Win, K. M. (1992) Complete nucleotide sequence of a hepatitis E virus isolated from the Xinjiang epidemic (1986–1988) of China. *Nucl. Acid Res.* **20,** 3512.
29. Bi, S. L., Purdy, M. A., McCaustland, K. A., Margolis, H. S., and Bradley, D. W. (1993) The sequence of hepatitis E virus isolated directly from a single source during an outbreak in China. *Virus Res.* **28,** 233–247.
30. Yin, S. R., Purcell, R. H., and Emerson, S. U. (1994) A new Chinese isolate of hepatitis E virus: comparison with strains recovered from different geographical regions. *Virus Genes* **9,** 23–32.
31. Ray, R., Jameel, S., Manivel, V., and Ray, R. (1992) Indian hepatitis E virus shows a major deletion in the small open reading frame. *Virology* **189,** 359–362.
32. Panda, S. K., Nanda, S. K., Zafrullah, M., Ansari, I., Ozdener, M. H., and Jameel, S. (1995) An Indian strain of hepatitis E virus (HEV): cloning, sequence, and expression of structural region and antibody responses in sera from individuals from an area of high-level HEV endemicity. *J. Clin. Microbiol.* **33,** 2653–2659.

33. Huang, C. C., Nguyen, D., Fernandez, J., Yun, K. Y., Fry, K. E., Bradley, D. W., Tam, A. W., and Reyes, G. R. (1992) Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). *Virology* **191,** 550–558.
34. Tsarev, S. A., Emerson, S. U., Reyes, G. R., Tsareva, T. S., Legters, L. J., Malik, I. A., Iqbal, M., and Purcell, R. H. (1992) Characterization of a prototype strain of hepatitis E virus. *Proc. Natl. Acad. Sci. USA* **89,** 559–563.
35. Fry, K. E., Tam, A. W., Smith, M. M., Kim, J. P., Luk, K. C., Young, L. M., Piatak, M., Feldman, R. A., Yun, K. Y., Purdy, M. A., McCaustland, K. A., Bradley, D. W., and Reyes, G. R. (1992) Hepatitis E virus (HEV): strain variation in the nonstructural gene region encoding consensus motifs for an RNA-dependent RNA polymerase and an ATP/GTP binding site. *Virus Genes* **6,** 173–185.
36. Koonin, E. V., Gorbalenya, A. E., Purdy, M. A., Rozanov, M. N., Reyes, G. R., and Bradley, D. W. (1992) Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. *Proc. Natl. Acad. Sci. USA* **89,** 8259–8263.
37. Dalgarno, L., Rice, C. M., and Strauss, J. H. (1983) Ross River virus 26S RNA: complete nucleotide sequence and deduced sequence of the encoded structural proteins. *Virology* **129,** 170–187.
38. Frey, T. K. and Marr, L. D. (1988) Sequence of the region coding for virion proteins C and E2 and the carboxyl terminus of the nonstructural proteins of the rubella virus: comparison with alphaviruses. *Gene* **62,** 85–99.
39. Khudyakov, Y. E., Khudyakova, N. S., Fields, H. A., Jue, D., Starling, C., Favorov, M. O., Krawczynski, K., Polish, L., Mast, E., and Margolis, H. (1993) Epitope mapping in proteins of hepatitis E virus. *Virology* **194,** 89–96.
40. Heller, T., Purcell, R. H., Klimpel, K. R., and Emerson, S. U. (1996) Assessment of the function of the third open reading frame protein of the hepatitis E virus, in *Abstracts, IX Trien. Intern. Symp. Viral Hep. Liver Dis.*, Rome, Italy, p. 79.
41. Purcell, R. H. (1996) Hepatitis E virus, in *Fields Virology* (Fields, B. N and Knipe, D. M., eds.), Raven, Philadelphia, pp. 2831–2843.
42. Yarbough, P. O. and Tam, A. W. (1997) Hepatitis E virus: biology, diagnosis and prevention. *Hepatologia Clinica*, in press.
43. Schlauder, G., Dawson, G., Kwo, P., Gabriel, G., and Mushahwar, I. (1996) Partial sequence comparison of a United States isolate of hepatitis E virus, in *Abstracts, IX Trien. Intern. Symp. Viral Hep. Liver Dis.*, Rome, Italy, p. 78.
44. Yarbough, P. O., Tam, A. W., Gabor, K., Garza, E., Moeckli, R. A., Palings, I., Simonsen, C., and Reyes, G. R. (1994) Assay development of diagnostic tests for hepatitis E, in *Viral Hepatitis and Liver Disease* (Nishioka, K., Suzuki, H., Mishiro, S., and Oda, T., eds.), Springer-Verlag, Tokyo, pp. 367–370.
45. Chauhan, A., Dilawari, J. B., Kaur, U., Ganguly, N. K., Bushnurmath, S., and Chawla, Y. K. (1994) Atypical strain of hepatitis E virus (HEV) from North India. *J. Med. Virol.* **44,** 22–29.
46. Arankalle, V. A., Chadha, M. S., Tsarev, S. A., Emerson, S. U., Risbud, A. R., Banerjee, K., and Purcell, R. H. (1994) Seroepidemiology of water-borne hepatitis in India and evidence for a third enterically-transmitted hepatitis agent. *Proc. Natl. Acad. Sci. USA* **91,** 3428–3432.
47. Tsarev, S. A., Tsareva, T. S., Emerson, S. U., Govindarajan, S., Shapiro, M., Gerin, J. L., Robinson, R., Gorbalenya, A. E., and Purcell, R. H. (1996) Prospect for pre-

vention of hepatitis E, in *Enterically-Transmitted Hepatitis Viruses* (Buisson, Y., Coursaget, P., and Kane, M., eds.), La Simarre, Tours, pp. 373–383.
48. Zhang, Y., McAtee, P., Yarbough, P. O., Tam, A. W., and Fuerst, T. R. (1997) Expression, characterization, and immunoreactivities of a soluble hepatitis E virus putative capsid protein species expressed in insect cells. *Clin. Diag. Lab. Immunol,* in press.
49. Kazachkov, Y. A., Balayan, M. S., Ivannikova, T. A., Panina, L. I., Orlova, T. M., Zamyatina, N. A., and Kusov, Y. Y. (1992) Hepatitis E virus in cultivated cells. *Arch. Virol.* **127,** 399–402.
50. Huang, R. T., Li, D. R., Wei, J., Huang, X. R., Yuan, X. T., and Tian, X. (1992) Isolation and identification of hepatitis E virus in Xinjiang, China. *J. Gen. Virol.* **73,** 1143–1148.
51. Li, D. R., Huang, R. T., Tian, X., Yin, S., Wei, J., Huang, X. R., Wang, B. Z., Li, R. X., and Li, Y. C. (1995) Morphology and morphogenesis of hepatitis E virus (strain 87A). *Chinese Med. J.* **108,** 126–131.
52. Tam, A. W., White, R., Reed, E., Short, M., Zhang, Y., Fuerst, T. R., and Lanford, R. E. (1996) In vitro propagation and production of hepatitis E virus from in vivo-infected primary macaque hepatocytes. *Virology* **215,** 1–9.
53. Jacob, J. R., Sureau, C., Burk, K. H., Eichberg, J. W., Dressman, G. R., and Lanford, R. E. (1991) In vitro replication of non-A, non-B hepatitis virus, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), Williams and Wilkins, Baltimore, pp. 387–392.
54. Lanford, R. E., Sureau, C., Jacob, J. R., White, R., and Fuerst, T. R. (1994) Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. *Virology* **202,** 606–614.
55. Sureau, C., Jacob, J. R., Eichberg, J. W., and Lanford, R. E. (1991) Tissue culture system for infection with human hepatitis delta virus. *J. Virol.* **65,** 3443–3450.
56. Sureau, C., Moriarty, A. M., Thorton, G. B., and Lanford, R. E. (1992) Production of infectious hepatitis delta virus in vitro and neutralization with antibodies directed against hepatitis B virus pre-S antigens. *J. Virol.* **66,** 1241–1245.
57. Sureau, C., Guerra, B., and Lanford, R. E. (1993) Role of large hepatitis B virus envelope protein in infectivity of the hepatitis delta virion. *J. Virol.* **67,** 366–372.
58. Purcell, R. H. and Ticehurst, J. R. (1988) Enterically transmitted non-A, non-B hepatitis: epidemiology and clinical characteristics, in *Viral Hepatitis and Liver Disease* (Zuckerman, A. J., ed.), Alan R. Liss, New York, pp. 131–137.
59. Velazquez, O., Stetler, H. C, Avila, C., Ornelas, G., Alvarez, C., Hadler, S. C., Bradley, D. W., and Sepulveda, J. (1990) Epidemic transmission of enterically transmitted non-A, non-B hepatitis in Mexico, 1986–1987. *JAMA* **263,** 3281–3285.
60. Hyams, K. C., Purdy, M. A., Kaur, M., McCarthy, M. C., Hussain, M. A. M., EL-Tigani, A., Krawczynski, K., Bradley, D. W. and Mitchell, C. (1992) Acute sporadic hepatitis E in Sudanese children: analysis based on a new western blot assay. *J. Inf. Dis.* **165,** 1001–1005.
61. Hyams, K. C., McCarthy, M. C., Kaur, M., Purdy, M. A., Bradley, D. W., Mansour, M. M., Gray, S., Watts, D. M., and Carl. M. (1992) Acute sporadic hepatitis E in children living in Cairo, Egypt. *J. Med. Virol.* **37,** 274–277.
62. Skidmore, S. J., Yarbough, P. O., Gabor, K. A., Tam, A. W., Reyes, G. R., and Flower, A. J. E. (1991) Imported hepatitis E in UK. *Lancet* **337,** 1541.
63. Zaiijer, H. L., Kok, M., Lelile, P. N., Timmerman, R. J., Chau, K., and van der Pal, H. J. H. (1993) Hepatitis E in the Netherlands: imported and endemic. *Lancet* **341,** 826.

64. Dawson, G. J., Mushahwar, I. K., and Chau, K. H. (1992) Detection of long-lasting antibody to hepatitis E virus in a US traveler to Pakistan. *Lancet* **340,** 426–427.
65. Tong, M. J., Roue, R., Nahor, M., Gawara N'., Desrame, J., Buisson, Y., and Coursaget, P. (1996) Clinical aspects of hepatitis E and hepatitis A, in *A Comparison in Enterically-Transmitted Hepatitis Viruses* (Buisson, Y., Coursaget, P., and Kane, M., eds.), La Simarre, Tours, pp. 1–10.
66. Longer, C. F., Elliot, J. E., Caudill, J. D., Binn, L. B., Bryan, J. P., Iqbal, M., Innis, B. L., Clayson, E. T., Shrestha, M. P., Tsarev, S. A., Zhang, H. Y., and Tichurst, J. (1996) Observations on subclinical hepatitis E virus (HEV) infection and protection against reinfection, in *A Comparison in Enterically-Transmitted Hepatitis Viruses* (Buisson, Y., Coursaget, P., and Kane, M., eds.), La Simarre, Tours, pp. 362–372.
67. Khuroo, M. S., Rustgi, V. K., Dawson, G. J., Mushahwar, I. K., Yattoo, G. N., Kamili, S., and Khan, B. A. (1994) Spectrum of hepatitis E virus infection in India. *J. Med. Virol.* **43,** 281–286.
68. Goldsmith, R., Yarbough, P. O., Reyes, G. R., Fry, K. E., Gabor, K. A., Kamel, M., Zakaria, S., Amer, S., and Gaffar, Y. (1992) Enzyme-linked immunosorbent assay for diagnosis of acute sporadic hepatitis E in Egyptian children. *Lancet* **339,** 328–331.
69. Song D. Y., Zhuang, H., Kang, X. C., Liu, X. M., Li, Z., Hao W., Shi, K. C., Hao, F. M., Jia, Q., Chen, D. G., He, Z. X., and Ai, Z. Z. (1991) Hepatitis E in Hetian City: a report of 562 cases, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H., eds.), Wilkins and Wilkins, Baltimore, pp. 528–529.
70. Chauhan, A., Jameel, S., Dilawari, J. B., Chawla Y. K., Kaur, U., and Ganguly, N. K. (1993) Hepatitis E virus transmission to a volunteer. *Lancet* **341,** 149–150.
71. Mast, E. E. and Krawczynski, K. (1996) Hepatitis E: an overview. *Annu. Rev. Med.* **47,** 257–266.
72. Arankalle, V. A., Chadha, M., Mehendale, S. M., and Banerjee, K. (1988) Outbreak of enterically transmitted non-A, non-B hepatitis among schoolchildren. *Lancet* **2,** 1199–1200.
73. Uchida, T. (1992) Hepatitis E review. *Gastroenterol. Jap.* **27,** 687–696.
74. Khuroo, M. S., Teli M. R., Skidmore, S., Sofi, M. A., and Khuroo, M. (1991) Incidence and severity of viral hepatitis in pregnancy. *Amer. J. Med.* **70,** 252–255.
75. Tsarev, S. A., Tsareva, T. S., Emerson, S. U., Rippy, M. K., Zack, P., Shapiro, M., and Purcell, H. (1995) Experimental hepatitis E in pregnant rhesus monkeys: failure to transmit hepatitis E virus (HEV) to offspring and evidence of naturally acquired antibodies to HEV. *J. Inf. Dis.* **172,** 31–37.
76. Khuroo, M. S. and Dar, M. Y. (1992) Hepatitis E: evidence for person-to-person transmission and inability of low dose immune serum globulin from an Indian source to prevent it. *Indian J. Gastroenterol.* **11,** 113–116.
77. Mannucci, P. M., Gringeri, A., Santagostino, E., Romano, L., and Zanetti, A. (1994) Low Risk of transmission of hepatitis E virus by large-pool coagulation factor concentrates. *Lancet* **343,** 597–598.
78. Khuroo, M. S., Kamili, S., and Jameel, S. (1995) Vertical transmission of hepatitis E virus. *Lancet* **345,** 1025–1026.
79. Longer, C. F., Denny, S. L., Caudill, J. D., Miele, T. A., Asher, L. V. S., Myint, K. S. A., Huang, C. C., Engler, W. F., LeDuc, J. W., Binn, L. N., and Ticehurst, J. R. (1993) Experimental hepatitis E: pathogenesis in cynomolgus macaques (Macaca fascicularis). *J. Infect. Dis.* **168,** 602–609.

80. Purdy, M. A., McCaustland, K. A., Krawczynski, K., Spelbring, J., Reyes, G. R., and Bradley, D. W. (1993) Preliminary evidence that a trpE-HEV fusion protein protects cynomolgus macaques against challenge with wild-type hepatitis E virus (HEV). *J. Med. Virol.* **41,** 90–94.
81. Tsarev, S. A., Tsareva, T. S., Emerson, S. U., Govindarajan, S., Shapiro, M., Gerin, J. L., and Purcell, R. H. (1994) Successful passive and active immunization of cynomolgus monkeys against hepatitis E. *Proc. Natl. Acad. Sci. USA* **91,** 10,198–10,202.
82. Fuerst, T. R., Yarbough, P. O., Zhang, Y., McAtee, P., Tam, A., Lifson, J., McCaustland, K., Spellbring, J., Bradley, D., Margolis, H. S., Francotte, M., Garcon, N., Slaoui, M., Prieels, J. P., and Krawczynsi, K. (1996) Prevention of hepatitis E using a novel ORF2 subunit vaccine, in *Enterically-Transmitted Hepatitis Viruses* (Bussion, Y. P., Coursaget, P., Kane, M., eds.), La Simarre, Tours, pp. 384–392.
83. McAtee, C. P., Zhang, Y., Yarbough, P. O., Bird, T., Fuerst, T. R., Stone, K. L., Samander, S., and Williams, K. R. (1996) Purification and characterization of a recombinant hepatitis E protein vaccine candidate by liquid chromatography-mass spectrometry. *J. Chromat. (B)* **685,** 91–104.

7

The GB Viruses

Discovery, Molecular Biology, Diagnosis, and Epidemiology

Thomas P. Leary and Isa K. Mushahwar

1. Introduction

At present, five distinct human hepatotropic viruses have been characterized *(1)*. These viruses differ significantly from each other in their genomic organization and are classified into distinct viral families. They are transmitted either parenterally or enterically. The parenterally transmitted viruses are hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D (delta) virus (HDV). The enterically transmitted viruses are hepatitis A virus (HAV) and hepatitis E virus (HEV).

Following the isolation and characterization of these five viruses and the development of highly sensitive and specific assays for their detection, it became clear that approx 5–15% of community-acquired and parenterally transmitted hepatitis cases were not from infections caused by these viruses. Hence, the term non-A–E hepatitis was introduced. Evidence for the existence of viral non-A–E hepatitis included both short and long incubation periods prior to disease onset *(2)*, multiple episodes of affliction *(3)*, and chronic or fulminant hepatitis not attributed to the known hepatotropic viruses *(4,5)*. Direct evidence of a cryptogenic virus included visualization of togavirus-like particles in cases of acute liver failure and severe hemorrhagic necrosis following liver transplantation *(6,7)*, paramyxovirus implicated giant-cell hepatitis *(8)*, and serial passage in primates of the filterable GB agent *(9)*.

The GB agent was one of the first hepatotropic agents serially passaged in primates. The initial inoculum was derived from a surgeon who developed acute hepatitis with icterus. Serum from the third day of jaundice was inoculated into four tamarins; each developed acute biochemical hepatitis

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

with hepatic lesions characteristic of viral infection *(9,10)*. Despite the fact that the disease-causing agent could be filtered and passaged, concerns regarding its human origin remained. Because both serologic and molecular diagnostic assays could distinguish the agent from the known hepatotropic viruses, the possibility remained that the GB agent was responsible for at least a portion of human hepatitis cases.

Initial studies to isolate the agent responsible for GB hepatitis were unsuccessful because of the failure to propagate the virus in cell culture. However, recent advances in molecular biology techniques, such as the expression and cloning of infectious agents from high-titered animal sera followed by immunoscreening *(11)*, differential hybridization *(12)*, and/or representational difference analysis (RDA), allowed the rapid identification of an unique sequence between two cloning sources. The RDA technique *(13)* allows differences between two closely matched samples to be identified by a series of nucleic acid subtraction cycles, each followed by PCR amplification. The products can be molecularly cloned and examined by nucleic acid sequence analysis for the presence of unique viral sequences.

2. Discovery of the GB Viruses

2.1. Isolation of GB Virus A and GB Virus B

In an attempt to isolate the virus responsible for GB hepatitis, a modification of the RDA procedure was used to analyze acute phase serum from an infected tamarin *(Saguinus labiatus)*. Preinoculation serum nucleic acids were utilized to subtract endogenous tamarin sequences from the infectious cloning source. A modification of the procedure was necessary to include sequences that are present only as RNA forms, as many viruses exist without DNA intermediates. Utilizing this technique, 10 distinct clones were obtained that appeared to be viral in nature on the basis of comparisons with nucleic acid and protein databases *(14)*. Each of the clones were exogenous to the genomes of tamarins, yeast, *Escherichia coli*, and humans, and were not present in preinoculation serum. When examining serum from infected animals, putative viral sequences could only be detected following reverse transcription of the nucleic acids, indicative of an RNA virus lacking a DNA intermediate. Further, several of these clones could detect the putative viral genome when used as probes in Northern blot analysis of liver RNAs derived from infected tamarins.

To obtain genome-length sequence of the putative virus, both specific and anchored-PCR were performed to extend the isolated sequences. Initially, several of these sequences were readily connected to one another. Comparisons of these novel sequences to those present in existing databases

revealed that they shared limited identity to distinct regions of the HCV polyprotein *(14)*. Moreover, regions of the extended sequences that should have overlapped based on comparisons to HCV were more similar to HCV than they were to one another. Thus, it became apparent that two novel, but related viruses were present in the cloning source used for these studies. Further extension of these sequences disclosed that each virus, termed GB Virus A (GBV-A) and GB Virus B (GBV-B), was in excess of 9000 nucleotides in length, and each was found to contain single long open reading frames of 2954 and 2865 amino acids, respectively. Nucleic acid sequence comparisons of the GBV-A, GBV-B, and HCV genomes showed that there was <44% identity among the three, demonstrating that GBV-A and GBV-B are not genotypes of HCV and that GBV-A and GBV-B are not genotypes of one another.

PCR-based assays designed to detect GBV-A and GBV-B were subsequently used to examine the passage history of the GB agent in the tamarin animal model *(15)*. These studies demonstrated that both GBV-A and GBV-B could pass a 0.1-μm filter and still be transmitted to the animals. Further passage studies were successful in separating GBV-A from GBV-B. Interestingly, only the presence of GBV-B paralleled the elevation in serum liver enzyme levels and resolved accordingly. Coinciding with resolution of the hepatitis, these animals developed specific antibodies to GBV-B recombinant proteins and were immune to disease on reinfection of the virus. On the other hand, GBV-A was not sufficient to cause biochemical hepatitis in tamarins. Additionally, these animals never developed a detectable antibody response to GBV-A recombinant proteins expressed in *E. coli*, and the virus was able to persist for long periods of time in the serum. PCR studies designed to determine the origin of GBV-A revealed the presence of GBV-A-like variants present in several species of new world monkeys not experimentally inoculated with an infectious source *(16,17)*. Nucleic acid sequence comparisons revealed that these GBV-A variants are species-specific isolates of GBV-A that appear to be distinct genotypes of one another.

2.2. Isolation of GB Virus C

Initial studies to determine the presence of GBV-A and GBV-B in humans concentrated on populations at risk for exposure to parenterally transmitted viruses that were also seroreactive to *E. coli* expressed recombinant proteins derived from these viruses *(18)*. These investigations, which utilized specific PCR systems for the detection of GBV-A and B, were unsuccessful. It was reasoned that heterogeneity within the virus populations could be sufficient to provide false negative results in these assay systems. Thus, degenerate PCR primers designed to highly conserved sequences within the

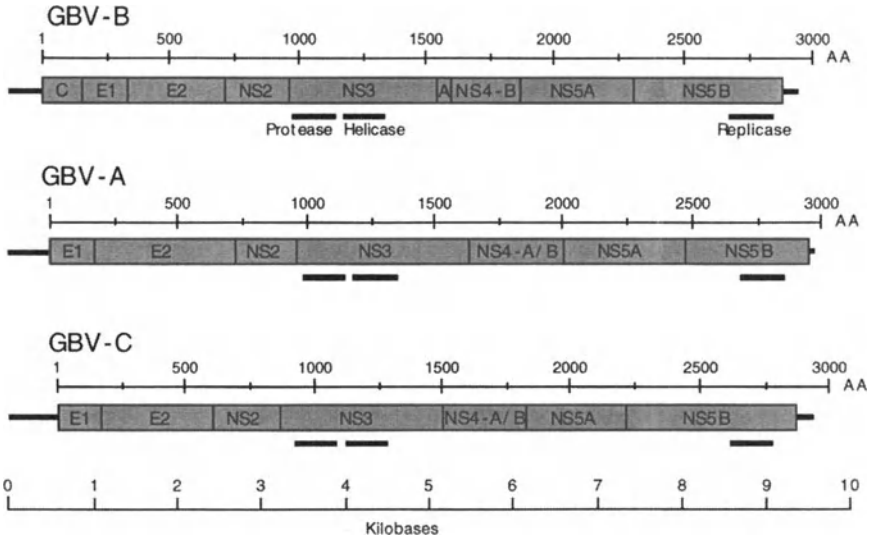


Fig. 1. Genomic organization of the GB viruses. Shaded boxes designate the putative polyproteins with the predicted viral proteins labeled, while the thick lines indicate the noncoding regions of the genomes. The relative positions of the putative protease, helicase, and replicase motifs are specified.

NS3 helicase domain of GBV-A, GBV-B, and HCV were utilized to analyze serum nucleic acids from these same human populations. Twelve individuals, including four with documented cases of cryptogenetic hepatitis, were found to have viral helicase sequences by this assay system *(18)*. Upon detailed analysis of the isolated sequences, it was apparent that they were derived from a virus distinct from GBV-A, GBV-B, or HCV. This novel sequence, later termed GB virus C (GBV-C), was most closely related to GBV-A, sharing 59% identity at the nucleic acid level and 64% identity at the amino acid level.

Ensuing studies have extended two of the initial GBV-C isolates to genome-length sequences *(19,20)*. Similar to GBV-A and GBV-B, GBV-C has an RNA genome that is in excess of 9300 nucleotides and also contains a single long open reading frame of 2842 amino acids. Amino acid sequence comparisons of the GBV-C long open reading frame to those of GBV-A, GBV-B, and HCV-1 exhibit identities of 48%, 28%, and 29%, respectively. Subsequent to the initial reports describing the isolation of GBV-C, another agent, hepatitis G virus (HGV) was reported *(21)*. Two independent isolates of this virus have been described, each derived from individuals with a his-

Table 1
Flaviviridae Polyprotein Percent Sequence Identity[a]

	GBV-C	GBV-A	GBV-B
GBV-A	48		
GBV-B	28	27	
HCV-1	29	26	32

[a]Comparisons were determined with the Wisconsin Sequence Analysis Package. Sequences are from GenBank: HCV-1, M62321; GBV-A, U22303; GBV-B, U22304; and GBV-C, U36380.

tory of liver disease. Similar to the GB viruses, HGV contains an RNA genome in excess of 9 kb that contains a long open reading frame. Comparisons of open reading frames of GBV-C and HGV demonstrate that these viruses are 86% identical at the nucleotide level and 95% identical at the amino acid level *(19)*. Additional localized comparisons of the open reading frames reveal even greater identity between these viruses. This suggests that GBV-C and HGV are simply distinct isolates of the same virus.

2.3. Genomic Organization of the GB Viruses

Each of the three GB viruses have RNA genomes in excess of 9 kb in length, which contain long open reading frames of greater than 2800 amino acids *(19,20,22)* (Fig. 1). These genomes are organized much like those of the *Flaviviridae*, whose members include the pesti- and flaviviruses, as well as HCV. The hallmark of this virus family is a single-stranded RNA genome that encodes a single large polyprotein that is posttranslationally cleaved into the individual viral components. A number of protein structural motifs are conserved across the polypeptides, including proteases, helicases, and replicases. These viruses encode their structural genes at the 5' one quarter of the genome and the nonstructural genes at the 3' three quarters. Additionally, the *Flaviviridae* encode a core protein that is encompassed by an envelope comprised of two or three virally encoded proteins.

Amino acid sequence comparisons of the large polyproteins illustrate the distinctions, as well as the relatedness, of the GB viruses (Table 1). GBV-A and GBV-C are most closely related (48% identical), while sharing only marginal identity to GBV-B and HCV1 (26–29%). GBV-B and HCV1 are slightly more related to one another at 32% identity across the polyproteins. Localized comparisons of the GBV-A, B, and C polyproteins define regions of much higher identity *(15,16,18)*. The greatest identity occurs within the helicase and replicase motifs, each of which cluster within the supergroup II helicases and replicases of the positive stranded RNA viruses, respectively.

Further, the GB viruses contain domains for two distinct proteases (thiol and serine). Thus it could be speculated that these polyproteins are processed in much the same manner as the other *Flaviviridae*, particularly HCV. Potential proteolytic cleavage sites have been identified within the polyprotein sequences of the GB viruses, again suggesting that these viruses process a precursor polyprotein into the individual viral subunits.

Another characteristic of the GB viruses is the presence of a long 5' nontranslated region (NTR) upstream of the open reading frames *(15,18)*. Regions such as these are also found in HCV and the pestiviruses, within which exists a significant amount of secondary structure. These structures are known to possess internal ribosome entry site (IRES) activity that allows for cap-independent translation of the long open reading frames. GBV-A and GBV-C also have been shown to exhibit IRES activity within the 5' NTR, though a portion of the coding region sequences have been found to be required for full activity *(23)*. Further, these studies have been used to identify the exact codon from which translation initiation occurs in GBV-A and GBV-C.

A distinguishing feature among GBV-A and GBV-C is the apparent lack of core-like sequences encoded within the polyprotein *(20)*. In GBV-B and HCV, a profoundly basic core protein is encoded at the N-terminus of the polyprotein, preceding the envelope glycoproteins. This protein is thought to associate with the viral genome during replication, allowing for the packaging of progeny virus. In GBV-A and GBV-C, the putative translation initiation codon occurs just upstream of the first envelope protein, in the absence of a core-like protein *(19)*. Examination of these genomes for potential open reading frames capable of encoding such a protein have not been successful. These data would suggest that GBV-A and GBV-C are clearly distinct within the *Flaviviridae* and may possibly be members of a unique viral family. What substitutes for the core protein during GBV-A and GBV-C replication is unclear at the time. The most simplistic reasoning is that a cellular RNA binding protein is utilized in the packaging of these viruses, though the possibilities are endless. Definitive answers to these questions will likely require the GB viruses to be propagated in vitro.

Phylogenetic analysis can be used to evaluate the evolutionary relationship of nucleic acid or amino acid sequences to one another. Such analyses, when performed on the GB viruses, clearly differentiates these viruses as members of the *Flaviviridae (18,20,22)*. When these comparisons are performed using the sequences within the large open reading frames of the GB viruses and HCV, an unrooted phylogenetic tree illustrates the relationship of these viruses. As shown in Fig. 2, the distinct HCV genotypes cluster on a single major branch of the tree, as does GBV-B on a separate branch.

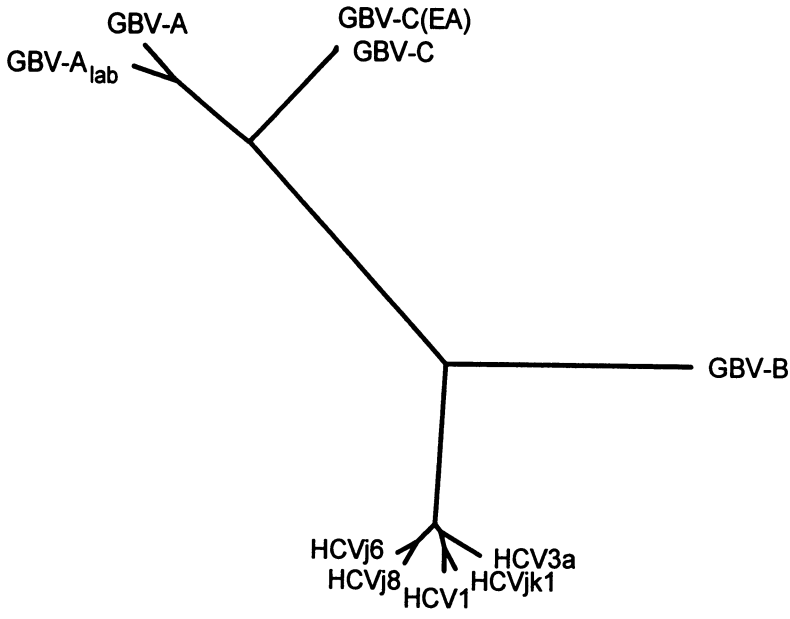


Fig. 2. Phylogenetic analysis of *Flaviviridae* polyproteins. Phylogenetic distances were determined using the PRODIST program of the PHYLIP package (v. 3.5). These distances were used for the construction of the unrooted tree using the program FITCH. TREEVIEW produced the final output.

GBV-A and GBV-C cluster to a third major branch, each of which are further subdivided. Currently, a number of genome-length GBV-C isolates have been reported in the literature. Amino acid sequence comparisons show these isolates to be remarkably conserved *(19)* as is evident from the phylogenetic tree. Interestingly, a number of geographically distinct genotypes of GBV-C can be defined when like comparisons are made within the 5' NTR *(24)*. Similar results have been reported when the NS3 helicase region is used for this analysis *(25)*, although concordant results were not obtained when this analysis was performed on each region with the same samples *(24)*. Presently, genome length sequence has only been determined for two isolates of GBV-A, viruses that have been determined to be genotypes of one another on the basis of 5' NTR sequence analysis *(16)*. This result has been confirmed with the polyprotein sequence as illustrated in Fig. 2. Distances observed between the GBV-A isolates is very similar to those found between the distinct HCV genotypes. Analyses such as these have in the past been the basis for classification of viruses within the individual families. Based on previous designations within the *Flaviviridae*, HCV and GBV-B would each

comprise separate genera within this family, while GBV-A and GBV-C would be distinct members of a third genera. An alternate classification for HCV has been proposed in which a separate family is created, the *Hepaciviridae (26)*. Within this designation, the GB viruses would again fall into two distinct genera.

2.4. Detection Systems for the Presence of GBV-C

Because of the inability to identify and express GBV-C immunodominant epitopes in procaryotic systems, several distinct probe-based assays have been developed as well as a serological assay utilizing a mammalian expressed envelope protein. The first of the probe-based assay systems utilize degenerate oligonucleotide primers designed within the highly conserved NS3 region of GBV-C *(27)*. Degenerate primers were employed to overcome possible sensitivity problems with regard to primer:template mismatches. Further, an amplification protocol that incrementally decreases the annealing temperature at each cycle was utilized, again in an attempt to circumvent possible sensitivity issues that could arise as a result of mutations that may occur within the virus population.

The second probe-based assay system was designed to detect sequences within the 5' NTR region of GBV-C *(28)*. As was the case for HCV, it was postulated that sequences within this region of the virus genome would be the best target for PCR-based assays. Relative to the NS3 region, less sequence variability is present in the 5' NTR of GBV-C. In fact, multiple regions are absolutely conserved between all known isolates of GBV-C. PCR assays designed within this region provide results virtually identical to those obtained with the NS3 region assay, in the absence of degenerate oligonucleotide primers or altered cycling conditions. The troublesome aspect with each of these PCR-based assay systems is that Southern hybridization must be performed to confirm the results. This is often time consuming and prone to error. Thus, an automated RT-PCR assay was developed to detect sequences within the 5' NTR of GBV-C. This assay system utilizes oligomer hybridization with a labeled probe to identify amplified viral sequences in an automated format.

The final detection system is a bead-based ELISA that utilizes a recombinant GBV-C second envelope protein as antigen *(29)*. This assay can be used to detect antibodies directed against this antigen that are present in serum or plasma. Seroprevalence studies have shown that GBV-C infection in volunteer blood donors is from 3 to 8% and is much greater in individuals at risk for exposure to parenterally transmitted viruses, including injectable drug users, plasmapheresis donors, and those infected with other hepatitis viruses. Additionally, it has been shown that seroconversion to the second

envelope protein is associated with clearance of circulating GBV-C *(29,30)*. Therefore, both probe- and serological-based assay systems are necessary to determine the true prevalence of GBV-C within the human population.

3. Epidemiology, Clinical Course and Natural History

Since the original report by Simons et al. *(18)*, several studies have shown GBV-C to be parenterally transmitted, globally distributed, and present in blood and blood products. These studies are summarized in Table 2. Other detailed studies concerning the natural history, clinical course, and persistence of GBV-C in a variety of populations are discussed below.

3.1. Population Studies

3.1.1. Fulminant Hepatic Failure

The role of GBV-C in the etiology of fulminant hepatitis is not as yet fully established. Further convincing studies are needed to confirm a definite link between fulminant hepatitis and GBV-C infection. One study carried out in Japan by Yoshiba et al. *(31)* documented the presence of GBV-C RNA in 3 out of 6 (50%) fulminant hepatitis patients without evidence of infection with known hepatitis viruses. Since that report, questions were raised concerning the association of GBV-C with acute liver failure *(32)*, specifically, whether GBV-C was an "innocent bystander" transmitted through transfusions given to the three patients prior to the onset of fulminant hepatitis. Additional studies by Yoshiba et al. *(33)*, however, showed that only a few of the 63 fulminant hepatitis patients so far studied had received therapeutic transfusions prior to the onset of fulminant hepatitis, but definitely not all.

In a similar study also carried out in Japan *(34)*, GBV-C RNA was detected in 3 out of 15 (20%) patients with HBV infection and 3 out of 25 (12%) patients without markers of hepatitis A-E virus infection. Overall, GBV-C RNA was detected in 6 out of 44 (14%) patients with fulminant hepatitis at a frequency significantly higher ($p < 0.001$) than that in 3 out of 326 (0.9%) blood donors matched for age with the patients. Of the 6 patients with GBV-C RNA, only 3 (50%) had a history of transfusion, and all of these were coinfected with HBV. These results according to the authors indicate a role of GBV-C in inducing fulminant hepatitis either by itself or in concert with other hepatitis viruses.

In a study carried out in Germany by Heringlake et al. *(35)*, 50% (11 out of 22) of fulminant hepatitis patients had positive tests for GBV-C RNA compared to only 4.7% (5 out of 106) of blood donors. Of these 11 fulminant

Table 2
GBV-C RNA Among a Variety of Sources/Patients

Sources/Patients	Country	Total No.	% RNA	Reference
Acute non-A-E	Italy	31	35	45
	US	16	7	46
Aplastic anemia	Taiwan	4	100	47
Chronic HCV	Germany	100	19	49
	Japan	189	11	48
	Taiwan	6	100	47
	US	8	25	46
Chronic liver disease	Japan	58	55	41
	Japan	203	5	50
	Pakistan	43	12	51
Chronic non-A-E	Brazil	13	8	52
	Italy	18	39	45
	US	48	13	42
Fulminant hepatitis	Germany	22	50	37
	Japan	6	50	31
	Japan	7	14	53
	Japan	44	14	34
	US	7	43	46
Hemodialysis	France	61	58	44
	Italy	100	19	43
	Japan	519	3	40
	Japan	149	5	41
Hemophiliacs	Italy	70	6	54
	Japan	63	24	56
	UK	95	14	55
	US	49	18	42
Hepatocellular carcinoma	S. Africa	167	11	38
	US	30	2	42
Homosexuals/Bisexual men	Germany	101	11	57
Intravenous drug abusers	China	85	75	59
	Germany	130	33	60
	Japan	49	24	58
	US	94	16	46
	US	60	33	42
Leprous patients	Japan	229	5	61
Plasma pools/products:	Europe	60	8	62
	US	46	54	62
Factor 8[a]	U.K.	17	94	55
Factor 9[a]	U.K.	6	100	55
Vertical transmission	Germany	9	33	63
	Italy[b]	50	48	63

Table 2
GBV-C RNA among a variety of sources/patients *(cont'd)*

Sources/Patients	Country	Total No.	% RNA	Reference
Volunteer blood donors	Australia	120	4	64
	China	50	2	59
	Germany	90	2	57
	Germany	92	3	60
	Japan	448	1	40
	US	769	2	42
	US	100	3	46
	Vietnam	81	7	65

[a]Not inactivated
[b]Personal communication, A. R. Zanetti

hepatitis patients, six had evidence of prior infection with HBV, and the remaining five had no evidence of infection with other known hepatitis viruses. No information however was given as to whether the 11 patients had received therapeutic transfusions prior to the onset of hepatitis. Further analysis of nucleic acid sequences of these cases showed six mutations at defined positions in all 11 patients with fulminant hepatic failure. None of these mutations were found in the five GBV-C RNA positive control-group of blood donors. However, these mutations were also found in 6 out of 19 GBV-C RNA positive German patients with chronic cryptogenic hepatitis.

In a fourth study, Sallie et al. *(36)* performed RT-PCR for GBV-C on RNA obtained from livers resected from 20 patients at the time of transplantation for fulminant hepatic failure. The 20 patients were seronegative for all known hepatitis viruses, and all liver RNA samples were shown to be negative for GBV-C RNA. This, however, is not surprising, since GBV-C has not yet been proven to be a hepatotropic virus.

3.1.2. Autoimmune Liver Disease

A study by Heringlake et al. *(37)* was carried out to investigate whether GBV-C is capable of triggering autoimmune hepatitis. They found a slightly higher prevalence of GBV-C in cryptogenic hepatitis (12%) and autoimmune hepatitis types I, II, and III (6.7%, 10.0%, and 12.0%) respectively, compared with 4.7% among blood donors. In contrast, patients with viral hepatitis B, C, and D were more frequently infected with GBV-C (16%, 20%, and 36% respectively). These results suggest that GBV-C is not a major cause for inducing autoimmunity and leading to autoimmune hepatitis.

3.1.3. Hepatocellular Carcinoma

The role of GBV-C in triggering hepatocellular carcinoma is not as yet fully established. Lightfoot et al. *(38)* compared the prevalence of GBV-C infection in 167 Southern African Blacks with hepatocellular carcinoma to 167 carefully matched hospital-based control subjects and tested for possible interactive effects between this virus, HBV, as well as HCV in the development of the tumor. They showed that individuals infected with GBV-C did not have an increased relative risk of developing hepatocellular carcinoma. Moreover, coinfection with GBV-C did not further the increased risk of tumor development in patients chronically infected with HBV or HCV alone.

In another study, the frequency of GBV-C infection was investigated by Berg et al. *(39)* in 74 patients with chronic HCV infection who had received orthotopic liver transplantation (OLT) because of decompensated liver cirrhosis. They found a significantly higher percentage of hepatocellular carcinoma in patients with preOLT GBV-C/HCV coinfection compared with patients with HCV infection alone.

3.1.4. Maintenance Hemodialysis

Among patients on maintenance hemodialysis, the prevalence rates of GBV-C, as expected, vary from country to country. Reported prevalences among Japanese hemodialysis subjects were 3–5% *(40,41)*, 20% in the United States *(42)* and Italy *(43)*, 55% in Indochina *(41)*, and 58% in France *(44)*. Transfusion, patient-to-patient spread and increasing years on dialysis may be responsible for the high prevalence of GBV-C among patients on maintenance hemodialysis. As has been already discussed *(40)*, infection transmitted by transfusion should result in unique sequences that do differ substantially among patients after sequencing a part of the GBV-C genome, whereas identical sequences would be expected with nosocomial infection within a dialysis unit.

3.2. Histopathology, Clinical Course and Persistence of GBV-C Infection

An elegant study *(45)* showing histological features in liver samples from patients with GBV-C infection has been reported. These investigators implicated GBV-C in a significant number of acute and chronic cases of non-A-E hepatitis. Among the six chronic hepatitis patients positive for GBV-C RNA, the histology of the liver samples revealed chronic active hepatitis in one patient and chronic persistent hepatitis in five others. All chronic patients had elevated ALT levels between 89 and 478 U/L. In contrast, among the 11 acute hepatitis cases positive for GBV-C RNA, the ALT levels varied between 615 and 2477 U/L.

In another study, Masuko et al. *(40)* reported that GBV-C RNA was detected in 3.1% of patients on maintenance hemodialysis as compared with 0.9% of healthy blood donors. None of the 16 patients had evidence of acute liver disease, although seven were also infected with HCV. Eight patients with GBV-C infection were followed for 7–16 yr. In two patients, the virus was present at the start of hemodialysis. One had a history of transfusion and GBV-C RNA persisted over a period of 16 yr, the other cleared GBV-C RNA after 10 yr. In five patients, GBV-C RNA was first detected 3–20 wk after blood transfusion and persisted for up to 13 yr.

In spite of the above-mentioned studies, the role of GBV-C in liver disease has yet to be investigated. Liver pathology of individuals infected with GBV-C alone has not been fully studied. It is possible that the main site of GBV-C replication is not the liver but rather other organ(s) such as the kidney, spleen, and lungs. Hence, tissue tropism and *in situ* hybridization studies are essential to be carried out in order to clarify the pathogenicity of GBV-C and its site of replication.

References

1. Mushahwar, I. K., Dawson, G. J., and Reyes, G. R. (1996) Hepatitis E virus: molecular biology and diagnosis. *Eur. J. Gastroenterol. Hepatol.* **8,** 312–318.
2. Hollinger, F. B., Mosley, J. W., Szmuness, W., Aach, R. D., Peters, R. L., and Stevens, C. (1980) Transfusion-transmitted viruses study: experimental evidence for two non-A, non-B hepatitis agents. *J. Infect. Dis.* **142,** 400–407.
3. Mosley, J. W., Redeker, A. G., Feinstone, S. M., and Purcell, R. H. (1977) Multiple hepatitis viruses in multiple attacks of acute viral hepatitis. *N. Engl. J. Med.* **296,** 75–80.
4. Alter, M. J., Margolis, H. S., Krawczynski, K., Judson, F. N., Mares, A., Alexander, W. J., Hu, P. Y., Miller, J. K., Gerber, M. A., Sampliner, R. E., Meeks, E. L., and Beach, M. J. (1992) The natural history of community-acquired hepatitis C in the United States. *N. Engl. J. Med.* **327,** 1899–1905.
5. Wu, J. C., Chen, C. L., Hou, M. C., Chen, T. Z., Lee, S. D., and Lo, K. L. (1994) Multiple viral infection as the most common cause of fulminant and subfulminant viral hepatitis in an area endemic for hepatitis B: application and limitations of the polymerase chain reaction. *Hepatology* **19,** 833–840.
6. Fagan, E. A., Ellis, D. S., Tovey, G. M., Lloyd, G., Smith, H. M., Portmann, B., Tan, K.-C., Zuckerman, A. J., and Williams, R. (1992) Toga virus-like particles in acute liver failure attributed to sporatic non-A, non-B hepatitis and recurrence after liver transplantation. *J. Med. Virol.* **38,** 71–77.
7. Fagan, E. A., Ellis, D. S., and Tovey, G. M. (1989) Toga-like virus as a cause of fulminant hepatitis attributed to sporatic non-A, non-B hepatitis. *J. Med. Virol.* **28,** 150–155.
8. Phillips, M. J., Blendis, L. M., Poucell, S., Patterson, J., Petric, M., Roberts, E., Levy, G. A., Superina, R. A., Greig, P. D., Cameron, R., Langer, B., and Purcell, R. H. (1991) Syncytial giant-cell hepatitis: sporadic hepatitis with distinctive pathological features, a severe clinical course, and paramyxoviral features. *N. Engl. J. Med.* **324,** 455–460.

9. Deinhardt, F., Holmes, A. W., Capps, R. B., and Popper, H. (1967) Studies on the transmission of disease of human viral hepatitis to marmoset monkeys. I. Transmission of disease, serial passage and description of liver lesions. *J. Exp. Med.* **125,** 673–687.
10. Deinhardt, F., and Holmes, A. W. (1968) Transmission of human viral hepatitis to non-human primates, in *Prospectives in Virology*, vol. 6. Academic, New York, pp. 89–103.
11. Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Isolation of a cDNA clone derived from blood-borne non-A, non-B viral hepatitis genome. *Science* **244,** 359–362.
12. Reyes, G. R., Purdy, M. A., Kim, J. P., Luk, K.-C., Young, L. M., Fry, K. E., and Bradley, D. W. (1990) Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science* **247,** 1335–1339.
13. Lisitsyn, N., Lisitsyn, N., and Wigler, M. (1993) Closing the differences between two complex genomes. *Science* **259,** 946–951.
14. Simons, J. N., Pilot-Matias, T. J., Leary, T. P., Dawson, G. J., Desai, S. M., Schlauder, G. G., Muerhoff, A. S., Erker, J. C., Buijk, S. L., Chalmers, M. L., Sant, C. L. V., and Mushahwar, I. K. (1995) Identification of two flavivirus-like genomes in the GB hepatitis agent. *Proc. Natl. Acad. Sci. USA* **92,** 3401–3405.
15. Schlauder, G. G., Dawson, G. J., Simons, J. N., Pilot-Matias, T. J., Guiterrez, R. A., Heynen, C. A., Knigge, M. F., Kurpiewski, G. S., Leary, T. P., Muerhoff, A. S., Desai, S. M., and Mushahwar, I. K. (1995) Molecular and serologic analysis in the transmission of the GB hepatitis agents. *J. Med. Virol.* **46,** 81–90.
16. Leary, T. P., Desai, S. M., Yamaguchi, J., Chalmers, M. L., Schlauder, G. G., Dawson, G. J., and Mushahwar, I. K. (1996) Species-specific variants of GB virus A (GBV-A) in captive monkeys. *J. Virol.* **70,** 9028–9030.
17. Schlauder, G. G., Pilot-Matias, T. J., Gabriel, G. S., Simons, J. N., Muerhoff, A. S., Dawson, G. J., and Mushahwar, I. K. (1995) Origin of GB-hepatitis viruses. *Lancet* **346,** 447.
18. Simons, J. N., Leary, T. P., Dawson, G. J., Pilot-Matias, T. J., Muerhoff, A. S., Schlauder, G. G., Desai, S. M., and Mushahwar, I. K. (1995) Isolation of novel virus-like sequences associated with human hepatitis. *Nat. Med.* **1,** 564–569.
19. Erker, J. C., Simons, J. N., Muerhoff, A. S., Leary, T. P., Chalmers, M. L., Desai, S. M., and Mushahwar, I. K. (1996) Molecular cloning and characterization of a GB virus C isolate from a patient with non-A-E hepatitis. *J. Gen. Virol.* **77,** 2713–2720.
20. Leary, T. P., Muerhoff, A. S., Simons, J. N., Pilot-Matias, T. J., Erker, J. C., Chalmers, M. C., Schlauder, G. G., Dawson, G. J., Desai, S. M., and Mushahwar, I. K. (1996) The sequence and genomic orginization of GBV-C: a novel member of the flaviviridae associated with human non-A-E hepatitis. *J. Med. Virol.* **48,** 60–67.
21. Linnen, J., Jr., J. W., Zhang-Keck, Z.-Y., Fry, K. E., Krawczynski, K. Z., Alter, H., Koonin, E., Gallagher, M., Alter, M., Hadziyannis, S., Karayiannis, P., Fung, K., Nakatsuji, Y., Shih, J. W.-K., Young, L., Jr., M. P., Hoover, C., Fernandez, J., Chen, S., Zou, J.-C., Morris, T., Hyams, K. C., Ismay, S., Lifson, J. D., Hess, G., Foung, S. K. H., Thomas, H., Bradley, D., Margolis, H., and Kim, J. P. (1996) Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. *Science* **271,** 505–508.
22. Muerhoff, A. S., Leary, T. P., Simons, J. N., Pilot-Matias, T. J., Erker, J. C., Chalmers, M. C., Schlauder, G. G., Dawson, G. J., Desai, S. M., and Mushahwar, I. K. (1995) Genomic organization of GBV-A and GBV-B: two new members of the flaviviridae associated with GB-agent hepatitis. *J. Virol.* **69,** 5621–5630.

23. Simons, J. N., Desai, S. M., Schultz, D. E., Lemon, S. M., and Mushahwar, I. K. (1996) Translation initiation in GB viruses A and C: evidence for internal ribosome entry and implications on genome organization. *J. Virol.* **70,** 6126–6135.
24. Muerhoff, A. S., Simons, J. N., Leary, T. P., Erker, J. C., Chalmers, M. L., Pilot-Matias, T. J., Dawson, G. J., Desai, S. M., and Mushahwar, I. K. (1996) Sequence heterogeneity within the 5'-terminal region of the hepatitis GB virus C genome and evidence for genotypes. *J. Hepatol.* **25,** 379–384.
25. Kao, J.-H., Chen, P.-J., Hsiang, S.-C., Chen, W., and Chen, D.-S. (1996) Phylogenetic analysis of GB virus C: comparison of isolates from Africa, North America and Taiwan. *J. Infect. Dis.* **174,** 410–413.
26. Shukla, D. D., Hoyne, P. A., and Ward, C. W. (1995) Evaluation of complete genome sequences and sequences of individual gene products for the classification of hepatitis C viruses. *Arch. Virol.* **140,** 1747–1761.
27. Leary, T. P., Muerhoff, A. S., Simons, J. N., Pilot-Matias, T. J., Erker, J. C., Chalmers, M. L., Schlauder, G. G., Dawson, G. J., Desai, S. M., and Mushahwar, I. K. (1996) Consensus oligonucleotide primers for the detection of GB virus C in human cryptogenic hepatitis. *J. Virol. Meth.* **56,** 119–121.
28. Muerhoff, A. S., Simons, J. N., Erker, J. C., Desai, S. M., and Mushahwar, I. K. (1996) Identification of conserved nucleotide sequences within the GB virus C 5' untranslated region: design of PCR primers for detection of viral RNA. *J. Virol. Meth.* **62,** 55–62.
29. Dille, B. J., Surowy, T. K., Gutierrez, R. A., Coleman, P. F., Knigge, M. F., Carrick, R. J., Aach, R. D., Hollinger, F. B., Stevens, C. E., Barbosa, L. H., Nemo, G. J., Mosley, J. W., Dawson, G. J., and Mushahwar, I. K. (1996) An ELISA for detection of antibodies to the E2 protein of GB virus C. *J. Infect. Dis.* **175,** 458–461.
30. Pilot-Matias, T. J., Carrick, R. J., Coleman, P. F., Leary, T. P., Simons, J. N., Muerhoff, A. S., Buijk, S. L., Chalmers, M. L., Dawson, G. J., Desai, S. M., and Mushahwar, I. K. (1996) Expression of the GB virus C E2 glycoprotein using the Semliki Forest virus vector system and its utility as a serologic marker. *Virology* **225,** 282–292.
31. Yoshiba, M., Okamoto, H., and Mishiro, S. (1995) Detection of the GBV-C hepatitis virus genome in serum from patients with fulminant hepatitis of unknown etiology. *The Lancet* **346,** 1131–1132.
32. Alter, H. J. (1996) The cloning and clinical implications of HGV and HGBV-C. *N. Engl. J. Med.* **334,** 1536–1537.
33. Yoshiba, M., Inoue, K., and Sekiyama, K. (1996) Hepatitis GB Virus C *N. Engl. J. Med.* **335,** 1392–1393.
34. Tameda, Y., Kosaka, Y., Tagawa, S., Takase, K., Sawada, N., Nakao, H., Tsuda, F., Tanaka, K., Okamoto, H., Miyakawa, Y., and Mayumi, M. (1966) Infection with GB virus C (GBV-C) in patients with fulminant hepatitis. *J. Hepatol.* **25,** 842–847.
35. Heringlake, S., Osterkamp, S., Trautwein, C., Tillmann, H. L., Boker, K., Muerhoff, S., Mushahwar, I. K., Hansmann, G., and Manns, M. P. (1996) Association between fulminant hepatic failure and a strain of GBVC? *Lancet* **348,** 1626–1629.
36. Sallie, R., Shaw, J., and Mutimer, D. (1996) GBV-C virus and fulminant hepatic failure. *Lancet* **347**, 1552.
37. Heringlake, S., Tillman, H. L., Cordes-Temme, P., Trautwein, C., Hunsmann, G., and Manns, M. P. (1996) GBV-C is not the major cause of autoimmune hepatitis. *J. Hepatol.* **25,** 980–984.

38. Lightfoot, K., Skelton, M., Kew, M. C., Yu, M. C., Kedda, M-A., Coppin, A., and Hodkinson, J. Does hepatitis GB virus C infection cause hepatocellular carcinoma in Black Africans? (Personal communication to IKM by Dr. Kew)
39. Berg, T., Naumann, U., Fukumoto, T., Bechstein, W. O., Neuhaus, P., Lobeck, H., Hohne, M., Schreier, T., and Hopf, U. (1996) GB virus C infection in patients with chronic hepatitis B and C before and after liver transplantation. *Transplantation* **62,** 711–714.
40. Masuko, K., Mitsui, T., Iwano, K., Yamazaki, C., Okuda, K., Meguro, T., Murayama, N., Inoue, T., Tsuda, F., Okamoto, H., Miyakawa, Y., and Mayumi, M. (1996) Infection with hepatitis GB virus C in patients on maintenance hemodialysis. *N. Engl. J. Med.* **334,** 1485–1490.
41. Tsuda, F., Hadiwandowo, S., Sawada, N., Fukuda, M., Tanaka, T., Okamoto, H., Mayumi, M. (1996) Infection with GB Virus C (GBV-C) in patients with chronic liver disease or on maintenance hemodialysis in Indonesia. *J. Med. Virol.* **49,** 248–252.
42. Linnen, J., Wages, J., Zhang-Keck, Z. K., Fry, K. E., Krawczynski, K. Z., Alter, H., Koonin, E., Gallagher, M., Alter, M., Hadziyannis, S., Karayiannis, P., Fung, K., Nakatsuji, Y., Shih, J., W.-K., Young, L., Piatak, M., Hoover, C., Fernandez, J., Chen, S., Zou, J.-C., Morris, T., Hyams, K. C., Ismay, S., Lifson, J. D., Hess, G., Foung, S. K. H., Thomas, H., Bradley, D., Margolis, H., and Kim, J. P. (1996) Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. *Science* **271,** 505–508.
43. Sampietro, M., Badalamenti, S., and Lunghi, G. (1996) Hepatitis GB virus C. *N. Engl. J. Med.* **335,** 1392.
44. DeLamballerie, X., Charrel, R. N., and Dussol, B. (1996) Hepatitis GB virus C in patients on hemodialysis. *Lancet* **334,** 1549.
45. Fiordalisi, G., Zanella, I., Mantero, G., Bettinardi, A., Stellini, R., Paraninfo, G., Cadeo, G., and Primi, D. (1996) High prevalence of GB virus C infection in a group of Italian patients with hepatitis of unknown etiology. *J. Infect. Dis.* **174,** 181–183.
46. Dawson, G. J., Schlauder, G. G., Pilot-Matias, T. J. Theile, D., Leary, T. P., Murphy, P., Rosenblatt, J. E., Simons, J. N., Martinson, F. E. A., Gutierrez, R. A., Lentino, J. R. Pachuki, C., Muerhoff, A. S., Widell, A., Tegtmeir, G., Desai, S. M., and Mushahwar, I. K. (1996) Prevalence of GB Virus C using reverse transcriptase-polymerase chain reaction. *J. Med. Virol.* **50,** 97–103.
47. Kao, J-H., Chen, P-J., Hsiang, S-C., Chen, W., and Chen, D-S. (1996) Phylogenetic analysis of GB virus C: comparison of isolates from Africa, North America and Taiwan. *J. Infect. Dis.* **174,** 410–413.
48. Tanaka, E., Alter, H. J., Nakatsuji, Y., Shih, W-K., Kim-J. P., Matsumoto, A., Kobayashi, M., and Kiyosawa, K. (1996) Effect of hepatitis G virus infection on chronic hepatitis C. *Ann. Int. Med.* **125,** 740–743.
49. Schleicher, S., Chaves, R. L., Dehmer, T., Gregor, M., Hess, G., and Fleming, B. (1996) Identification of GBV-C hepatitis G RNA in chronic hepatitis C patients. *J. Med. Virol.* **50,** 71–74.
50. Orito, E., Mizokami, M., Nakano, T., Wu, R-R., Cao, K., Ohba, K-I., Ueda, R., Mukaide, M., Hikiji, K., Matsumoto, M., and Iino, S. (1996) GB Virus C/hepatitis G virus infection among Japanese patients with chronic liver disease and blood donors. *Virus Res.*, in press.
51. Moatter, T., Tariq, S. A., Fisher-Hoch, S., Haroon, S., and McCormick, J. B. (1996) Hepatitis G virus in Karachi, Pakistan. *Lancet* **348,** 1318–1319.

52. Pinho, J. R. R., Cappacci, M. L., da Silva, L. C., Carrilho, F. J., Santos, C. A., Pugliese, V., Guz, B., Levi, J. E., Ballarati, C. A. F., and Bernardini (1996) Hepatitis G virus/GB virus C in Brazil. Preliminary report. *Rev. Inst. Med. Trop. S. Paulo* **38,** 243–246.
53. Kuroki, T., Nishiguchi, S., Tanaka, M., Enomoto, M., and Kobayashi, K. (1996) Does GBV-C cause fulminant hepatitis in Japan? *Lancet* **347,** 908.
54. Tagariello, G., Infantolini, D., Biasin, M. R., Davoli, P. G., and Traldi, A. (1996) Hepatitis G Viral RNA in Italian hemophiliacs with and without hepatitis C infection. *Lancet* **348,** 760–761.
55. Jarvis, L. M., Davidson, F., Hanley, J. P., Yap, P. L., Ludlam, C. A., and Simmonds, P. (1996) Infection with hepatitis G virus among recipients of plasma products. *Lancet* **348,** 1352–1355.
56. Kinoshita, T., Miyake, K., Nakao, H., Tanaka, T., Tsuda, F., Okamoto, H., Miyakawa, Y., and Mayumi, M. (1997) Molecular investigation of GB virus C infection in Japan. *J. Infect. Dis.* **175**, 454–457.
57. Startk, K., Bienzle, U., Hess, G., Engel, A. M., Hegenscheid, B., and Schluter, V. (1996) Detection of the hepatitis G virus genome among injecting drug users, homosexual and bisexual men, and blood donors. *J. Infect. Dis.* **174,** 1320–1323.
58. Aikawa, T., Sugai, Y., and Okamoto, H. (1996) Hepatitis G infection in drug abusers with chronic hepatitis C. *N. Engl. J. Med.* **334,** 195–196.
59. Wu, R.-R., Mizokami, M., Cao, K., Nakano, T., Ge, X.-M., Wang, S.-S., Orito, E., Ohba, K.-I., Mukaide, M., Hikiji, K., Lau, J. Y. N., and Iino, S. (1997) GB virus C/ hepatitis G virus infection in Southern China. *J. Infect. Dis.* **175,** 168–171.
60. Schlueter, V., Schmolke, S., Stark, K., Hess, G., Openloch-Haehnle, B., and Engel, A. (1996) Reverse transcription-PCR detection of hepatitis G virus. *J. Clin. Microbiol.* **34,** 2660–2664.
61. Egawa, K., Yukawa, T., Arakawa, S., Nakao, H., Inoue, T., Tanaka, T., Tsuda, F., Okamoto, H., Miyakawa, Y., and Mayumi, M. (1966) Infection with GB virus C in leprous patients in Japan. *J. Med. Virol.* **49,** 110–114.
62. Nubling, C. M. and Lower, J. (1996) GB-C genomes in a hig-risk group, in plasma pools, and in intravenous immunoglobulin. *Lancet* **347,** 68.
63. Feucht, H-H., Zollner, B., Polywka, S., and Laufs, R. (1996) Vertical transmission of hepatitis G. *Lancet* **347,** 615.
64. Moaven, L. D., Hyland, C. A., Young, I. F. Bowden, D. S., Mccaw, R., Mison, L., and Locarnini, S. A. (1996) Prevalence of hepatitis G virus in Queensland blood donors. *Med. J. Australia* **165,** 369–371.
65. Brown, K. E., Wong, S., Buu, M., Binh, t. V., Be, T. V., and Young, N. S. (1997) High prevalence of GB virus C/hepatitis G virus in healthy persons in Ho Chi Minh City, Vietnam. *J. Infect. Dis.* **175,** 450–453.

8

Laboratory Diagnosis of Viral Hepatitis

Richard L. Hodinka

1. Introduction

Viral hepatitis is a disease of major concern, resulting in significant morbidity and mortality worldwide. Although a variety of viruses can cause hepatitis, the five most common viruses that are associated with liver disease include hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). These viruses represent a heterogeneous group of genetically unrelated agents which are hepatotropic. While HAV and HEV cause only acute liver disease, infection with HBV, HCV, and HDV can lead to either acute or chronic hepatitis. In 1994, Deka and colleagues *(1)* described an enteric agent, designated hepatitis F virus (HFV), which is thought to be associated with sporadic non-A–E hepatitis in humans. More recently, two additional viruses, hepatitis GB virus (GBV) and hepatitis G virus (HGV), have been described and linked to acute viral hepatitis *(2,3)*.

Clinical illness with these viruses can be difficult to distinguish, and the selection and use of specific laboratory studies is imperative for the accurate diagnosis and management of patients with viral hepatitis. Because hepatitis viruses do not readily grow in cell culture, serological testing is most often used for the laboratory diagnosis of these infections. Enzyme immunoassays (EIAs) are the most commonly used commercial tests, replacing earlier methods such as radioimmunmoassays (RIAs), hemagglutination tests, and latex agglutination assays. Multiple EIAs are currently available for the detection of specific antigen or antibody markers that distinguish the different hepatitis viruses. In combination, results from these tests can provide information for determining the virus type, stage of disease, degree of infectivity, and prognosis or for identifying those patients who are susceptible to or have had previous hepatitis infection. In certain clinical situations,

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

however, it also may be necessary to examine tissues and body fluids for viral antigens, nucleic acids, or intact viral particles. Histological techniques, *in situ* hybridization, the polymerase chain reaction (PCR), and electron microscopy can be quite useful as selective tools in the diagnosis and management of infections with hepatitis viruses.

This review describes current laboratory tests available for the diagnosis of infections with hepatitis viruses, the appropriate use and understanding of these methods, collection and handling of specimens for testing, and newly developed assays. The article should serve as an informative guide for clinicians and scientists working with this group of viruses.

2. General Concepts

2.1. Collection and Storage of Specimens

2.1.1. Specimens for Serology

Serum is the specimen of choice for the immunodiagnosis of hepatitis viruses, although plasma can be used as well. Remove the serum or plasma from the clotted blood or red cells, respectively, as soon as possible to avoid hemolysis. Accurate test results generally can be obtained using lipemic, icteric, or hemolyzed specimens. When possible, however, specimens that are clear and nonhemolyzed should be used to avoid any possible inconsistencies. Specimens containing particulate matter may give inconsistent results and should be clarified by centrifugation before testing. *Heat-treated specimens should not be used.* The serum or plasma should be refrigerated shortly after collection and during transport to the laboratory. Specimens may be stored at 2–8°C for up to 5 d. If an extended delay in transport or testing of a specimen is anticipated, freeze the serum or plasma to at least –20°C. Repeated freezing and thawing of specimens should be avoided.

2.1.2. Specimens for Histology

Impression smears, frozen sections, or formaldehyde-fixed and paraffin-embedded material can be used for the histological examination of tissue specimens for the detection of hepatitis virus antigens. Frozen or paraffin-embedded sections of tissue cut to 3–5 μm in thickness should be fixed to glass slides for staining. Impression smears can be prepared from fresh tissue by first slicing the tissue to expose a fresh surface and then firmly pressing the tissue to a glass slide. Cells from the tissue should be evenly distributed over a small area of the slide; avoid smearing the tissue onto the slide. All tissue specimens for impression smears or frozen sections should be placed in sterile saline or viral transport medium and refrigerated (2–8°C) immediately after collection and during storage or transport. Fresh tissue

should be frozen to at least –60°C if extended delays in transport are anticipated and should be transported to the laboratory on dry ice. Fixed tissue should not remain in formaldehyde for more than 24 h before being embedded in paraffin.

2.1.3. Specimens for Detection or Quantitation of Nucleic Acids

2.1.3.1. *In situ* Hybridization

Either frozen or paraffin-embedded tissue specimens can be successfully employed for *in situ* hybridization of specific nucleic acid probes. Freezing tissue offers the advantages of readily preserving RNA and DNA and inhibits the activity of tissue RNases. Frozen sections can be obtained from the tissue blocks as described above and placed on slides for processing. Disadvantages of using frozen specimens include the need to store tissue blocks at –70°C before sectioning and the possible distortion of tissue morphology during freezing of the specimen. Paraffin-embedded tissue is more widely used for *in situ* hybridization and offers the distinct advantage that specimens can be stored for extended periods. However, the sensitivity of *in situ* hybridization using paraffin-embedded tissues is greatly affected by the type of fixative employed, the length of time the tissue is fixed, and the time of fixation after specimen collection. As a general rule, tissue should be fixed immediately after excision and fixation should not exceed 24 h for buffered formalin or 12–16 h for Bouin's solution.

2.1.3.2. Molecular Amplification and Quantitation

Serum, plasma, tissue, and stool specimens can be employed for the qualitative detection of hepatitis virus nucleic acids using molecular amplification assays (e.g., PCR). Serum is the specimen of choice for molecular quantitation of HBV or HCV, although plasma can be used as well. The majority of the hepatitis viruses possess single-stranded RNA genomes, with the exception of HBV, which contains double-stranded DNA. Because RNA is especially vulnerable to degradation, improper collection, handling, and/or storage of specimens can adversely affect the results of molecular amplification methods *(4–6)*. For serum, collect 3–5 mL of blood in a sterile tube without anticoagulants. Allow the blood to clot at room temperature, and centrifuge within 1 h to separate serum from cells. Aseptically remove the serum from the clot within 4 h of blood collection. Plasma should be obtained from anticoagulated whole blood collected in EDTA, sodium citrate, or acid citrate dextrose and separated and removed from the cells as described for serum. Tissue specimens should be placed in viral transport medium following collection. For optimum results, all specimens for molecular amplification of hepatitis virus nucleic acids should be immediately frozen to –70°C

and, if necessary, transported to the laboratory on dry ice. It has been shown that loss of HBV DNA or HCV RNA levels in separated serum or plasma specimens is lowest at storage temperatures ≤4°C and increases with rises in temperature *(4–7)*. Holding serum or plasma at room temperature for extended times or repeated freezing and thawing of specimens should be avoided.

2.1.4. Oral Fluid Specimens

The use of whole saliva and oral mucosal transudates rich in gingival crevicular fluid has been advocated as a noninvasive alternative to the collection of blood for the detection of antibodies to hepatitis A, B, and C viruses *(8–10)*. Unstimulated saliva can be obtained by tilting the head forward and dribbling saliva from the lower lip into a graduated test tube fitted with a funnel. After 5 min, the subject expectorates any remaining saliva from the mouth. Dribbled saliva has a stability of 5 d at room temperature but can be stored for longer times at 4°C to –20°C *(9)*. Mechanical stimuli such as parafilm, paraffin wax, neutral gum base, or rubber bands can be used to collect stimulated saliva. Saliva from the parotid, submandibular, and sublingual glands can be obtained directly from the glandular ducts using specially designed collectors. Several commercial devices have been developed for the collection of oral mucosal transudate specimens. The devices provide a homogeneous specimen rich in plasma-derived IgG and IgM that is passively transferred to the mouth across the mucosa and through the gingival crevices (for a detailed description of the devices, *see* ref. *11*). Oral mucosal transudate specimens may be stored for 21 d at temperatures of 4–37°C or at –20°C for longer periods of time *(12)*.

3. Hepatitis A Virus

3.1. Direct Detection

Direct detection of hepatitis A virus in clinical specimens is not necessary for diagnosis, although finding virus in stools may be beneficial during epidemic outbreaks of HAV to identify individuals who may be shedding virus and are potential reservoirs of spread during the incubation or prodromal phases of infection. Such methods may also be beneficial for the identification of environmental sources of the virus. HAV appears in the blood and stool prior to the development of clinical symptoms (Fig. 1). Viremia is short and minimal, however, and it is unproductive to examine blood for HAV particles or antigen. Electron microscopy has been used to observe negatively stained hepatitis A virus particles in the stools of infected patients, although differentiating these small viruses (27–32 nm in size) from sur-

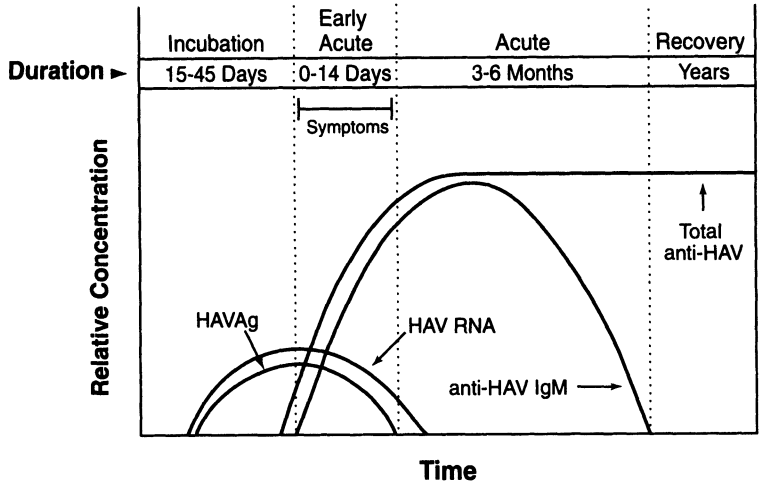


Fig. 1. Serologic patterns observed during acute hepatitis A virus infection. Abbreviations: HAVAg, hepatitis A virus antigen; HAV RNA, hepatitis A virus RNA; anti-HAV IgM, immunoglobulin M class antibody to hepatitis A virus; total anti-HAV; total antibody to hepatitis A virus. (Information courtesy of Abbott Laboratories.)

rounding particulate matter can be difficult. Immune electron microscopy has been used to enhance visualization of HAV through the formation of immune aggregates when specific antibody is complexed with virus particles *(13)*. However, this method is time consuming, expensive, and requires special equipment and a high level of technical expertise. Sensitive and specific EIAs and RIAs also have been developed for detecting HAV antigen in fecal specimens *(14–16)*. In general, none of these methods are practical in a clinical setting, however, since low concentrations of virus particles are present in feces during overt illness with HAV.

Isolation of HAV from clinical specimens can be accomplished, but the procedure is technically difficult. In vitro propagation of HAV was first accomplished in primary explant cultures of marmoset hepatocytes and in a fetal rhesus monkey kidney cell line (FRhK6) *(17)*. A number of primary and continuous cells of human or primate origin, including primary African green monkey kidney cells, primary human fibroblast cells, continuous human diploid embryonic lung fibroblasts, and human hepatoma cells have since been used to grow HAV in cell culture *(18–21)*. Most wild-type strains of HAV do not adapt well to cell culture, growing very slowly and requiring multiple blind passages before virus can be detected from clinical specimens *(18,21)*. Also, HAV produces little to no cytopathic effect in infected host cells, and growth must be detected using immunologic methods for viral

antigen or molecular hybridization assays for HAV RNA. Culture for HAV has been primarily used as a research tool for vaccine preparation and studies of viral replication.

More recently, molecular hybridization of HAV RNA with cloned HAV cDNA or HAV-specific RNA probes has been applied to the detection of HAV from clinical and environmental specimens *(22–24)*. PCR has also been described for the identification of HAV from clinical and environmental specimens and for defining the genetic relatedness of HAV strains recovered from different geographical locations *(25–28)*. Detection of HAV RNA by PCR has also been used to establish the transmission of hepatitis A virus to hemophiliac patients by contaminated factor VIII and factor IX concentrates *(29–31)*.

3.2. Serology

Acute or past infection with HAV is best diagnosed by detecting specific antibodies in the sera or plasma of individual patients. Commercial solid-phase antibody capture EIAs and RIAs are used for the detection of IgM antibodies to HAV *(32,33)*; a positive test provides presumptive evidence of acute or recent HAV infection. The solid phase is coated with a goat antihuman antibody specific for the μ-chain of human IgM. Anti-HAV IgM from a patient's serum is captured on the solid phase and detected by the sequential binding of HAV antigen and labeled human anti-HAV antibody to the bound IgM antibody. Hemadsorption of erythrocytes to antigen-antibody complexes captured to a solid phase has also been used to indicate the presence of HAV-specific IgM antibodies *(34)*. IgM-specific anti-HAV is present from the onset of symptoms and declines to nondetectable levels within 3–6 mo in most patients (Fig. 1) *(35,36)*. Low levels of anti-HAV IgM may persist for more than 1 yr in some individuals. IgG and IgA anti-HAV rises simultaneously with the production of IgM anti-HAV, and all three antibodies can be detected in competitive RIAs or EIAs for total anti-HAV *(37,38)*. In the total anti-HAV assay, anti-HAV present in test sera competes with a known amount of added labeled anti-HAV antibody for binding to HAV antigen attached to the solid phase. The more anti-HAV in the test sample, the less labeled anti-HAV bound to the solid support. In the absence of acute HAV infection, IgG anti-HAV is the predominate antibody detected in the total anti-HAV assay and persists for life *(36)*. Testing of serum for total anti-HAV is used to determine the immune status of an individual, to assess a person's risk for traveling to an endemic region or working in a high-risk area, and to assist in making decisions to administer HAV vaccine or immune globulin prophylaxis following exposure to HAV. A positive result for total anti-HAV in the absence of IgM anti-HAV indicates

previous infection with HAV (or successful immunization) and protection against reinfection. Recipients of intraveneous immunoglobulin, newborn infants possessing passively acquired maternal antibody, or patients who have received recent blood transfusions may be positive for total anti-HAV.

Testing oral fluids for antibody to hepatitis A virus has been shown to be a simple and convenient alternative to using serum or plasma, with wide application in the management of patients and community outbreaks *(8–10,39)*. Screening assays primarily intended for serum or plasma have been modified for use with oral fluid specimens and can reliably detect IgM or total anti-HAV antibodies in the saliva of patients with acute or past hepatitis A infection. Since the concentration of immunoglobulins in oral fluids is less than that in blood, assay modifications have included increasing the sample volume, decreasing the sample diluent volume, increasing the specimen incubation time, and lowering the optical density cutoff from that used with serum or plasma. When such changes are made and assay protocols are optimized to accept oral fluid specimens, the sensitivities and specificities of these assays are equal to those from testing serum or plasma. Blood spots dried on filter paper and urine specimens have also been shown to be acceptable specimens for the detection of antibodies to HAV *(40,41)*.

4. Hepatitis B Virus

4.1. Serologic Diagnosis

Infection with HBV is complex and normally results in the sequential production of HBV-specific antigens and antibodies in the serum of an infected patient. The most practical and reliable way of making a laboratory diagnosis of HBV infection involves the detection of these serologic markers (*see* refs. *42,43* for extensive reviews). A number of commercial solid-phase antibody capture and competitive inhibition RIAs and EIAs are available and are well established for the identification of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), total antibody to hepatitis B core antigen (anti-HBc Total), IgM antibody to hepatitis B core antigen (anti-HBcIgM), antibody to HBeAg (anti-HBe), and antibody to HBsAg (anti-HBs). These assays are routinely used to differentiate acute from chronic HBV hepatitis, to evaluate the infectivity or immune status of a patient, and to screen blood products and organ donors. Tests for the direct detection of HBV core antigen are not available, since HBcAg is rarely detected during acute infection, and its presence has not been documented during chronic illness. Some assays have been adapted to automated instruments, making them convenient and simpler to perform.

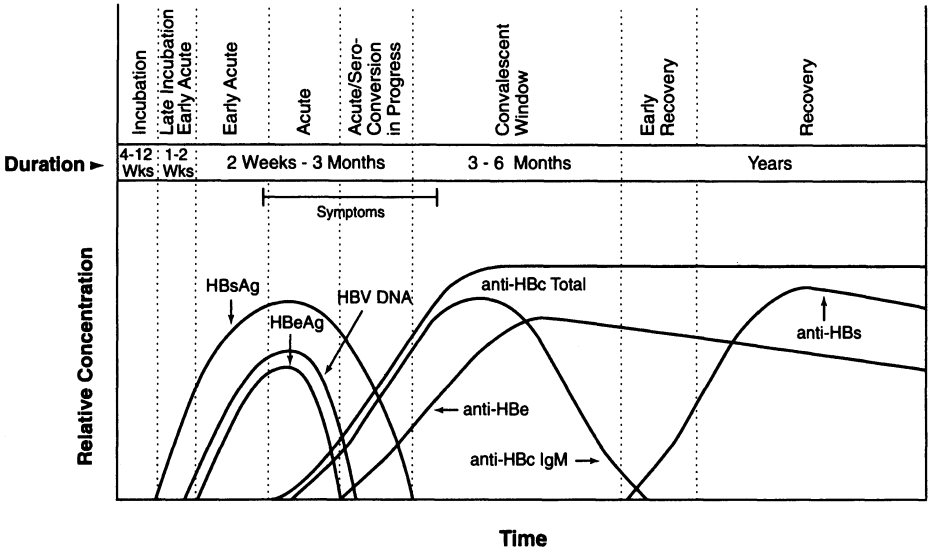


Fig. 2. Serologic patterns observed during acute hepatitis B virus infection. Abbreviations: HBsAg, hepatitis B surface antigen; HBV DNA, hepatitis B virus DNA; HBeAg, hepatitis B e antigen; anti-HBc IgM, immunoglobulin M class antibody to hepatitis B core antigen; anti-HBc total, total antibody to hepatitis B core antigen; anti-HBe, antibody to hepatitis B e antigen; anti-HBs, antibody to hepatitis B surface antigen. (Information courtesy of Abbott Laboratories.)

4.1.1. Acute Infection

The typical serological course of acute HBV infection can be seen in Fig. 2. HBsAg appears 3–5 wk before the onset of symptoms, peaks during the acute phase of the disease, and slowly declines to undetectable levels within 4–6 mo *(44)*. Detection of HBsAg indicates the presence of HBV but does not distinguish between acute and chronic infection. In rare cases of acute fulminant hepatitis caused by HBV, HBsAg may be rapidly cleared from the bloodstream and may not be detected in the serum at the time of presentation *(45,46)*. HBeAg appears concurrently with HBsAg and usually disappears during clinical illness and before the disappearance of HBsAg *(47,48)*. Measuring the presence of HBeAg in the serum of infected patients indicates active viral replication, high concentrations of virus in the blood, and high infectivity *(49)*. HBeAg is not detected in the absence of HBsAg, but a negative result for HBeAg in a patient with detectable HBsAg may indicate lower communicability. Appearance of antibodies to HBeAg indicates a decrease in viral replication and is prognostic for resolution of the

disease *(50)*. Anti-HBeAg levels decline with time and are no longer detectable within 6 mo in one third of patients. A positive test for IgM anti-HBc is diagnostic for acute or recent HBV infection *(51)* and may be the only marker present in acutely ill neonates and patients with acute fulminant HBV infection who may not have detectable HBsAg. IgM anti-HBc is present at the onset of acute hepatitis and persists for 3–12 mo before declining to indetectable levels *(52)*. Total antibody (IgG and IgM) to HBV core antigen develops within 1–4 wk of the appearance of HBsAg, rapidly rises to high levels, and is detectable for the remainder of a patient's life *(49)*. This marker is an indicator of current or previous HBV infection. Following the loss of IgM anti-HBc during acute HBV infection, the total anti-HBc response primarily consists of IgG anti-HBc. Anti-HBs generally appears during early convalescence and usually after HBsAg is no longer detectable. This antibody persists for life and is a marker of recovery and immunity *(50,53,54)*. Anti-HBs also develops following administration of HBV vaccine. Both anti-HBsAg and total anti-HBc antibody test results are needed to determine the immune status or to access the recovery of an individual following natural HBV infection, while only the anti-HBs result is required to monitor vaccine efficacy. Negative results for total anti-HBc and anti-HBsAg help identify those persons who are susceptible to HBV infection and who may benefit from vaccination. Positive results may be observed for anti-HBsAg and total anti-HBc because of passive transfer of antibodies in patients receiving immune globulin and clotting factors and in neonates born to mothers with recent or past HBV infection. A lack of or loss of detectable levels of anti-HBs may occur in some patients following acute HBV infection despite continued immunity *(55)*. In the absence of anti-HBs, the presence of anti-HBe and anti-HBc total antibodies can also indicate convalescence of the disease and prior HBV infection.

4.1.2. Chronic Infection

The serological patterns for chronic HBV infection are shown in Fig. 3. Persistence of HBsAg for 6 mo or longer or the persistence of HBeAg for more than 8–10 wk after acute illness is indicative of chronic infection *(48)*. HBsAg can be detectable for life in most individuals, and only 1–2% of chronic HBV carriers become negative for HBsAg each year *(56,57)*. Patients with chronic HBV infection who are positive for HBeAg are highly infectious and are at increased risk of eventually developing severe liver disease. Screening HBsAg-positive pregnant women for HBeAg can identify those individuals who may transmit HBV to their newborn infants. If an HBsAg-positive woman is also HBeAg positive, her infant will have a 90% chance of aquiring chronic HBV infection compared to fewer than 10% of

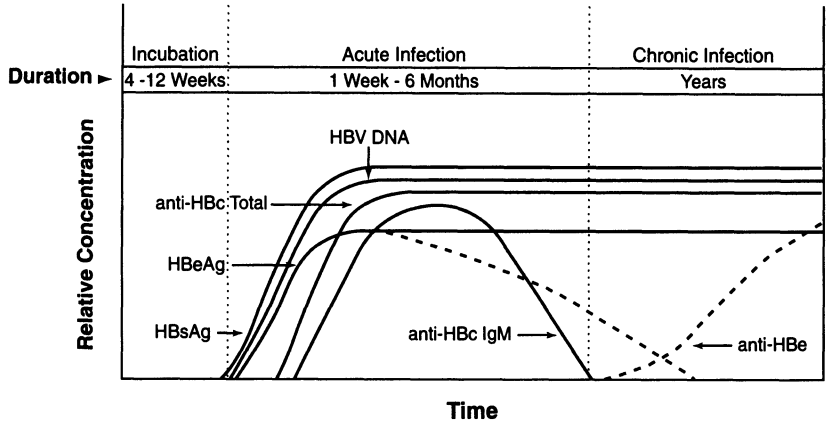


Fig. 3. Serological patterns observed during chronic hepatitis B virus infection. Abbreviations: HBsAg, hepatitis B surface antigen; HBV DNA, hepatitis B virus DNA; HBeAg, hepatitis B e antigen; anti-HBc IgM, immunoglobulin M class antibody to hepatitis B core antigen; anti-HBc total, total antibody to hepatitis B core antigen; anti-HBe, antibody to hepatitis B e antigen. (Information courtesy of Abbott Laboratories.)

infants whose mothers are anti-HBe positive. HBeAg is spontaneously lost over time in approx 50% of patients with the subsequent development of anti-HBe antibody and cessation of active viral replication and a decrease in viremia *(58–60)*. Treatment of patients with interferon (IFN) α accelerates the clearance of HBeAg and reduces the duration of active liver disease *(61)*. HBsAg may persist for years after the loss of HBeAg and may subsequently clear in a small percentage of patients. Total anti-HBc antibody is present in virtually all patients with chronic HBV infection and persists at high levels. IgM anti-HBc can persist at low levels during chronic infection, but this response goes unnoticed because commercial assays for IgM anti-HBc have been designed to only detect high concentrations of this antibody during acute disease *(51,52)*. Anti-HBs is absent in the majority of chronic HBV carriers, although low concentrations of anti-HBs in the presence of HBsAg can be observed in some individuals. The clinical and diagnostic significance of the coexistence of HBsAg and anti-HBs during chronic infection is unclear. In some patients it is thought to represent heterotypic antibody against HBsAg subdeterminants not present in serum *(62)*, whereas in others it may represent the formation of immune complexes *(45)*, advanced chronic liver disease *(63)*, or an association with renal disease *(64)*.

Patients positive for HBsAg and anti-HBe antibody who have cleared their HBeAg may spontaneously reactivate their chronic infection or undergo

reactivation following treatment with chemotherapeutic agents *(65,66)*. Both IgM anti-HBc and HBeAg may reappear at detectable levels in these patients.

4.1.3. Significance of Isolated Antibody to Hepatitis B Core Antigen

Testing for HBV infection may lead to the detection of an isolated positive result for total anti-HBc antibodies with negative results for HBsAg and anti-HBs antibody. This may happen in 10–55% of all positive total anti-HBc samples *(67)*. There are several reasons why this phenomenon may occur, including

1. a false-positive reaction in the serologic assay for total anti-HBs,
2. passive transfer of total anti-HBc antibody as described above,
3. chronic infection without detectable HBsAg as a result of the development of viral mutants and the subsequent production of undetectable levels of antigen in serum,
4. suppression of HBsAg production following HDV superinfection, and
5. acute infection involving the "core window" period between the decline of detectable HBsAg and the subsequent rise in anti-HBs antibody.

Low titers of total anti-HBc antibody usually represent nonspecific reactions and account for a large number of the isolated total HBc results, particularly in individuals at low risk for HBV infection. However, detection of high titers of isolated total anti-HBc may truly represent HBV infection and the potential for HBsAg-negative transmission of the virus through blood or organ donation. Analysis of other markers, including IgM anti-HBc, HBeAg, anti-HBe and HBV DNA may assist in better defining specific total anti-HBc reactions and preventing the spread of HBV infection.

4.2. Direct Detection

4.2.1. Detection of Hepatitis B Virus DNA.

4.2.1.1. Qualitative Determinations

Detection of HBV DNA in serum or tissue of infected patients has been shown to be a more direct and sensitive means of measuring viremia and viral infectivity than conventional serological tests for either HBsAg or HBeAg *(68,69)* and is an early predictor of the effects of antiviral agents on HBV replication *(70)*. Conventional Southern blot, dot blot and slot blot nucleic acid hybridization techniques *(49,68,71–77)*, liquid hybridization assays *(70,78,79)*, and PCR *(69,80–82)* have been developed for the detection of HBV DNA. The liquid hybridization method has a lower limit of detection of 1.6 pg/mL (4.5×10^5 genome equivalents/mL) *(68)* and is considered to be more sensitive than slot blot and dot blot hybridization assays, which have a sensitivity of 10 to 500 pg/mL (2.8×10^6 to 1.4×10^7 genome

equivalents/mL) *(49)*. PCR is considered to be the most sensitive method and may be positive when other HBV DNA tests are negative *(49,83,84)*. HBV DNA can be detected in serum before biochemical or serological evidence of hepatitis *(69)* and can be found in patients with either acute or chronic disease *(78,84)*. HBV DNA has been demonstrated in blood, liver, or mononuclear cells of HBsAg-negative patients with acute and chronic HBV infection *(85–88)* and has also been found in healthy blood donors having no HBV serological markers or with combinations of seropositivity to anti-HBs, total anti-HBc and anti-HBe *(89)*. In addition, a significant number of apparently healthy people with normal liver function who are either seronegative for HBsAg or seropositive for anti-HBs also possess levels of detectable HBV DNA *(90)*, suggesting that negative serologic testing may not be sufficient to exclude HBV infection. Active replication of HBV DNA also has been documented in a variety of extrahepatic sources, including a number of solid organs, cervicovaginal cells, saliva, urine, and seminal fluid *(76,91,92)*. Thiers et al. *(87)* used PCR to identify and characterize HBV DNA sequences in three patients negative for all serological markers of HBV. Transmission of virus from the sera of these patients with subsequent development of acute hepatitis was demonstrated in inoculated chimpanzees. Finally, when HBV DNA is present in the serum of HBsAg/anti-HBe-positive HBV carriers, active chronic liver disease is often found and appears to be related to continued replication and secretion of HBV *(93,94)*.

4.2.1.2. Quantitative Determinations

Various quantitative assays have been developed and evaluated for measuring levels of HBV DNA in sera of infected patients *(95–99)*. These assays are commercially available and include several solution hybridization kits *(70,78,79)*, a dot blot assay *(77)*, and methods involving target *(100)* and signal amplification *(101)*.

Quantitation of HBV DNA may be the best marker of active viral replication and disease progression *(102)*, as HBV DNA levels in serum, unlike the traditional serologic markers of HBV infection, have been shown to correlate with biochemical and histological measures of disease *(103–105)*. Quantitation of HBV DNA has been used to predict a response to IFN treatment *(101,106,107)* as a loss of detectable HBV DNA levels correlates with improved clinical outcome and reappearance of HBV DNA predicts relapse. High initial levels of HBV DNA are predictive of a poor response to IFNα therapy and for a poor prognosis following transplantation. Quantitative assays have also allowed for a more accurate detection of certain genetic variants of HBV *(95,101,108–110)*. The concentration of HBV DNA in serum also correlates with the infectivity of blood products and the potential

infectiousness of HBV carriers *(96)*. With the availability of new treatment options and combination therapy for chronic hepatitis, the value of monitoring quantitative levels of HBV in infected patients will continue to grow.

4.2.2. Other Methods for Direct Detection

HBV has been successfully propagated in primary cultures of adult or fetal human hepatocytes *(111–113)*, and such culture systems have been used in studies of virus-host cell interactions. The availability of a constant supply of human liver tissue, however, limits the utility of cell culture for laboratory diagnosis of HBV infection. Cloned HBV DNA has been transfected into established cell lines with the replication of HBV DNA, expression of viral proteins, and the production of viral particles *(114–118)*. These transfected cells have been used to study intracellular events of HBV replication and for the evaluation of developed antiviral agents *(119,120)*.

HBV-associated antigens or particles can be directly detected in clinical specimens using a variety of techniques, including electron microscopy, immunofluorescence, immunohistochemistry, and *in situ* hybridization *(121–126)*. Similar to the described cell culture methods, these procedures are mainly being used to study the natural history of HBV infection and to provide information on viral replication and the degree of gene expression within infected cells.

4.2.3. Hepatitis B Virus Variants

Mutations in the precore/core gene of HBV in patients with hepatitis have led to the development of precore genetic variants that do not produce HBeAg *(73,93)*. These variants are thought to be associated with severe chronic active hepatitis or acute fulminate hepatitis *(127–129)*, although these same strains have been found in patients without active liver disease *(130,131)*. Patients possessing a precore variant will have negative results for both HBeAg and anti-HBeAg in their serum. Replication of these variants can be demonstrated by detection of HBV DNA in the serum or HBcAg in a liver biopsy *(127)*. Also, the development of mutations in the preS/S region of the surface antigen gene of HBV results in the production of genetic variants, termed escape mutants, that are no longer neutralized by anti-HBs and, therefore, are capable of evading the immune response induced by HBV vaccines *(132,133)*. Patients harboring these escape mutants may experience a loss of detectable HBsAg, but they will continue to have circulating HBV DNA. Therefore, it is important to look not only for HBsAg but other HBV markers in the serum of patients with suspected hepatitis. Genotyping procedures have been developed to identify and characterize both precore/core and preS/S variants from serum samples of patients with acute and chronic HBV hepatitis *(134–138)*.

5. Hepatitis C Virus

5.1. Serology

Detecting HCV-specific IgG antibody by enzyme immunoassay and recombinant immunoblots *(139–141)* remains the first choice for diagnosis of HCV infection and to screen blood products and organ donors. Antibodies to HCV become detectable during the course of illness, with 50–70% of patients having anti-HCV antibodies at the onset of symptoms and approx 90% of patients having an HCV-specific antibody response within 3 mo after onset of infection. Anti-HCV antibodies persist in the serum of most patients that have been infected *(142,143)*, but loss of antibody may occur in individuals with resolved acute infection *(142,143)*, immunosuppressed organ recipents and those with HIV *(144–146)* and following IFN therapy *(147,148)*. Antibodies to HCV can be demonstrated in virtually all patients with chronic HCV hepatitis. The presence of IgM antibodies in patients infected with HCV is intermittent, and detection of this class of antibodies is limited *(149,150)*.

5.1.1. Enzyme Immunoassays

The complete HCV genome has been cloned and sequenced and encodes for a nucleocapsid, an envelope, and five nonstructural proteins *(151,152)*. The original clone, termed 5-1-1, was derived from the nonstructural (NS) region 4 of the viral genome and led to the development of a recombinant polypeptide (c100-3), composed of the gene product of three overlapping clones from a portion of the NS3 region and nearly all of the NS4 region of the HCV genome *(139)*. This polypeptide was expressed in yeast cells as a fusion protein with human superoxide dismutase (SOD) and used as the antigen for a first-generation EIA for the detection of antibodies to HCV. Although this test was an important advance in the diagnosis of HCV infections, the first-generation assay had several problems, including a lack of acceptable sensitivity and specificity *(153,154)*. Using this test, a sensitivity of 80–90% was seen in patients with risk factors and elevated liver aminotransferase (ALT) levels, suggesting chronic hepatitis *(155)* and approx 60% in volunteer blood donors *(156)*. False-positive results were obtained from stored sera *(157)* and sera from tropical communities *(158)* and observed in patients with antibodies to superoxide dismutase, rhematoid factors, hyperglobulinemia, and paraproteinemia *(154,159,160)*. The first-generation assay also was of little use in the diagnosis of acute HCV infection, as antibody to HCV was detected, on average, 10–15 wk after onset of hepatitis, but as long as 6–12 mo in some cases *(161,162)*.

This led to the production of a second-generation assay that was approved by the Food and Drug Administration in 1992 and is currently

used for the detection of HCV antibodies. This assay contains multiple recombinant HCV antigens, including the c100-3 polypeptide and the c22-3 and c33c epitopes from the nucleocapsid (core) and NS3 regions of the HCV genome, respectively. The addition of these antigens resulted in an increased sensitivity for the identification of HCV infection in blood donors and patients with acute and chronic non-A, non-B hepatitis and has resulted in detection of antibody to HCV as early as 1–6 wk after the onset of clinical symptoms *(141,153,155,156,163–167)*. The second-generation assay detects approx 10–30% more anti-HCV antibody positive individuals than the first-generation assay, because of the identification of antibodies to c22-3 and c33c in persons who lack a response to the c100-3 polypeptide. The specificity of the second generation assay has also been improved over that previously observed for the first-genration test *(154,166–168)*. Recently, a third-generation assay has been developed, which incorporates an additional recombinant antigen corresponding to the NS5 region *(169,170)*. The third-generation assay has been shown to be comparable with the second-generation test, without a significant improvement in sensitivity or specificity. This assay is currently used in Europe, but is not available in the United States.

5.1.2. Recombinant Immunoblot and Dot Blot Assays

Recombinant strip immunoblot assays (RIBA) have been used as supplemental procedures for testing samples that are positive for HCV antibodies by EIA to help in distinguishing specific from nonspecific reactivity. The first-generation assay contained two recombinant HCV antigens (c100-3, produced in yeasts, and 5-1-1, produced in *Escherichia coli*) fused to human superoxide dismutase and immobilized as individual bands onto nitrocellulose strips *(140)*. A second-generation RIBA has been developed and utilizes two additional recombinant antigens from the virus core (c22-3, produced in yeast) and the NS3 region (c33c, produced in E. coli) *(171)*. The second-generation RIBA has been reported to be more sensitive and specific than the first-generation assay and has subsequently replaced this test *(171,172)*. Examples of results using the second-generation strip immunoblot assay are depicted in Fig. 4. Positive results are represented by antibody reactions with two or more bands representing at least two different HCV gene regions with intensities equal to or greater than that of the low-level IgG control and no reaction with the SOD band. Specimens having no visible HCV-specific bands or only an SOD band are considered to be negative. A specimen is considered to be indeterminate for antibody to HCV if a reaction occurs with only one HCV gene region or if the SOD band is seen in addition to reactivity to HCV antigens. Indeterminate reactions are common using the second-generation assay, and most of these represent

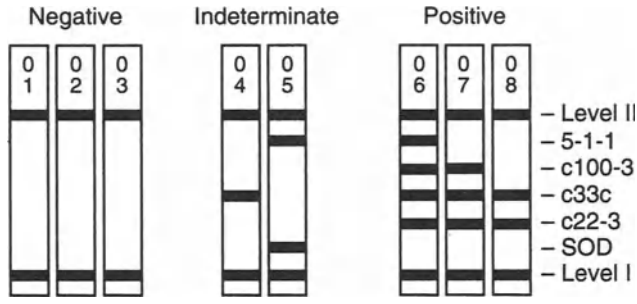


Fig. 4. Results of the RIBA HCV 2.0 strip immunoblot assay for the qualitative detection of antibody to hepatitis C virus. Strips 01–03: negative; the visible bands represent reactions with low- and moderate-level IgG controls. Strip 04: indeterminate; reaction with only one HCV gene region. Strip 05: indeterminate; reaction with the SOD carrier protein and one HCV gene region. Strips 6–8: positive; reactions with two or more bands representing at least two different HCV gene regions with intensities equal to or greater than that of the low-level IgG control and no reaction with the SOD band.

immunoreactivity to c22-3 only *(173,174)*. A third-generation assay has been developed and uses synthetic peptides for c100-3 and c22-3 to prevent nonspecific cross-reactivity *(175,176)*. In addition, recombinant polypeptides from c33c and the NS5 region are present, while the 5-1-1 epitope has been removed. This assay is available on the international market but not in the United States. The current manual procedures for performing either the second- or third-generation assays are labor intensive and time consuming. However, a new system has recently been developed for automated processing and analysis of the RIBA immunoblot strips *(177,178)*.

Two additional supplemental assays have been developed and evaluated for detection of HCV antibody *(176)*. The first is a dot blot assay containing four recombinant antigen dots representing the core, NS3, and NS4 regions of the HCV genome. The second test is an immunoblot assay with five synthetic bands (two from the viral core and one each from the E2/NS1, NS4, and NS5 regions) and one recombinant (NS3) antigen band. These assays are available commercially in Canada and Europe.

Supplemental testing is indicated for volunteer blood donors with positive EIA results and individuals with normal serum ALT levels and no risk factors, but it is unnecessary in patients who are HCV antibody positive by EIA, are at risk for the virus, and have clinical and/or biochemical signs of liver disease. If both screening and supplemental tests are positive, however, the likelihood that a patient is infected with HCV is high *(179)*.

5.2. Direct Detection

The "window period" from infection to seroconversion can be quite long for HCV, as antibody responses may not be detectable until ≥12 wk after the onset of hepatitis *(180)*. Also, approx 15% of patients who are antibody positive for HCV may represent acute rather than chronic infection. Therefore, sensitive molecular assays have been developed for the direct detection and quantitation of HCV RNA in serum and liver. Following exposure, HCV RNA can be detected in blood within 1–3 wk. Recovery is characterized by disappearance of HCV RNA from the blood and return of liver enzymes to normal. Approximately 85% of HCV-infected individuals fail to clear the virus by 6 mo and develop chronic hepatitis with persistent viremia.

5.2.1. Detection of Hepatitis C RNA

5.2.1.1. Qualitative Determinations

Detection of HCV RNA is considered to be the most reliable and direct marker for viral replication and infectivity of the virus. Hepatitis C virus possesses a single-stranded, positive-sense RNA which is 9.4 kb in length. The 5' untranslated region of the virus is highly conserved among the HCV genotypes that have been identified to date *(181–183)* and has been selected by most investigators as the site for developing oligonucleotide primers and probes for detection of HCV using reverse transcriptase (RT)-PCR *(184–188)*. RT-PCR has proven to be a rapid, sensitive, and useful method for the detection of HCV infections *(179,184–196)* and is considered to be the gold standard for identifying this agent in clinical specimens. The assay can detect HCV-infected individuals prior to specific antibody production and can discriminate chronic HCV infections from resolved acute infections in patients who are positive for HCV antibody. The procedure can also be used to

1. diagnose HCV infections in newborns of HCV-infected women,
2. resolve indeterminate serologic results,
3. select and monitor patients that are most likely to benefit from antiviral therapy, and
4. identify HCV infection in high-risk, seronegative individuals.

RT-PCR has also made significant contributions to our understanding of the natural history and pathogenesis of this unculturable infectious agent.

With RT-PCR, HCV viremia is detected within 10–19 d after infection *(197)*, well before the onset of hepatitis, and generally persists throughout chronic infection. HCV RNA may not be detectable in serum during all stages of infection, as fluctuating levels of RNA are commonly observed. Therefore, a single negative HCV RNA test does not always indicate that an

individual is uninfected or has responded to appropriate antiviral therapy. Sustained loss of detectable HCV RNA in serum may indicate response to treatment. HCV RNA can be detected in 50–75% of serum samples from random, seropositive blood donors *(190,191)* and can be detected in as many as 100% of sera from seropositive symptomatic patients and from seropositive blood donors with elevated ALT levels *(179,187,192–194)*. HCV RNA also has been detected in liver tissue *(198,199)* and in peripheral blood mononuclear cells *(200,201)*. A commercial HCV RT-PCR assay has been developed and has performed favorably in preclinical and clinical trials *(202–204)*. More recently, this assay has been successfully adapted to an automated instrument *(205,206)*. A novel, ligation-dependent PCR assay using two DNA capture probes for RNA isolation and two DNA hemiprobes for subsequent target amplification also has been described *(207)*.

5.2.1.2. Quantitative Determinations

The development of molecular assays to quantitate the levels of HCV in infected patients has proven to be a valuable tool to assess the progression of acute and chronic HCV hepatitis, identify and monitor those patients who will derive the most benefit from IFNα therapy, recognize nonresponders and relapsing infection, and to facilitate our understanding of the natural history and pathogenesis of this virus. Molecular methods, such as quantitative competitive PCR, quantitative RT-PCR, nucleic acid sequence-based amplification, and branched-chain technology are now available for the accurate quantitation of HCV *(208)*.

Studies using molecular technology to quantitate serum levels of HCV RNA have shown that the degree of HCV viremia correlates with the stage of disease *(209)*, and that HCV RNA levels are significantly higher in patients coinfected with HIV than in those patients with HCV alone *(210)*. The level of circulating HCV may play a role in viral transmission, as mothers coinfected with HIV and HCV are more likely to transmit HCV to their infants than mothers infected only with HCV *(211)*. Measurements of serum HCV RNA concentrations have also provided useful information about the in vivo viral dynamics and the pathogenesis of HCV infection *(212)*. Quantitative determination of HCV in serum may also provide a marker for monitoring therapeutic intervention. Shindo et al. *(213)* have shown that a clinical response to IFNα in patients with chronic hepatitis C infection is associated with a loss of detectable HCV RNA from serum. Other investigators have demonstrated that the presence of greater amounts of HCV RNA prior to the initiation of therapy is associated with patients who either show an initial response with relapse or show no response to IFNα, and that patients with low HCV RNA levels show a more sustained, positive response to treatment

(214–222). Davis et al. *(218)* have suggested that monitoring HCV RNA levels in serum during IFN treatment may allow for logical changes in therapy through dose adjustments and determination of appropriate end points for treatment. A method has been developed for quantitating HCV RNA levels in the liver of patients with chronic hepatitis, but the clinical use of such an assay remains to be defined *(223)*.

Comparisons of different quantitative assays for measuring HCV RNA levels have been reported *(224–232)*, with differences observed in sensitivity, linearity, precision, and dynamic range of quantification.

5.2.2. Other Methods for Direct Detection

Additional methods have been developed for the direct detection and diagnosis of HCV infection, although such procedures have been used solely as research tools. Limited replication of HCV has been documented following propagation of the virus in the human T-cell lines MOLT-4 and HPB-Ma *(233,234)*. Primary cultures of human fetal and adult chimpanzee hepatocytes *(235,236)* and a human bone-marrow-derived B-cell line *(237)* also can be infected in vitro with HCV. Recently, Yoo et al. *(238)* have established a long-term culture persistently infected with HCV following transfection of a human hepatoma cell line (Huh7) with in vitro-transcribed HCV RNA. Such culture systems may prove useful in studies of the replication, persistence, and pathogenesis of HCV and for further development of diagnostic tests. HCV antigens can be detected within hepatocytes and mononuclear cells using immunological techniques *(239,240)* and *in situ* hybridization assays *(241,242)* have been developed to detect HCV RNA within these cells. The development of antigen assays for the detection of HCV in serum has been hampered by the low levels of circulating virus found in the blood of infected patients.

5.2.3. Hepatitis C Virus Genotypes

Hepatitis C virus demonstrates considerable nucleic acid sequence variability *(243)*, and the development of molecular and serological genotyping assays for distinguishing genetic variants of this agent has been shown to be clinically important for the management of infected patients *(244–246)*. Viral genetic variation may impact on the overall course of disease with this virus and may affect detection of HCV infection and the response of patients to antiviral therapy. Infections with HCV genotype 1b, for instance, may be associated with more severe liver disease and progression to hepatocellular carcinoma *(247–249)*. Evidence is accumulating to suggest that the reactivity of current HCV antibody assays is dependent on the particular viral genotype *(250–253)*. Future assays may need to be designed to include multiple genotype-specific antigens or be targeted to specific genotypes observed in

a given geographic region or population. Also, failure of patients with HCV infection to respond to α-IFN therapy appears to be the result of the presence of a particular viral genotype *(221,254–257)*. Infection with either genotype 1a or 1b is associated with a poor response to IFN treatment compared with infection with types 2a, 2b, 3a, and 3b.

Molecular assays for HCV genotyping have relied on the examination of sequence polymorphisms in variable regions throughout the 5'-untranslated, core, or NS5 regions of the viral genome. Techniques such as direct nucleic acid sequence analysis *(258)*, restriction fragment-length polymorphism analysis *(259,260)*, RT-PCR with type-specific or subtype-specific primers *(261,262)*, and allele-specific oligonucleotide hybridization *(263,264)* have been developed. More recently, cleavase fragment-length polymorphism (CFLP) analysis *(265)* and a heteroduplex tracking assay (HTA) *(266)* have been introduced. The CFLP assay relies on the formation of unique secondary structures that result when DNA is allowed to cool following brief heat denaturation. These structures serve as substrates for the structure-specific Cleavase I enzyme, resulting in the generation of unique collections of cleavage products or structural fingerprints for each sequence analyzed. Use of CFLP analysis on amplicons generated by RT-PCR amplification of the HCV 5'-untranslated region allows for differentiation of the viral genotypes. The HTA involves hybridizing single-stranded probes from the core region of the virus to amplicons generated by RT-PCR and electrophoresis of the hybridization products on gels. The formation of a heteroduplex band on a gel indicates genotype and subtype.

An enzyme immunoassay for serological determination of HCV genotypes also has been produced and can differentiate HCV genotypes 1–6 using peptides from the NS4 region of the viral genome *(267–270)*. Serologic assays based on peptides from the core and recombinant polypeptides from the NS4 region that only distinguish between genotypes 1, 2, and 3 have also been described *(271–274)*. Serotyping for HCV has limited utility, because virus subtypes cannot be differentiated with this method and the assays are not suitable for use in immunocompromised patients *(269)*.

Comparative studies of the concordance between various HCV genotyping and serotyping systems have been accomplished *(269,270,273–277)*.

6. Hepatitis D Virus

Hepatitis delta virus is a defective RNA virus that requires the helper function of HBV for replication and expression *(278–281)*. HDV infection can occur as an acute coinfection with HBV or as an acute or chronic superinfection of an HBV chronic carrier (Fig. 5). HDV hepatitis should be con-

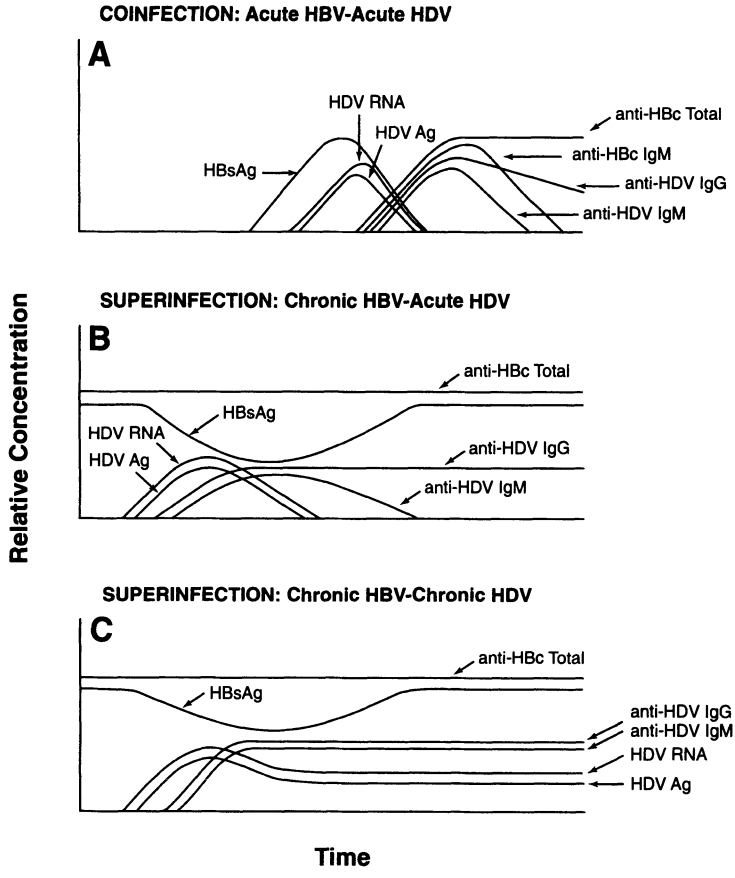


Fig. 5. Serological patterns observed during hepatitis D virus infection. **(A)** Acute coinfection with hepatitis B and hepatitis D viruses. **(B)** Acute superinfection of hepatitis D virus in a chronic carrier of hepatitis B virus. **(C)** Chronic superinfection of hepatitis D virus in a chronic carrier of hepatitis B virus. Abbreviations: HBsAg, hepatitis B surface antigen; anti-HBc IgM, immunoglobulin M class antibody to hepatitis B core antigen; anti-HBc total, total antibody to hepatitis B core antigen; HDV Ag, hepatitis D virus antigen; HDV RNA, hepatitis D virus RNA; anti-HDV IgM, immunoglobulin M class antibody to hepatitis D virus antigen; anti-HDV IgG, immunoglobulin G class antibody to hepatitis D virus antigen.

sidered in any person who is HBsAg positive or who has evidence of recent HBV infection, especially when there is fulminant acute HBV hepatitis or chronic HBV infection that suddenly becomes more progressive and severe. The diagnosis of HDV is primarily made on the basis of serologic tests for the detection of total (IgG and IgM) anti-HDV antibody, IgM anti-HDV antibody, and HDV antigen in the serum of infected individuals. Solid-phase

antibody capture RIAs and EIAs are used for the detection of IgM antibodies to HDV *(282–285)*, while total anti-HDV antibodies are identified using competitive inhibition and blocking immunoassays *(286–288)*. The HDV antigen for these assays is derived from either infected liver or infectious serum of humans or animals and a reliable source of quality reagent can be difficult to maintain. More recently, EIAs using recombinant expressed HDV antigen have been developed *(289,290)*, and a dipstick immunobinding assay *(291)* has been used as a simple and economical method for detection of HDV antibodies. The monitoring of serum for HDV antigen has been accomplished using solid-phase immunoassays *(286,292)* and by Western blot analysis *(293–295)*. The Western blot assay has been advocated for use in patients with chronic HDV infection, since HDV antigen is difficult to detect by EIA or RIA in this group because of the formation of antigen-antibody complexes in the presence of high titers of anti-HDV antibodies. Assays for total anti-HDV are commercially available in the United States and Europe, while tests for IgM anti-HDV and HDV antigen are available internationally but not in the United States *(296–299)*. Conventional dot blot and Northern blot nucleic acid hybridization assays *(300–305)* and RT-PCR *(306–309)* have been developed for the detection of HDV RNA in serum and can augment the serologic diagnosis of HDV infection. These assays can be used for the early diagnosis and follow-up of HDV infection in patients with acute or chronic hepatitis, to study the natural history of HDV hepatitis and the genetic variability of the HDV genome, and for monitoring response to antiviral therapy *(310–313)*. HDV antigen can be detected in liver using direct immunofluorescence or immunoperoxidase assays *(314–316)*, and *in situ* hybridization assays have been developed for use on both frozen and formalin-fixed, paraffin-embedded tissue sections *(317–319)* to monitor the clinical course of patients with chronic HDV infection. Assays for HDV RNA in serum and liver, and tests for HDV antigen in liver are currently available as research tools. Limited replication of HDV occurs in primary hepatocyte culture from woodchucks and chimpanzees *(320,321)*, but propagation of HDV using an in vitro cell culture system is impractical.

6.1. Acute Coinfection

Patients with acute coinfection with HDV and HBV will have a serological pattern characteristic of acute HBV hepatitis and detectable markers of HDV infection in their serum *(322–324)* (Fig. 5A). HDV RNA and HDV antigen appears during the late incubation period, remains for several weeks at low levels, and disappears in early convalescence. In immunodeficient patients who have not produced antibodies to HDV, levels of HDV antigen may persist for extended times *(325)*. The presence of HDV RNA and/or

antigen is evidence of active viral replication and acute HDV infection. Total and IgM-specific HDV antibodies develop in the acute phase of infection and are also transient and present only in low titers *(283)*. Anti-HDV IgM disappears several months after recovery; while anti-HDV IgG declines more gradually and generally persist at low levels for a longer period of time. The presence of anti-HDV IgM, especially when associated with rising titers of total anti-HDV, confirms the diagnosis of acute HDV hepatitis *(324)*. The temporal appearance and quantity of anti-HDV antibodies and HDV antigen can be variable, and multiple specimens may need to be tested over a period of several weeks to make a diagnosis of HDV infection *(326)*. Alternatively, a diagnosis of HDV coinfection can be most effectively made by testing acute-phase sera for all of the available HDV markers *(327)*. The prevalence of HDV coinfection with HBV is thought to be underestimated in the United States, since only the total anti-HDV assay is licensed for diagnostic use. In a patient with HBsAg and anti-HDV IgM or total anti-HDV antibodies, the presence of anti-HBc IgM indicates acute coinfection of HDV and HBV.

6.2. Acute and Chronic Superinfection

Patients with acute HDV superinfection (Fig. 5B) will have demonstrable levels of HDV RNA and antigen in their serum during the late incubation period. This is followed by the appearance of anti-HDV IgM and IgG during the acute phase of illness. Anti-HDV IgM is transient and at low levels, while IgG levels are high and persistent *(284)*. Progression to chronic superinfection (Fig. 5C) results in the persists of high levels of anti-HDV IgM and IgG as well as HDV antigen and RNA *(283,284,313,328,329)*. The presence of anti-HDV IgM appears to correlate with the severity and clinical course of liver disease *(282,284)*. All patients superinfected with HDV will be positive for HBsAg and anti-HBc total but negative for anti-HBc IgM. Therefore, HDV superinfection is differentiated from coinfection by the absence of anti-HBc IgM. HBV replication is usually suppressed during the acute phase of HDV superinfection, resulting in decreased levels of HBsAg.

7. Hepatitis E Virus

Diagnosis of hepatitis caused by HEV has been limited primarily to developing countries where the virus is endemic, including Mexico and those in Africa, Asia, and certain parts of South America and southern Europe. Routine testing for HEV infection in the United States is not necessary but should be considered in individuals with hepatitis who have recently traveled in known areas of endemicity or have recently emigrated from a country where HEV is endemic and in whom other causes of hepatitis have been excluded.

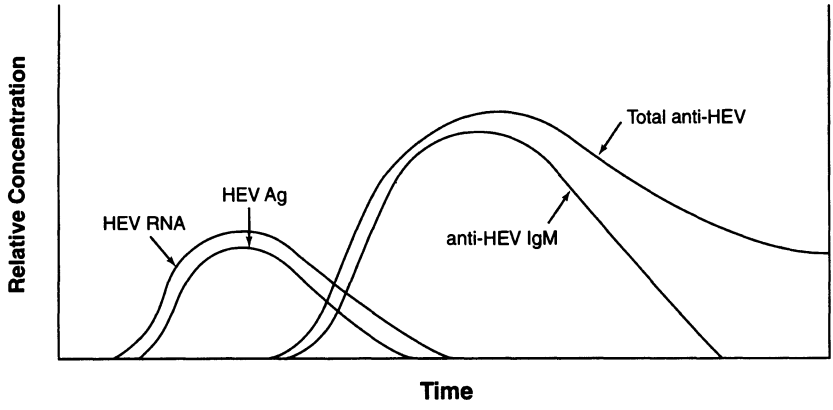


Fig. 6. Serological patterns observed during acute hepatitis E virus infection. Abbreviations: HEV Ag, hepatitis E virus antigen; HEV RNA, hepatitis E virus RNA; anti-HEV IgM, immunoglobulin M class antibody to hepatitis E virus; total anti-HEV; total antibody to hepatitis E virus.

7.1. Direct Detection

Direct detection of HEV in clinical specimens is not practical, as standard methods have not been developed and current procedures are only available as research tools. Also, the highest concentrations of HEV are normally found in the blood and feces of infected patients before clinical symptoms, with only low amounts of virus present during acute disease (Fig. 6) *(330–331)*. Detecting HEV in clinical material, however, may be beneficial in further understanding the transmission, pathogenesis, and epidemiology of this virus and may provide useful information for the diagnosis of acute HEV infection. The presence of HEV in a clinical specimen clearly indicates acute infection; the absence of HEV does not exclude the possibility of infection, since virus is not detectable in many infected patients.

Immune electron microscopy has been used to identify HEV particles in the stools and bile of infected humans and experimental animals *(330)*. This test is highly specific but lacks sesnsitivity, detecting only 10% of cases associated with an outbreak of HEV in Pakistan *(332)*. A more sensitive immune electron microscopic method has been developed and uses grids coated with protein A or antiimmunoglobulin to capture HEV particles within immune complexes that form as a specific immune response occurs early in the disease *(333)*. HEV antigen can be detected in liver tissue using immunofluorescence microscopy *(334,335)*, but assays for the detection of HEV antigen in feces or blood have not been developed.

HEV has not been reproducibly isolated and grown in cell culture, as the necessary cultured cells are not readily available and serial propogation has not been well established. In general, inoculation of various species of primates has been used by most research laboratories to isolate and propogate virus. In 1987, Pillot et al. *(336)* showed that PLC/PRF/5 cells, a carcinomatous hepatocyte cell line, was able to replicate HEV. The virus also has been propogated in diploid human embryonic lung cells *(337)*, human lung carcinoma cells (A549) *(338)*, and by cocultivation of fetal rhesus monkey kidney (FRhK) cells with primary cynomolgus monkey kidney cells obtained from a monkey experimentally infected with HEV *(339)*. More recently, HEV has been grown in primary hepatocyte cultures derived from cynomolgus macaques *(340,341)*. Replication of HEV in cultured cells is low and produces little to no cytopathic effects on primary culture. Viral growth in cell culture can be detected using indirect immunofluorescence for viral antigens or by detecting HEV RNA using molecular hybridization assays or PCR.

RT-PCR for HEV RNA appears to be a sensitive method for the direct detection of HEV in clinical and environmental specimens. HEV RNA has been detected by RT-PCR in stool and serum specimens collected from humans with acute HEV infections and from bile and liver tissue collected during experimental HEV infection in monkeys *(342–347)*. This method has also been used for the detection of HEV in raw and treated wastewater *(348)* and for estimating the duration of viremia and fecal shedding of HEV in individual cases *(331,349)*. In a recent study by Clayson et al. *(349)*, viremia and/or fecal shedding could be detected by RT-PCR in 14 patients with acute hepatitis who had no detectable levels of IgM and IgG antibody to HEV.

7.2. Serology

Similar to hepatitis A virus, serology is the most practical way of making a diagnosis of HEV infection. The first assays used for the detection of antibody to HEV were immune electron microscopy *(330)* and immunofluorescence microscopy *(334,335)*. These methods involve the use of native HEV antigens; available quantities of HEV have been insufficient for the development of practical and reproducible assays. Such assays are only performed in a few research laboratories. More recently, several major antigens encoded by portions of open reading frames 2 and 3 of the HEV genome have been identified using molecular cloning *(350)*. Serologic assays based on recombinant proteins expressed from cloned HEV cDNA and synthetic peptides have been developed for the detection of both IgG and IgM antibodies *(351–354)*. HEV antigens expressed in *E. coli* and insect cells have also been used for the detection of antibody to HEV by EIA and Western blot *(355–361)*. Commercial EIAs for detection of IgM and total antibodies

(IgG + IgM) to HEV are available in Europe and Asia *(352,362)*, but have not been marketed in the United States. The sensitivities and specificities of these assays have not been fully established.

Specific IgM and IgG anti-HEV develops early in the course of infection and usually by the onset of clinical disease (Fig. 6). IgM anti-HEV reaches peak titers within 4 wk after onset of hepatitis and declines to nondetectable levels by 3 mo in greater than 50% of infected patients *(363–365)*. Currently available assays can detect IgM anti-HEV in up to 90% of acute infections if serum is collected 1–4 wk after onset of disease *(353,363,365,366)*. Titers of IgG anti-HEV peak between 2 and 4 wk after onset of hepatitis and rapidly decline to lower levels shortly after infection *(352,364,365)*. It is unclear how long IgG anti-HEV can persist at detectable levels. Levels of detectable IgG anti-HEV have been reported to persist in adults for longer than 20 mo to 14 yr *(365–367)*, but Goldsmith et al. *(366)* found that two thirds of children infected with HEV were seronegative by 9 mo after onset of disease. IgA anti-HEV also has been detected in the serum of patients with HEV infection *(368)*. Detection of anti-HEV IgM or IgA indicates acute or recent infection; absence of these antibodies does not exclude acute infection since current assays may not be sufficiently sensitive to detect responses in all patients. A positive result for total anti-HEV in the absence of IgM anti-HEV, therefore, indicates infection with HEV but does not define when the infection may have occurred; absence of detectable total anti-HEV antibody does not exclude past infection, since titers may wane with time. Examination of acute and convalescent sera for seroconversion or a diagnostic rise in titer of specific anti-HEV is impractical since antibody levels reach their peak so quickly after onset of disease. The detection of anti-HEV antibody in low-risk populations with no history of exposure to HEV or travel to endemic areas should be interpreted with caution, since the specificity of serologic assays for anti-HEV is not completely defined *(369)*. When using currently available techniques, it may be necessary in certain clinical situations to use a combination of serologic assays and direct detection methods to provide the most accurate diagnosis of acute HEV infection.

8. Hepatitis F Virus

Hepatitis F virus has been isolated from stool extracts of French patients with enterically transmitted hepatitis and has been successfully transmitted to rhesus monkeys *(1)*. The genome of HFV was determined to be composed of double-strand DNA. The authors also used molecular hybridization techniques to demonstrate the presence of HFV genome in stool samples from infected patients and in the liver and stool of infected monkeys. In addition,

an EIA for the detection of HFV antigen was developed to monitor the presence of this virus in fecal material from experimentally infected monkeys. To date, this work has not been confirmed by others.

9. Hepatitis GB Virus

Hepatitis GB virus originated from the serum of a surgeon (initials GB) with acute hepatitis and was successfully isolated following transmission to and development of hepatitis in nonhuman primates *(370)*. Additional passage and studies of this virus in New World monkeys (tamarins) led to extensive virological characterization *(371–373)*. Recently, representational difference analysis has been used to clone specific nucleotide sequences present in the infectious plasma of an experimentally infected tamarin, and GB virus has been shown to actually consist of two novel flavi-like viruses designated GB virus A (GBV-A) and GB virus B (GBV-B) *(374,375)*. Schlauder et al. *(376)* subsequently developed an RT-PCR using primers from nonstructural (NS) genes for the detection of GBV-A and GBV-B RNA and enzyme immunoassays to detect antibodies associated with infection of the two agents in tamarins. Recombinant proteins encoded by nonstructural genes NS3/4 and NS5 of GBV-A and the core and NS3/4 and NS5 genes of GBV-B were used in the newly developed EIAs. Both GBV-A and GBV-B were shown to be transmissible to tamarins, and neither virus was found in uninfected animals. Only infection with GBV-B, however, led to a detectable seroconversion and elevations in liver enzyme levels, suggesting that GBV-B is the putative etiological agent associated with GB virus-related human hepatitis. Simons et al. *(377)* used the above mentioned EIAs to determine the seroprevalence of GBV-A and GBV-B in various human populations. Antibodies to GBV-A and GBV-B were found in 0.3% and 1.2% of volunteer blood donors, 3.0% and 11.0% of intravenous drug users, and 8.4% and 14.6% of a high-risk West African population. A total of 3.1% (40/1300) of the specimens from West Africa were immunoreactive to both GBV-A and GBV-B. Many of the tested sera were positive for both IgG and IgM class antibodies, suggesting recent infection. However, RT-PCR using GBV-A- and GBV-B-specific primers failed to detect genomic sequences of either virus in these human sera. As such, a direct link between infection with GBV-A and/or GBV-B and human hepatitis could not be fully established. Viruses similar to GBV-A also have been recently identified in some uninoculated tamarins and may suggest that GBV-A is a virus indigenous to New World monkeys that was adventitiously acquired during continuous passage and pooling of the GB viruses in tamarins *(378,379)*. Only with the continued development and use of more-sensitive and more-specific diag-

nostic reagents will it be possible to determine the role of the GB viruses in human non-A–E hepatitis.

10. Hepatitis G Virus

In 1995, an RNA virus termed hepatitis G virus (HGV) was cloned from the plasma of a patient with chronic posttransfusion hepatitis who was also infected with HCV, and a second clone was obtained from a phlebotomist with a 4-yr history of mildly elevated levels of liver transaminases who was negative for markers of other recognized hepatotropic viruses *(3)*. In an independent study using RT-PCR and degenerate oligonucleotides capable of amplifying a segment of the helicase genes from GBV-A, GBV-B, or HCV, novel sequences from a virus designated GB virus C (GBV-C) were identified in sera from 12 West African individuals, including four with hepatitis *(377)*. Three of the four patients with hepatitis had no evidence of infection with hepatitis A to E viruses. Nucleotide and amino acid sequence homology between HGV and GBV-C has been shown to be 85.5% and 100%, respectively, indicating that HGV and GBV-C are the same virus *(3)*. GBV-C is thought to be a West African strain of HGV, so the term HGV will be used in this chapter for further discussion of these two agents.

HGV is a positive-sense, single-stranded RNA virus shown to belong to the family *Flaviviridae*, but to a genus different from that of HCV *(3,380,381)*. The virus has a worldwide distribution and appears to induce acute and chronic infections in humans. Parenteral exposure to blood and blood products is the main mode of transmission for HGV, but vertical transmission has been documented *(382)*. High prevalence rates of HGV have been reported among selected risk groups, including intravenous drug users, multiply transfused patients, hemodialysis patients, hemophiliacs, and individuals receiving pooled plasma or intraveneous immunoglobulin *(3,383–386)*. The virus has also been detected in volunteer blood donors and in patients with cryptogenic hepatitis. Coinfection with HBV, HCV, and HIV is common *(385,387)*. The actual role of this virus in liver disease is still unclear, and debate remains whether blood donations and patients with hepatitis should be screened for HGV. HGV has recently been associated with cases of non-A–E fulminant hepatic failure *(388–390)* and aplastic anemia *(391–393)*, but there is limited evidence to suggest that this virus causes chronic liver disease *(380,387,394–396)*.

The diagnosis of HGV has been based entirely on the detection of viral RNA by RT-PCR, using primers derived from the 5' noncoding region or the nonstructural regions NS3 and NS5a of the viral genome *(397–401)*. Both commerical and in-house PCR-based diagnostic assays have been devel-

oped *(398,402)*. HGV RNA has been detected in serum, saliva, peripheral blood mononuclear cells and liver of infected patients *(403–406)*. More recently, an EIA for the detection of antibody to HGV envelope protein E2 has been developed *(407–411)*. The replication of HGV also has been demonstrated in MT-2C cells, a human T-cell leukemia virus type I-infected T cell line, and PH5CH, a nonneoplastic human hepatocyte line *(412)*. The combined use of these assays should further the understanding of the natural history and pathogenesis of this virus and assist in studies of the relationship of HGV infection to liver disease of unknown etiology *(413–416)*.

11. Conclusions

The last decade has seen considerable advances in the laboratory diagnosis and evaluation of acute and chronic viral hepatitis. Existing serologic assays have been improved, and new immunologic tests have been added. Newer molecular methods have been developed for accurate viral detection, and quantitation of viral nucleic acids is now available for predicting disease progression, monitoring the efficacy of antiviral therapy, and for understanding the natural history and pathogenesis of the hepatitis viruses.

References

1. Deka, N., Sharama, M. D., and Mukerjee, R. (1994) Isolation of the novel agent from human stool samples that is associated with sporadic non-A, non-B hepatitis. *J. Virol.* **68,** 7810–7815.
2. Simons, J. N., Leary, T. P., Dawson, G. J., Pilot-Matias, T. J., Muerhoff, A. S., Schlauder, G. G., Desai, S. M., and Mushahwar, I. K. (1995) Isolation of novel virus-like sequences associated with human hepatitis. *Nature Med.* **1,** 564–569.
3. Linnen, J., Wages, Jr., J., Zhang-Keck, Z.-Y., Fry, K. E., Krawczynski, K. Z., Alter, H., Koonin, E., Gallagher, M., Alter, M., Hadziyannis, S., Karaylannis, P., Fung, K., Nakatsuji, Y., Wai-Kuo Shih, J., Young, L., Piatak, Jr., M., Hoover, C., Fernandez, J., Chen, S., Zou, J.-C., Morris, T., Hyams, K. C., Ismay, S., Lifson, J. D., Hess, G., Foung, S. K. H., Thomas, H., Bradley, D., Margolis, H., Kim, J. P. (1996) Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. *Science* **271,** 505–508.
4. Busch, M. P., Wilber, J. C., Johnson, P., Tobler, L., and Evans, C. S. (1992) Impact of specimen handling and storage on detection of hepatitis C virus RNA. *Transfusion* **32,** 420–425.
5. Wang, J.-T., Wang, T.-H., Sheu, J.-C., Lin, S.-M., Lin, J.-T., and Chen, D.-S. (1992) Effects of anticoagulants and storage of blood samples on efficacy of the polymerase chain reaction assay for hepatitis C virus. *J. Clin. Microbiol.* **30,** 750–753.
6. Cuypers, H. T. M., Bresters, D., Winkel, I. N., Reesink, H. W., Weiner, A. J., Houghton, M., van der Poel, C. L., and Lelie, P. N. (1992) Storage conditions of blood samples and primer selection affect the yield of cDNA polymerase chain reaction products of hepatitis C virus. *J. Clin. Microbiol.* **30,** 3220–3224.

7. Krajden, M., Comanor, L., Rifkin, O., Grigoriew, A., Minor, J. M., and Kapke, G. F. (1998) Assessment of hepatitis B virus DNA stability in serum by the chiron quantiplex branched-DNA assay. *J. Clin. Microbiol.* **36,** 382–386.
8. Parry, J. V., Perry, K. R., Panday, S., and Mortimer, P. P. (1989) Diagnosis of hepatitis A and B by testing saliva. *J. Med. Virol.* **28,** 255–260.
9. Parry, J. V. (1993) Simple and reliable salivary tests for HIV and hepatitis A and B virus diagnosis and surveillance. *Ann. NY Acad. Sci.* **694,** 216–233.
10. Thieme, T., Yoshihara, P., Piacentini, S., and Beller, M. (1992) Clinical evaluation of oral fluid samples for diagnosis of viral hepatitis. *J. Clin. Microbiol.* **30,** 1076–1079.
11. Hodinka, R. L., Nagashunmugam, T., and Malamud, D. (1998) Detection of human immunodeficiency virus antibodies in oral fluids: a review. *Clin. Diag. Lab. Immunol.* **5,** 419–426.
12. Gaudette, D., North, L., Hindahl, M., Griffin, K., Klimkow, N., and Thieme, T. (1994) Stability of clinically significant antibodies in saliva and oral fluid. *J. Clin. Immunol.* **17,** 171–175.
13. Feinstone, S. M., Kapikian, A. Z., and Purcell, R. H. (1973) Hepatitis A: detection by immune electron microscopy of a viruslike antigen associated with acute illness. *Science* **182,** 1026–1028.
14. Mathiesen, L. R., Feinstone, S. M., Wong, D. C., Skinhoej, P., and Purcell, R. H. (1978) Enzyme-linked immunosorbent assay for detection of hepatitis A antigen in stool and antibody to hepatitis A antigen in sera: comparison with solid-phase radioimmunoassay, immune electron microscopy, and immune adherence hemagglutination assay. *J. Clin. Microbiol.* **7,** 184–193.
15. Purcell, R. H., Wong, D. C., Moritsugu, Y., Dienstag, J. L., Routenberg, J., and Boggs, J. D. (1976) A microtiter solid-phase radioimmunoassay for hepatitis A antigen and antibody. *J. Immunol.* **116,** 349–356.
16. Nadala, E. C. B. and Loh, P. C. (1990) A nitrocellulose-enzyme immunoassay method for the detection of hepatitis A virus. *J. Virol. Meth.* **28,** 155–164.
17. Provost, P. J. and Hilleman, M. R. (1979) Propagation of human hepatitis A virus in cell culture in vitro. *Proc. Soc. Exp. Biol. Med.* **160,** 213–221.
18. Daemer, R. J., Feinstone, S. M., Gust, I. D., and Purcell, R. H. (1981) Propagation of human hepatitis A virus in African green monkey kidney cell culture: primary isolation and serial passage. *Infect. Immun.* **32,** 388–393.
19. Fleming, B., Vallbracht, A., and Wurster, G. (1981) Hepatitis A virus in cell culture. III. Propagation of hepatitis A virus in human embryo kidney and human embryo fibroblasts. *Med. Microbiol. Immunol.* **170,** 83–89.
20. Frosner, G. G., Deinhardt, F., Scheid, R., Gauss-Muller, V., Holmes, N., Messelberger, V., Siegl, G., and Alexander, J. J. (1979) Propagation of human hepatitis A virus in a hepatoma cell line. *Infection* **7,** 303–305.
21. Binn, L. N., Lemon, S. M., Marchwicki, R. H., Redfield, R. R., Gates, N. L., and Bancroft, W. H. (1984) Primary isolation and serial passage of hepatitis A virus strains in primate cell cultures. *J. Clin. Microbiol.* **20,** 28–33.
22. Jansen, R. W., Newbold, J. E., and Lemon, S. M. (1985) Combined immunoaffinity cDNA-RNA hybridization assay for detection of hepatitis A virus in clinical specimens. *J. Clin. Microbiol.* **22,** 984–989.
23. Ticehurst, J. R., Feinstone, S. M., Chestnut, T., Tassopoulos, N. C., Popper, H., and Purcell, R. H. (1987) Detection of hepatitis A virus by extraction of viral RNA and molecular hybridization. *J. Clin. Microbiol.* **25,** 1822–1829.

24. Zhou, Y.-J., Estes, M. K., Jiang, X., and Metcalf, T. G. (1991) Concentration and detection of hepatitis A virus and rotavirus from shellfish by hybridization tests. *Appl. Environ. Microbiol.* **57,** 2963–2968.
25. Jansen, R. W., Siegl, G., and Lemon, S. M. (1990) Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. *Proc. Natl. Acad. Sci. USA* **87,** 2867–2871.
26. Robertson, B. H., Jansen, R. W., Khanna, B., Totsuka, A., Nainan, O. V., Siegl, G., Widell, A., Margolis, H. S., Isomura, S., Ito, K., Ishizu, T., Moritsugu, Y., and Lemon, S. M. (1992) Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. *J. Gen. Virol.* **73,** 1365–1377.
27. Birkenmeyer, L. G. and Mushahwar, I. K. (1994) Detection of hepatitis A, B and D virus by the polymerase chain reaction. *J. Virol. Meth.* **49,** 101–112.
28. Divizia, D., Gnesivo, C., Amore, R. B., Morace, G., Pisani, G., and Pana, A. (1993) Hepatitis A virus identification in an outbreak by enzymatic amplification. *Eur. J. Epidemiol.* **9,** 203–208.
29. Normann, A., Graff, J., and Flehmig, B. (1994) Detection of hepatitis A virus in a factor VIII preparation by antigen capture/PCR. *Vox. Sang.* **67**(Suppl. 1), 57–60.
30. Robertson, B. H., Normann, A., Graff, J., Flehmig, B., Friedberg, D., and Shouval, D. (1995) Hepatitis A virus and polymerase chain reaction amplification: methodology and results. *Blood Coagul. Fibrinol.* **6**(Suppl. 2), S27-S31.
31. Lawlor, E., Graham, S., Davidson, E., Yap, P. L., Cunningham, C., Daly, H., and Temperley, I. J. (1996) Hepatitis A transmission by factor IX concentrates. *Vox. Sang.* **71,** 126–128.
32. Dvemeyer, W., Wielaard, F., and van der Veen, J. (1979) A new principle for the detection of specific IgM antibodies applied in an ELISA for hepatitis A. *J. Med. Virol.* **4,** 25–32.
33. Lemon, S. M., Brown, C. D., Brooks, D. S., Simms, T. E., and Bancroft, W. H. (1980) Specific immunoglobulin M response to hepatitis A virus determined by solid-phase radioimmunoassay. *Infect. Immun.* **28,** 927–936.
34. Summers, P. L., Dubois, D. R., Cohen, W. H., Macarthy, P. O., Binn, L. N., Sjogren, M. H., Snitbhan, R., Innis, B. L., and Eckels, K. H. (1993) Solid-phase antibody capture hemadsorption assay for detection of hepatitis A virus immunoglobulin M antibodies. *J. Clin. Microbiol.* **31,** 1299–1302.
35. Kao, H. W., Ashcavai, M., and Redeker, A. G. (1984) The persistence of hepatitis A IgM antibody after acute clinical hepatitis A. *Hepatol.* **4,** 933–936.
36. Locarnini, S. A., Ferris, A. A., Lehmann, N. I., and Gust, I. D. (1977) The antibody response following hepatitis A infection. *Intervirol.* **8,** 309–318.
37. Storch, G. A., Bodicky, C., Parker, M., Blecka, L. J., and Aach, R. D. (1982) Use of conventional and IgM-specific radioimmunoassays for anti-hepatitis A antibody in an outbreak of hepatitis A. *Amer. J. Med.* **73,** 663–668.
38. Robbins, D. J., Krater, J., Kiang, W., Alcalde, X., Helgesen, S., Carlos, J., and Mimms, L. (1991) Detection of total antibody against hepatitis A virus by an automated microparticle enzyme immunoassay. *J. Virol. Meth.* **32,** 255–263.
39. Stuart, J. M., Majeed, F. A., Cartwright, K. A., Room, R., Parry, J. V., Perry, K. R., and Begg, N. T. (1992) Salivary antibody testing in a school outbreak of hepatitis A. *Epidemiol. Infect.* **109,** 161–166.
40. Gil, A., Gonzalez, A., Dal-Re, R., Dominguez, V., Astasio, P., and Aguilar, L. (1997) Detection of antibodies against hepatitis A in blood spots dried on filter paper. Is this a reliable method for epidemiological studies? *Epidemiol. Infect.* **118,** 189–191.

41. Perry, K. R., Parry, J. V., Vandervelde, E. M., and Mortimer, P. P. (1992) The detection in urine specimens of IgG and IgM antibodies to hepatitis A and hepatitis B core antigens. *J. Med. Virol.* **38,** 265–270.
42. Hollinger, F. B. and Dienstag, J. L. (1995) Hepatitis B and D viruses, in *Manual of Clinical Microbiology,* 6th ed. (Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C., and Yolken, R. H., eds.), Amer. Soc. Microbiol., Washington, pp. 1033–1049.
43. Hollinger, F. B. (1996) Hepatitis B virus, in *Virology,* 3rd ed. (Fields, B. N., Kniper, D. M., and Howley, P. M., eds.), Raven, New York, pp. 2739–2807.
44. Frosner, G. G., Schomerus, H., Wiedman, K. H., Zachoval, R., Bayerl, B., Backer, U., Gathof, G. A., and Sugg, U. (1982) Diagnostic significance of quantitative determination of hepatitis B surface antigen in acute and chronic hepatitis B infection. *Eur. J. Clin. Microbiol.* **1,** 52–58.
45. Trepo, C. G., Robert, D., Motin, J., Trepo, D., Sepetjian, M., and Prince, A. M. (1976) Hepatitis B antigen (HBsAg) and or antibodies (anti-HBs and anti-HBc) in fulminant hepatitis: pathogenic and prognostic significance. *Gut* **17,** 10–13.
46. Kryger, P., Aldershville, J., Mathiesen, L. R., and Nielsen, J. O. (1982) Acute type B hepatitis among HBsAg negative patients detected by anti-HBc IgM. *Hepatology* **2,** 50–53.
47. Aikawa, T., Sairenji, H., Furuta, S., Kiyosawa, K., Shikata, T., Imai, M., Miyakawa, Y., Yanase, Y., and Mayumi, M. (1978) Seroconversion from hepatitis B e antigen to anti-HBe in acute hepatitis B virus infection. *N. Engl. J. Med.* **298,** 439–441.
48. Frosner, G. G., Brodersen, M., Papaevangelou, G., Sugg, U., Haas, H., Mushahwar, I. K., Ling, C. M., Overby, L. R., and Deinhardt, F. (1978) Detection of HBeAg and anti-HBe in acute hepatitis B by a sensitive radioimmunoassay. *J. Med. Virol.* **3,** 67–76.
49. Hoofnagle, J. H. and Di Bisceglie, A. M. (1991) Serologic diagnosis of acute and chronic viral hepatitis. *Semin. Liver Dis.* **11,** 73–83.
50. Krugman, S., Overby, L. R., Mushahwar, I. K., Ling, C. M., Frosner, G. G., and Deinhardt, F. (1979) Viral hepatitis, type B. Studies on natural history and prevention re-examined. *N. Engl. J. Med.* **300,** 101–106.
51. Perrillo, R. P., Chau, K. H., Overby, L. R., and Decker, R. H. (1983) Anti-hepatitis B core immunoglobulin M in the serologic evaluation of hepatitis B virus infection and simultaneous infection with type B, delta agent, and non-A, non-B viruses. *Gastroenterology* **85,** 163–167.
52. Chau, K. H., Hargie, M. P., Decker, R. H., Mushahwar, I. K., and Overby, L. R. (1983) Serodiagnosis of recent hepatitis B infection by IgM class anti-HBc. *Hepatology* **3,** 142–149.
53. Lander, H. J., Holland, P. V., Alter, H. J., Chanock, R. M., and Purcell, R. H. (1972) Antibody to hepatitis-associated antigen. Frequency and pattern of response as detected by radioimmunoprecipitation. *JAMA* **220,** 1079–1082.
54. Seeff, L. B., Beebe, G. W., Hoofnagle, J. H., Norman, J. E., Buskell-Bales, Z., Waggoner, J. G., Kaplowitz, N., Koff, R. S., Petrini, J. L., Jr., and Schiff, E. R. (1987) A serologic follow-up of the 1942 epidemic of post-vaccination hepatitis in the United States Army. *N. Engl. J. Med.* **316,** 965–970.
55. Hoofnagle, J. H. and Shafer, D. F. (1986) Serologic markers of hepatitis B virus infection. *Semin. Liver Dis.* **6,** 1–10.
56. Sampliner, R. E., Hamilton, F. A., Iseri, O. A., Tabor, E., and Boitnott, J. (1979) The liver histology and frequency of clearance of the hepatitis B surface antigen (HBsAg) in chronic carriers. *Amer. J. Med. Sci.* **277,** 17–22.

57. Alward, W. L., McMahon, B. J., Hall, D. B., Heyward, W. L., Francis, D. P., and Bender, T. R. (1985) The long-term serological course of asymptomatic hepatitis B virus carriers and the development of primary hepatocellular carcinoma. *J. Infect. Dis.* **151,** 604–609.
58. Viola, L. A., Barrison, I. G., Coleman, J. C., Paradinas, F. J., and Murray-Lyon, I. M. (1981) The HBe antigen-antibody system and its relationship to clinical and laboratory findings in 100 chronic HBsAg carriers in Great Britian. *J. Med. Virol.* **8,** 169–175.
59. Hoofnagle, J. H., Dusheiko, G. M., Seeff, L. B., Jones, E. A., Waggoner, J. G., and Bales, Z. B. (1981) Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. *Ann. Int. Med.* **94,** 744–748.
60. Liaw, Y. F., Chu, C. M., Su, I. J., Huang, M. J., Lin, D. Y., and Chang-Chien, C. S. (1983) Clinical and histological events preceding hepatitis B e antigen seroconversion in chronic type B hepatitis. *Gastroenterology* **84,** 216–219.
61. Wong, D. K. H., Cheung, A. M., O'Rourke, K., Naylor, C. D., Detsky, A. S., and Heathcote, J. (1993) Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. *Ann. Int. Med.* **119,** 312–323.
62. Tsang, T. K., Blei, A. T., O'Reilly, D. J., and Decker, R. (1986) Clinical significance of concurrent hepatitis B surface antigen and antibody positivity. *Dig. Dis. Sci.* **31,** 620–624.
63. Heijtink, R. A., van Hattum, J., Schalm, S. W., and Masurel, N. (1982) Co-occurrence of HbsAg and anti-HBs: two consecutive infections or a sign of advanced chronic liver disease. *J. Med. Virol.* **10,** 83–90.
64. Foutch, P. G., Carey, W. D., Tabor, E., Cianflocco, A. J., Nakamoto, S., Smallwood, L. A., and Gerety, R. J. (1983) Concomitant hepatitis B surface antigen and antibody in thirteen patients. *Ann. Int. Med.* **99,** 460–463.
65. Galbraith, R. M., Eddleston, A. L., Williams, R., and Zuckerman, A. J. (1975) Fulminant hepatic failure in leukaemia and choriocarcinoma related to withdrawal of cytotoxic drug therapy. *Lancet* **2,** 528–530.
66. Davis, G. L., Hoofnagle, J. H., and Waggoner, J. G. (1984) Spontaneous reactivation of chronic hepatitis B virus infection. *Gastroenterology* **86,** 230–235.
67. Kroes, A. C. (1996) Role of testing for antibody to hepatitis B core antigen to prevent transmission of hepatitis B virus in organ and tissue transplantation. *Transplant Proc.* **28,** 2937–2938.
68. Krogsgaard, K. (1988) Hepatitis B virus DNA in serum. Applied molecular biology in the evaluation of hepatitis B infection. *Liver* **8,** 257–280.
69. Kaneko, S., Miller, R. H., Di Bisceglie, A., Feinstone, S. M., Hoofnagle, J. H., and Purcell, R. H. (1990) Detection of hepatitis B virus DNA in serum by polymerase chain reaction. Application for clinical diagnosis. *Gastroenterology* **99,** 799–804.
70. Kuhns, M. C., McNamara, A. L., Perrillo, R. P., Cabal, C. M., and Campbel, C. R. (1989) Quantitation of hepatitis B viral DNA by solution hybridization: comparison with DNA polymerase and hepatitis B e antigen during antiviral therapy. *J. Med. Virol.* **27,** 274–281.
71. Scotto, J., Hadchouel, M., Hery, C., Alvarez, F., Yvart, J., Tiollais, P., Bernard, O., and Brechot, C. (1983) Hepatitis B virus DNA in children's liver diseases: detection by blot hybridisation in liver and serum. *Gut* **24,** 618–624.
72. Scotto, J., Hadchouel, M., Hery, C., Yvart, J., Tiollais, P., and Brechot, C. (1983) Detection of hepatitis B virus DNA in serum by a simple spot hybridization technique: comparison with results for other viral markers. *Hepatology* **3,** 279–284.

73. Chu, C. M., Karayiannis, P., Fowler, M. J., Monjardino, J., Liaw, Y. F., and Thomas, H. C. (1985) Natural history of chronic hepatitis B virus infection in Taiwan: studies of hepatitis B virus DNA in serum. *Hepatology* **5,** 431–434.
74. Yoffe, B., Noonan, C. A., Melnick, J. L., and Hollinger, F. B. (1986) Hepatitis B virus DNA in mononuclear cells and analysis of cell subsets for the presence of replicative intermediates of viral DNA. *J. Infect. Dis.* **153,** 471–477.
75. Lin, H. J., Chung, H. T., Lai, C. L., Leong, S., and Tam, O. S. (1989) Detection of supercoiled hepatitis B virus DNA and related forms by means of molecular hybridization to an oligonucleotide probe. *J. Med. Virol.* **29,** 284–288.
76. Yoffe, B., Burns, D. K., Bhatt, H. S., and Combes, B. (1990) Extrahepatic hepatitis B virus DNA sequences in patients with acute hepatitis B infection. *Hepatology* **12,** 187–192.
77. Valentine-Thon, E., Steinmann, J., and Arnold, W. (1990) Evaluation of the commercially available HepProbe kit for detection of hepatitis B virus DNA in serum. *J. Clin. Microbiol.* **28,** 39–42.
78. Kuhns, M. C. (1990) Monitoring hepatitis B virus replication. *J. Hepatol.* **11,** S90–S94.
79. Janssen, H. L., Schoenmaker-Weber, Y. A., Kruining, H., Schalm, S. W., and Heijtink, R. A. (1993) Quantitative assessment of hepatitis B virus DNA in chronic hepatitis B: comparison of two solution hybridization assays. *J. Med. Virol.* **40,** 307–312.
80. Larzul, D., Guigue, F., Sninsky, J. J., Mack, D. H., Brechot, C., and Guesdon, J. L. (1988) Detection of hepatitis B virus sequences in serum by using in vitro enzymatic amplification. *J. Virol. Meth.* **20,** 227–237.
81. Kaneko, S., Feinstone, S. M., and Miller, R. H. (1989) Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. *J. Clin. Microbiol.* **27,** 1930–1933.
82. Keller, G. H., Huang, D.-P., Shih, J. W.-K., and Manak, M. M. (1990) Detection of hepatitis B virus DNA in serum by polymerase chain reaction amplification and microtiter sandwich hybridization. *J. Clin. Microbiol.* **28,** 1411–1416.
83. Monjardino, J., Velosa, J., Thomas, H. C., and de Moura, M. C. (1991) Serum HBV DNA detected by PCR in dot blot negative HBV chronic carriers with active liver disease. *J. Hepatol.* **13,** 44–48.
84. Kaneko, S., Miller, R. H., Feinstone, S. M., Unoura, M., Kobayashi, K., Hattori, N., and Purcell, R. H. (1989) Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay. *Proc. Natl. Acad. Sci. USA* **86,** 312–316.
85. Noonan, C. A., Yoffe, B., Mansell, P. W., Melnick, J. L., and Hollinger, F. B. (1986) Extrachromosomal sequences of hepatitis B virus DNA in peripheral blood mononuclear cells of acquired immune deficiency syndrome patients. *Proc. Natl. Acad. Sci. USA* **83,** 5698–5702.
86. Pao, C. C., Yang, W.-L., Wu, S.-Y., Lai, G.-M., Shih, L.-Y., Sun, C.-F., and Liaw, Y.-F. (1987) Presence of hepatitis B virus DNA in serum of surface-antigen-seronegative immunocompromised patients. *J. Clin. Microbiol.* **25,** 449–451.
87. Thiers, V., Nakajima, E., Kremsdorf, D., Mack, D., Schellekens, H., Driss, F., Goudeau, A., Wands, J., Sninsky, J., Tiollais, P., and Brechot, C. (1988) Transmission of hepatitis B from hepatitis-B-seronegative subjects. *Lancet* **2,** 1273–1276.
88. Fagan, E. A., Davison, F. D., Trowbridge, R., Carman, W. F., Smith, H. M., Tedder, R., and Williams, R. (1991) Detection of hepatitis B virus DNA sequences in liver

in HBsAg aeronegative patients with liver disease with and without anti-HBc antibodies. *Q. J. Med.* **78,** 123–134.

89. Sun, C.-F., Pao, C. C., Wu, S.-Y., and Liaw, Y.-F. (1988) Screening of hepatitis B virus in healthy blood donors by molecular DNA hybridization analysis. *J. Clin. Microbiol.* **26,** 1848–1852.
90. Pao, C. C., Yao, D. S., Lin, C. Y., Kao, S. M., Tsao, K. C., Sun, C. F., and Liaw, Y. F. (1991) Serum hepatitis B virus DNA in hepatitis B virus seropositive and seronegative patients with normal liver function. *Amer. J. Clin. Pathol.* **95,** 591–596.
91. Karayiannis, P., Novick, D. M., Lok, A. S., Fowler, M. J., Monjardino, J., and Thomas, H. C. (1985) Hepatitis B virus DNA in saliva, urine, and seminal fluid of carriers of hepatitis B e antigen. *Br. Med. J.* **290,** 1853–1855.
92. Pao, C. C., Yao, D. S., Lin, M. Y., Lin, C. Y., and Hsieh, T. T. (1991) Hepatitis B virus DNA in cervicovaginal cells. *Arch. Pathol. Lab. Med.* **115,** 607–609.
93. Hadziyannis, S. J., Lieberman, H. M., Karvountzis, G. G., and Shafritz, D. A. (1983) Analysis of liver disease, nuclear HBcAg, viral replication, and hepatitis B virus DNA in liver and serum of HBeAg vs. anti-HBe positive carriers of hepatitis B virus. *Hepatology* **3,** 656–662.
94. Karayiannis, P., Fowler, M. J., Lok, A. S., Greenfield, C., Monjardino, J., and Thomas, H. C. (1985) Detection of serum HBV-DNA by molecular hybridisation. Correlation with HBeAg/anti-HBe status, racial origin, liver histology and hepatocellular carcinoma. *J. Hepatol.* **1,** 99–106.
95. Zaaijer, H. L., ter Borg, F., Cuypers, H. T. M., Hermus, M. C. A. H., and Lelie, P. N. (1994) Comparison of methods for detection of hepatitis B virus DNA. *J. Clin. Microbiol.* **32,** 2088–2091.
96. Gerlich, W. H., Heermann, K. H., Thomssen, R., and The Eurohep Group (1995) Quantitative assays for hepatitis B virus DNA: standardization and quality control. *Virol. Hep. Rev.* **1,** 53–57.
97. Quint, W. G. V., Heijtink, R. A., Schirm, J., Gerlich, W. H., and Niesters, H. G. M. (1995) Reliability of methods for hepatitis B virus DNA detection. *J. Clin. Microbiol.* **33,** 225–228.
98. Butterworth, L.-A., Prior, S. L., Buda, P. J., Faoagali, J. L., and Cooksley, W. G. E. (1996) Comparison of four methods for quantitative measurement of hepatitis B viral DNA. *J. Hepatol.* **24,** 686–691.
99. Kapke, G. F., Watson, G., Sheffler, S., Hunt, D., and Frederick, C. (1997) Comparison of the Chiron Quantiplex branched DNA (bDNA) assay and the Abbott Genostics solution hybridization assay for quantitation of hepatitis B viral DNA. *J. Viral Hep.* **4,** 67–75.
100. Valentine-Thon, E. (1995) Evaluation of SHARP signal system for enzymatic detection of amplified hepatitis B virus DNA. *J. Clin. Microbiol.* **33,** 477–480.
101. Hendricks, D. A., Stowe, B. J., Hoo, B. S., Kolberg, J., Irvine, B. D., Neuwald, P. D., Urdea, M. S., and Perrillo, R. P. (1995) Quantitation of HBV DNA in human serum using a branched DNA (bDNA) signal amplification assay. *Amer. J. Clin. Pathol.* **104,** 537–546.
102. Yoffe, B. and Noonan, C. A. (1992) Hepatitis B virus: new and evolving issues. *Dig. Dis. Sci.* **37,** 1–9.
103. Lieberman, H. M., LaBrecque, D. R., Kew, M. C., Hadziyannis, S. J., and Shafritz, D. A. (1983) Detection of hepatitis B virus DNA directly in human serum by a

simplified molecular hybridization test: comparison to HBeAg/anti-HBe status in HBsAg carriers. *Hepatology* **3,** 285–291.

104. Fattovich, G., Rugge, M., Brollo, L., Pontisso, P., Noventa, F., Guido, M., Alberti, A., and Realdi, G. (1986) Clinical, virologic and histologic outcome following seroconversion from HBeAg to anti-HBe in chronic hepatitis type B. *Hepatology* **6,** 167–172.
105. Hess, G. and Reuschling, M. (1993) Toward routine diagnosis of hepatitis B virus desoxyribonucleic acid. *Clin. Biochem.* **26,** 289–293.
106. Perrillo, R. P., Schiff, E. R., Davis, G. L., Bodenheimer, H. C., Lindsay, K., Payne, J., Dienstag, J. L., O'Brien, C., Tamburro, C., Jacobson, I. M., Sampliner, R., Feit, D., Lefkowitch, J., Kuhns, M., Meschievitz, C., Sanghvi, B., Albrecht, J., Gibas, A., and The Hepatitis Interventional Therapy Group (1990) A randomized, controlled trial of interferon alfa–2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. *N. Engl. J. Med.* **323,** 295–301.
107. Ryff, J. C. (1993) To treat or not to treat? The judicious use of interferon-α-2a for the treatment of chronic hepatitis B. *Hepatology* **17**(suppl. 3), S42–S46.
108. Carmen, W. F., Jacyna, M. R., Hadziyannis, Karayiannis, P., McGarvey, M., Makris, A., and Thomas, H. C. (1989) Mutation preventing formation of e antigen in patients with chronic HBV infection. *Lancet* **336,** 588–591.
109. Paterlini, P., Gerken, G., Nakajima, E., Terre, S., D'Errico, A., Grigioni, W., Nalpas, B., Franco, D., Wands, J., Kew, M., Pisi, E., Tiollais, P., and Brechot, C. (1990) Polymerase chain reaction to detect hepatitis B virus DNA and RNA sequences in primary liver cancers from patients negative for hepatitis B surface antigen. *N. Engl. J. Med.* **323,** 80–85.
110. Ljunggren, K. K., Nordenfelt, E., and Kidd, A. (1993) Correlation of HBeAG/anti-HBe, ALT levels, and HBV DNA PCR results in HBsAg-positive patients. *J. Med. Virol.* **39,** 297–302.
111. Ochiya, T., Tsurimoto, T., Ueda, K., Okubo, K., Shiozawa, M., and Matsubara, K. (1989) An in vitro system for infection with hepatitis B virus that uses primary human fetal hepatocytes. *Proc. Natl. Acad. Sci. USA* **86,** 1875–1879.
112. Gripon, P., Diot, C., and Guguen-Guillouzo, C. (1993) Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. *Virology* **192,** 534–540.
113. Galle, P. R., Hagelstein, J., Kommerell, B., Volkmann, M., Schranz, P., and Zentgraf, H. (1994) In vitro experimental infection of primary human hepatocytes with hepatitis B virus. *Gastroenterology* **106,** 664–673.
114. Sureau, C., Romet-Lemonne, J. L., Mullins, J. I., and Essex, M. (1986) Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. *Cell* **47,** 37–47.
115. Tsurimoto, T., Fujiyama, A., and Matsubara, K. (1987) Stable expression and replication of hepatitis B virus genome in an integrated state in a human hepatoma cell line transfected with the cloned viral DNA. *Proc. Natl. Acad. Sci. USA* **84,** 444–448.
116. Yaginuma, K., Shirakata, Y., Kobayashi, M., and Koike, K. (1987) Hepatitis B virus (HBV) particles are produced in a cell culture system by transient expression of transfected HBV DNA. *Proc. Natl. Acad. Sci. USA* **84,** 2678–2682.
117. Sells, M. A., Chen, M. L., and Acs, G. (1987) Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc. Natl. Acad. Sci. USA* **84,** 1005–1009.

118. Muller, C. Bergmann, K. F., Gerin, J. L., and Korba, B. E. (1992) Production of hepatitis B virus by stably transfected monocytic cell line U-937, a model for extrahepatic hepatitis B virus replication. *J. Infect. Dis.* **165,** 929–933.
119. Korba, B. E. and Milman, G. (1991) A cell culture assay for compounds which inhibit hepatitis B virus replication. *Antiviral Res.* **15,** 217–228.
120. Korba, B. E. and Gerin, J. L. (1992) Use of a standardized cell culture assay to assess activities of nucleoside analogs against hepatitis B virus replication. *Antiviral Res.* **19,** 55–70.
121. Ray, M. B., Desmet, V. J., Bradburne, A. F., Desmyter, J., Fevery, J., and De Groote, J. (1976) Differential distribution of hepatitis B surface antigen and hepatitis B core antigen in the liver of hepatitis B patients. *Gastroenterology* **71,** 462–469.
122. Huang, S. N. and Neurath, A. R. (1979) Immunohistologic demonstration of hepatitis B viral antigens in liver with reference to its significance in liver injury. *Lab. Invest.* **40,** 1–17.
123. Blum, H. E., Haase, A. T., and Vyas, G. N. (1984) Molecular pathogenesis of hepatitis B virus infection: simultaneous detection of viral DNA and antigens in paraffin-embedded liver sections. *Lancet* **2,** 771–775.
124. Blum, H. E., Figus, A., Haase, A. T., and Vyas, G. N. (1985) Laboratory diagnosis of hepatitis B virus infection by nucleic acid hybridization analyses and immunohistologic detection of gene products. *Dev. Biol. Stand.* **59,** 125–139.
125. Gowans, E. J., Burrell, C. J., Jilbert, A. R., and Marmion, B. P. (1985) Cytoplasmic (but not nuclear) hepatitis B virus (HBV) core antigen reflects HBV DNA synthesis at the level of the infected hepatocyte. *Intervirology* **24,** 220–225.
126. Chu, C. M. and Liaw, Y. F. (1995) Membrane staining for hepatitis B surface antigen on hepatocytes: a sensitive and specific marker of active viral replication in hepatitis B. *J. Clin. Pathol.* **48,** 470–473.
127. Carman, W. F., Jacyna, M. R., Hadziyannis, S., Karayiannis, P., McGarvey, M. J., Makris, A., and Thomas, H. C. (1989) Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* **2,** 588–591.
128. Carman, W. F., Fagan, E. A., Hadziyannis, S., Karayiannis, P., Tassopoulos, N. C., Williams, R., and Thomas, H. C. (1991) Association of a precore genomic variant of hepatitis B virus with fulminant hepatitis. *Hepatology* **14,** 219–222.
129. Omata, M., Ehata, T., Yokosuka, O., Hosoda, K., and Ohto, M. (1991) Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N. Engl. J. Med.* **324,** 1699–1704.
130. Tur-Kaspa, R., Klein, A., and Aharonson, S. (1992) Hepatitis B virus precore mutants are identical in carriers from various ethnic origins and are associated with a range of liver disease severity. *Hepatology* **16,** 1338–1342.
131. Wu, J. C., Chen, C. M., Chen, T. Z., Lee, S. D., Yen, F. S., and Choo, K. B. (1996) Prevalence and type of precore hepatitis B virus mutants in hepatitis D virus superinfection and its clinical implications. *J. Infect. Dis.* **173,** 457–459.
132. Carman, W. F., Zanetti, A. R., Karayiannis, P., Waters, J., Manzillo, G., Tanzi, E., Zuckerman, A. J., and Thomas, H. C. (1990) Vaccine-induced escape mutant of hepatitis B virus. *Lancet* **336,** 325–329.
133. Gerken, G., Kremsdorf, D., Capel, F., Petit, M. A., Dauguet, C., Manns, M. P., Meyer zum Buschenfelde, K. H., and Brechot, C. (1991) Hepatitis B defective virus with rearrangements in the preS gene during chronic HBV infection. *Virology* **183,** 555–565.

134. Shih, J. W.-K., Cheung, L. C., Alter, H. J., Lee, L. M., and Gu, J. R. (1991) Strain analysis of hepatitis B virus on the basis of restriction endonuclease analysis of polymerase chain reaction products. *J. Clin. Microbiol.* **29,** 1640–1644.
135. Repp, R., Rhiel, S., Heermann, K. H., Schaefer, S., Keller, C., Ndumbe, P., Lampert, F., and Gerlich, W. H. (1993) Genotyping by multiplex polymerase chain reaction for detection of endemic hepatitis B virus transmission. *J. Clin. Microbiol.* **31,** 1095–1102.
136. Rodriguez-Frias, F., Buti, M., Arranz, J. A., Cotrina, M., Jardi, R., Esteban, R., and Guardia, J. (1994) Detection of hepatitis B precore mutants by the fluorescent linear polymerase chain reaction sequencing method. *J. Hepatol.* **21,** 934–939.
137. Rodriguez-Frias, F., Buti, M., Jardi, R., Cotrina, M., Viladomiu, L., Esteban, R., and Guardia, J. (1995) Hepatitis B virus infection: precore mutants and its relation to viral genotypes and core mutations. *Hepatology* **22,** 1641–1647.
138. Chu, , C.-M., Yeh, C.-T., Chiu, C.-T., Sheen, I.-S., and Liaw, Y.-F. (1996) Precore mutant of hepatitis B virus prevails in acute and chronic infections in an area in which hepatitis B is endemic. *J. Clin. Microbiol.* **34,** 1815–1818.
139. Kuo, G., Choo, Q.-L., Alter, H. J., Gitnick, G. L., Redeker, A. G., Purcell, R. H., Miyamura, T., Dienstag, J. L., Alter, M. J., Stevens, C. E., Tegtmeier, G. E., Bonino, F., Colombo, M., Lee, W.-S., Kuo, C., Berfer, K., Shuster, J. R., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* **244,** 362–364.
140. Ebeling, F., Naukkarinen, R., and Leikola, J. (1990) Recombinant immunoblot assay for hepatitis C antibody as predictor of infectivity. *Lancet* **335,** 982–983.
141. McHutchison, J. G., Person, J. L., Govindarajan, S., Valinluck, B., Gore, T., Lee, S. R., Nelies, M., Polito, A., Chien, D., DiNello, R., Quan, S., Kuo, G., and Redeker, A. (1992) Improved detection of hepatitis C virus antibodies in high risk population. *Hepatology* **15,** 19.
142. Alter, M. J., Margolis, H. S., Krawczynski, K., Judson, F. N., Maree, A., Alexander, W. J., Hu, P. Y., Miller, J. K., Gerber, M. A., Sampliner, R. E., Meeks, E. L., and Beach, M. J. (1992) The nautral histroy of community-acquired hepatitis C in the United States. *N. Eng. J. Med.* **327,** 1899–1905.
143. Dittman, S., Roggendorf, M., Durkop, J., Wiese, M., Lorbeer, B., and Deinhardt, F. (1991) Long-term persistence of hepatitis C virus antibodies in a single source outbreak. *J. Hepatol.* **13,** 323–327.
144. Chamot, E., Hirschel, B., Wintsch, J., Robert, C. F., Gabriel, J., Deglon, J., Yerly, S., and Perrin, L. (1990) Loss of antibodies against hepatitis C virus in HIV-seropositive intravenous drug users. *AIDS* **4,** 1275–1277.
145. Lok, A. S. F., Chien, D., Choo, Q.-L., Chan, T. M., Chiu, E. K. W., Cheng, I. K. P., Houghton, M., and Kuo, G. (1993) Antibody response to core, envelope and nonstructural hepatitis C virus antigens—comparison of immunocompetent and immunosuppressed patients. *Hepatology* **18,** 497–502.
146. Hsu, H. H., Wright, T. L., Tsao, S. C., Combs, C., Donets, M., Feinstone, S. M., and Greenberg (1994) Attenuated antibody response to hepatitis C virus infection after liver transplantation. *Amer. J. Gastroenterol.* **89,** 1169–1174.
147. Yuki, N., Hayashi, N., Hagiwara, H., Takehara, T., Katayama, K., Kasahara, A., Fusamoto, H., and Kamada, T. (1993) Quantitative analysis of antibodies to hepatitis C virus during interferon-a therapy. *Hepatology* **17,** 960–965.
148. Saracco, G., Rosina, F., Abate, M. L., Chiandussi, L., Gallo, V., Cerutti, E., Dinapoli, A., Solinas, A., Deplano, A., Tocco, A., Cossu, P., Chien, D., Kuo, G., Polito, A.,

Weiner, A. J., Houghton, M., Verme, G., Bonino, F., and Rizzetto, M. (1993) Long-term follow-up of patients with chronic hepatitis C treated with different doses of interferon-a2b. *Hepatol*ogy **18,** 1300–1305.

149. Brillanti, S., Foli, M., Perini, P., Masci, C., Miglioli, M., and Barbara, L. (1991) Long-term persistence of IgM antibodies to HCV in chronic hepatitis C. *Hepatology* **14,** 969–974.
150. Chen, M., Sonnerborg, A., and Sallberg, M. (1995) Levels of hepatitis C virus (HCV) RNA in serum and their relationship to levels of immunoglobulin M and G antibodies against HCV core protein. *J. Clin. Microbiol.* **33,** 778–780.
151. Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244,** 359–362.
152. Houghton, M., Weiner, A., Han, J., Kuo, G., and Choo, Q.-L. (1991) Molecular biology of the hepatitis C viruses: Implications for diagnosis, development and control of viral disease. *Hepatology* **14,** 381–388.
153. Alter, H. J. (1992) New kit on the block: Evaluation of second generation assays for detection of antibody to hepatitis C virus. *Hepatology* **15,** 350–353.
154. Kleinman, S., Alter, H., Busch, M., Holland, P., Tegtmeier, G., Nelles, M., Lee, S., Page, E., Wilber, J., and Polito, A. (1992) Increased detection of hepatitis C virus (HCV)-infected blood donors by a multiple-antigen HCV enzyme immunoassay. *Transfusion* **32,** 805–813.
155. Bresters, D., Cuypers, H. T., Reesink, H. W., Schaasberg, W. P., van der Poel, C. L., Mauser-Bunschoten, E. P., Houghton, M., Choo, Q.-L., Kuo, G., Lesniewski, R., Troonen, H., and Lelie, P. N. (1992) Enhanced sensitivity of a secon generation ELISA for antibody to hepatitis C virus. *Vox Sang.* **62,** 213–217.
156. Watanabe, J., Matsumoto, C., Fujimura, K., Shimada, T., Yoshizawa, H., Okamoto, H., Iizuka, H., Tango, T., Ikeda, H., Endo, N., Mazda, T., Nojiri, T., Aoyama, K., Kanemitsu, K., Yamano, H., Mizui, M., Yokoishi, F., Tokunaga, K., and Nishioka, K. (1993) Predictive value of screening tests for persistent hepatitis C virus infection evidence by viraemia—Japanese experience. *Vox Sang.* **65,** 199–203.
157. Lok, A. S., Ma, O. C., Chan, T. M., Lai, C. L., Chung, H. T., Ng, C. P., and Lam, J. S. (1991) Overestimation of the prevalence of antibody to hepatitis C virus in retrospective studies on stored sera. *Hepatology* **14,** 756–762.
158. Tibbs, C. J., Palmer, S. J., Coker, R., Clark, S. K., Parsons, G. M., Hojvat, S. Peterson, D. and Banatvala, J. E. (1991) Prevalence of hepatitis C in tropical communities: the importance of confirmatory assays. *J. Med. Virol.* **34,** 143–147.
159. Schvarcz, R., von Sydow, M., and Weiland, O. (1990) Autoimmune chronic active hepatitis: changing reactivity for antibodies to hepatitis C virus after immunosuppresive treatment. *Scand. J. Gastroenterol.* **25,** 1175–1180.
160. McFarlane, I. G., Smith, H. M., Johnson, P. J., Bray, G. P., Vergani, D., and Williams, R. (1990) Hepatitis C virus antibodies in chronic active hepatitis: pathogenetic factor or false-positive result? *Lancet* **335,** 754–757.
161. Alter, H. J., Purcell, R. H., Shih, J. W., Melpolder, J. C., Houghton, M., Choo, Q.-L., and Kuo, G. (1989) Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N. Engl. J. Med.* **321,** 1494–1500.
162. van der Poel, C. L., Reesink, H. W., Lelie, P. N. R., Leentvaar-Kuypers, A., Choo, Q.-L., Kuo, G., and Houghton, M. (1989) Anti-hepatitis C antibodies and non-A, non-B post-transfusion hepatitis in the Netherlands. *Lancet* **2,** 297–298.

163. Aach, R. D., Stevens, C. E., Hollinger, F. B., Mosley, J. W., Peterson, D. A., Taylor, P. E., Johnson, R. G., Barbosa, L. H., and Nemo, G. J. (1991) Hepatitis C virus infection in post-transfusion hepatitis. An analysis with first- and second-generation assays. *N. Engl. J. Med.* **325,** 1325–1329.
164. Sugitani, M., Inchauspe, G., Shindo, M., and Prince, A. M. (1992) Sensitivity of serological assays to identify blood donors with hepatitis C viraemia. *Lancet* **339,** 1018–1019.
165. van der Poel, C. L., Bresters, D., Reesink, H. W., Plaisier, A. A., Schaasberg, W., Leentvaar-Kuypers, A., Choo, Q.-L., Quan, S., Polito, A., Houghton, M., Kuo, G., Lelie, P. N. R., and Cuypers, H. T. M. (1992) Early anti-hepatitis C virus response with second-generation C200/C22 ELISA. *Vox Sang.* **62,** 208–212.
166. Lee, S., McHutchinson, J., Francis, B., DiNello, R., Polito, A., Quan, S., and Nelles, M. (1992) Improved detection of antibodies to hepatitis C virus using a second generation ELISA. *Adv. Exp. Med. Biol.* **312,** 183–189.
167. Prohaska, W., Schroeter, E., Kaars-Wiele, P., and Kleesiek, K. (1992) Enzyme immunoassays for anti-hepatitis C virus antibodies improved specificity and analytical sensitivity by combination of three different recombinant viral proteins in second generation tests. *Eur. J. Clin. Chem. Clin. Biochem.* **30,** 397–404.
168. Sayers, M. H. and Gretch, D. R. (1993) Recombinant immunoblot and polymerase chain reaction testing in volunteer whole blood donors screened by a multi-antigen assay for hepatitis C virus antibodies. *Transfusion* **33,** 809–813.
169. Marcellin, P., Martinot-Peignox, M., Gabriel, F., Branger, M., Degott, C., Elias, A., Xu, L. Z., Larzul, D., Erlinger, S., and Benhamou, J. P. (1993) Chronic non-B, non-C hepatitis among blood donors assessed with HCV 3rd generation tests and polymerase chain reaction. *J. Hepatol.* **19,** 167–170.
170. Lavanchy, D., Mayerat, C., Morel, B., Schneider, P., Zufferey, C., Gonvers, J.-J., Pecoud, A., and Frei, P. C. (1994) Evaluation of third-generation assays for detection of anti-hepatitis C virus (HCV) antibodies and comparison with presence of HCV RNA in blood donors reactive to c100–3 antigen. *J. Clin. Microbiol.* **32,** 2272–2275.
171. van der Poel, C. L., Cuypers, H. T., Reesink, H. W., Weiner, A. J., Quan, S., DiNello, R., Van, B. J. J., Winkel, I., Mulder-Folkerts, D., Exel-Oehlers, P. J., Schaasberg, W., Leentvaar-Kuypers, A., Polito, A. Houghton, M., and Lelie, P. N. (1991) Confirmation of hepatitis C virus infection by new four-antigen recombinant immunoblot assay. *Lancet* **337,** 317–319.
172. Chaudhary, R. K. and MacLean, C. (1991) Evaluation of first- and second-generation RIBA kits for detection of antibody to hepatitis C virus. *J. Clin. Microbiol.* **29,** 2329–2330.
173. Tobler, L. H., Busch, M. P., Wilber, J., DiNello, R., Quan, S., Polito, A., Kochesky, R., Bahl, C., Nelles, M., and Lee, S. R. (1994) Evaluation of indeterminate c22–3 reactivity in volunteer blood donors. *Transfusion* **34,** 130–134.
174. Zein, N. N., Germer, J. J., Wendt, N. K., Schimek, C. M., Thorvilson, J. N., Mitchell, P. S., and Persing, D. H. (1997) Indeterminate results of the second-generation hepatitis C virus (HCV) recombinant immunoblot assay: significance of high-level c22–3 reactivity and influence of HCV genotypes. *J. Clin. Microbiol.* **35,** 311–312.
175. Pawlotsky, J.-M., Fleury, A., Choukroun, V., Deforges, L., Roudot-Thoraval, F., Aumont, P., Duval, J., and Dhumeaux, D. (1994) Significance of highly positive c22–3 "indeterminate" second-generation hepatitis C virus (HCV) recombinant immunoblot assay (RIBA) and resolution by third-generation HCV RIBA. *J. Clin. Microbiol.* **32,** 1357–1359.

176. Chaudhary, R. K. and Jacobsen, H. (1994) Performance of third-generation confirmatory tests for detection of antibody to hepatitis C virus. *J. Clin. Microbiol.* **32,** 2606–2608.
177. Icardi, G., Bonanni, P., Raffo, A. M., Masini, A., Orione, L., De Martini, M., and Crovari, P. (1997) Evaluation of performance of the RIBA processor system for automated analysis of the strip immunoblot assay for detection of antibodies to hepatitis C virus. *J. Clin. Microbiol.* **35,** 2331–2336.
178. Martin, P., Fabrizi, F., Dixit, F. V., Quan, S., Brezina, M., Kaufman, E., Sra, K., DiNello, R., Polito, A., and Gitnick, G. (1998) Automated RIBA hepatitis C virus (HCV) strip immunoblot assay for reproducible HCV diagnosis. *J. Clin. Microbiol.* **36,** 387–390.
179. Gretch, D. R., Wilson, J. J., Carithers, R. L., dela Rosa, C., Han, J. H., and Corey, L. (1993) Detection of hepatitis C virus RNA: Comparison of one-stage polymerase chain reaction (PCR) with nested-set PCR. *J. Clin. Microbiol.* **31,** 289–291.
180. Lemon, S. M. and Brown, E. A. (1995) Hepatitis C virus, in *Principles and Practice of Infectious Diseases*, 4th ed. (Mandell, G. L., Douglas, R. G. J., and Benett, J. E., eds.), Churchill Livingston, New York, pp. 1474–1486.
181. Han, J., Shyamala, V., Richman, K. H., Brauer, M. H., Tekamp-Olson, P., Irvine, B., Urdea, M. S., Kuo, G., Choo, Q.-L., and Houghton, M. (1991) Characterization of the terminal regions of hepatitis C viral RNA: Identification of conserved sequences in the 5'-untranslated region and poly A tails at the 3'-end. *Proc. Natl. Acad. Sci. USA* **88,** 1711–1715.
182. Cha, T.-A., Beall, E., Irvine, B., Kolberg, J., Chien, D., Kuo, G., and Urdea, M. S. (1992) At least five related, but distinct, hepatitis C viral genotypes exist. *Proc. Natl. Acad. Sci. USA* **89,** 7144–7148.
183. Simmonds, P., Holmes, E. C., Cha, T. A., Chan, S. W., McOmish, F., Irvine, B., Beall, E., Yap, P. L., Kolberg, J., and Urdea, M. S. (1993) Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS–5 region. *J. Gen Virol.* **74,** 2391–2399.
184. Cha, T.-A., Kolberg, J., Irvine, B., Stempien, M., Beall, E., Yano, M., Choo, Q.-L., Houghton, M., Kuo, G., Han, J. H., and Urdea, M. S. (1991) Use of a signature nucleotide sequence of hepatitis C virus for detection of viral RNA in human serum and plasma. *J. Clin. Microbiol.* **29,** 2528–2534.
185. Bukh, J., Purcell, R. H., and Miller, R. H. (1992) Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. *Proc. Natl. Acad. Sci. USA* **89,** 187–191.
186. Wilber, J. C., Johnson, P. J., and Urdea, M. S. (1993) Reverse transcriptase-PCR for hepatitis C virus RNA, in *Diagnostic Molecular Microbiology: Principles and Applications* (Persing, D. H., Smith, T. F., Tenover, F. C., and White, T. J., eds.), Amer. Soc. Microbiol., Washington, pp. 327–331.
187. Okamoto, H., Okada, S., Sugiyama, Y., Yotsumoto, S., Tanaka, T., Sugai, Y., Akahane, Y., Machida, A., Mishiro, S., Yoshizawa, H., Miyakawa, Y., and Mayumi, M. (1990) Detection of hepatitis C virus RNA by a two stage polymerase chain reaction with two pairs of primers and deduced from the 5' noncoding region. *Japan J. Exp. Med.* **60,** 215–222.
188. Okamoto, M., Baba, M., Kodama, E., Sekine, K., Takagi, T., Kasukawa, R., and Shigeta, S. (1993) Detection of hepatitis C virus genome in human serum by multi-targeted polymerase chain reaction. *J. Med. Virol.* **41,** 6–10.

189. Weiner, A. J., Kuo, G., Bradley, D. W., Bonino, F., Saracco, G., Lee, C., Rosenblatt, J., Choo, Q.-L., and Houghton, M. (1990a) Detection of hepatitis C viral sequences in non-A, non-B hepatitis. *Lancet* **335,** 1–3.
190. Weiner, A. J., Truett, M. A., Rosenblatt, J., Han, J., Quan, S., Polito, A. J., Kuo, G., Choo, Q.-L., Houghton, M., Agius, C., Page, E., and Nelles, M. J. (1990b) HCV testing in a low-risk population. *Lancet* **336,** 695.
191. Garson, J. A., Preston, F. E., Makris, M., Tuke, P., Ring, C., Machin, S. J., and Tedder, R. S. (1990) Detection by PCR of hepatitis C virus in factor VIII concentrates. *Lancet* **335,** 1473–1474.
192. Garson, J. A., Tedder, R. S., Briggs, M., Tuke, P., Glazebrook, J. A., Trute, A., Parker, D., Barbara, J. A. J., Contreras, M., and Aloysius, S. (1990) Detection of hepatitis C viral sequences in blood donations by "nested" polymerase chain reaction and prediction of infectivity. *Lancet* **335,** 1419–1422.
193. Ulrich, P. P., Romeo, J. M., Lane, P. K., Kelly, I., Daniel, L. J., and Vyas, G. N. (1990) Detection, semiquantitation, and genetic variation in hepatitis C virus sequences amplified from the plasma of blood donors with elevated alanine aminotransferase. *J. Clin. Invest.* **86,** 1609–1614.
194. Kaneko, A., Unoura, M., Kobayashi, K., Kuno, K., Murakami, S., and Hattori, N. (1990) Detection of serum hepatitis C virus RNA (letter). *Lancet* **335,** 976.
195. Gretch, D., Lee, W., and Corey, L. (1992) Use of aminotransferase, hepatitis C antibody, and hepatitis C polymerase chain reaction RNA assays to establish the diagnosis of hepatitis C virus infection in a diagnostic virology laboratory. *J. Clin. Microbiol.* **30,** 2145–2149.
196. Marin, M. G., Bresciani, S., Puoti, M., Rodella, A., Gussago, A., Ravaggi, A., Pizzocolo, G., Albertini, A., and Cariani, E. (1994) Clinical significance of serum hepatitis C virus (HCV) RNA as marker of HCV infection. *J. Clin. Microbiol.* **32,** 3008–3012.
197. Young, K. K., Resnick, R. M., and Myers, T. W. (1993) Detection of hepatitis C virus RNA by a combined reverse transcription-polymerase chain reaction assay. *J. Clin. Microbiol.* **31,** 882–886.
198. Bresters, D., Cuypers, H. T. M., Reesink, H. W., Chamuleau, R. A. F. M., Schipper, M. E. I., Boesernunnink, B. D. M., Lelie, P. N., and Jansen, P. L. M. (1992) Detection of hepatitis C viral RNA sequences in fresh and paraffin-embedded liver biopsy specimens of non-A hepatitis, non-B hepatitis patients. *J. Hepatol.* **15,** 391–395.
199. Hosoda, K., Omata, M., Yokosuka, O., Kato, N. R., and Ohto, M. (1992) Non-A, non-B chronic hepatitis is chronic hepatitis C: a sensitive assay for detection of hepatitis C virus RNA in the liver. *Hepatology* **15,** 777–781.
200. Hsieh, T. T., Yao, D. S., Sheen, I. S., Liaw, Y. F., and Pao, C. C. (1992) Hepatitis C virus in peripheral blood mononuclear cells. *Amer. J. Clin. Pathol.* **98,** 392–396.
201. Wang, J. T., Sheu, J. C., Lin, J. T., Wang, T. H., and Chen, D. S. (1992) Detection of replicative form of hepatitis C virus RNA in peripheral blood mononuclear cells. *J. Infect. Dis.* **166,** 1167–1169.
202. Prati, D., Capelli, C., Bosoni, P., Mozzi, F., Zanella, A., and Sirchia, G. (1994) Determination of hepatitis C virus RNA in the serum by the Amplicor HCV PCR kit. *Vox Sang.* **67,** 112–114.
203. Zeuzem, S., Ruster, B., and Roth, W. K. (1994) Clinical evaluation of a new polymerase chain reaction assay (Amplicor HCV) for detection of hepatitis C virus. *Gastroenterology* **32,** 342–347.

204. Nolte, F. S., Thurmond, C., and Fried, M. W. (1995) Preclinical evaluation of AMPLICOR hepatitis C virus test for detection of hepatitis C virus RNA. *J. Clin. Microbiol.* **33,** 1775–1778.
205. Jungkind, D., DiRenzo, S., Beavis, K. G., and Silverman, N. S. (1996) Evaluation of automated COBAS AMPLICOR PCR system for detection of several infectious agents and its impact on laboratory management. *J. Clin. Microbiol.* **34,** 2778–2783.
206. Poljak, M., Seme, K., and Koren, S. (1997) Evaluation of the automated COBAS AMPLICOR Hepatitis C virus PCR system. *J. Clin. Microbiol.* **35,** 2983–2984.
207. Hsuih, T. C. H., Park, Y. N., Zaretsky, C., Wu, F., Tyagi, S., Kramer, F. R., Sperling, R., and Zhang, D. Y. (1996) Novel, ligation-dependent PCR assay for detection of hepatitis C virus in serum. *J. Clin. Microbiol.* **34,** 501–507.
208. Hodinka, R. L. (1998) The clinical utility of viral quantitation using molecular methods. *Clin. Diag. Virol.* **10,** 25–47.
209. Gretch, D., Corey, L., Wilson, J., dela Rosa, C., Willson, R., Carithers, R. Jr., Busch, M., Hart, J., Sayers, M., and Han, J. (1994) Assessment of hepatitis C virus RNA levels by quantitative competitive RNA polymerase chain reaction: high-titer viremia correlates with advanced stage of disease. *J. Infect. Dis.* **169,** 1219–1225.
210. Sherman, K. E., O'Brien, J., Gutierrez, A. G., Harrison, S., Urdea, M., Neuwald, P., and Wilber, J. (1993) Quantitative evaluation of hepatitis C virus RNA in patients with concurrent human immunodeficiency virus infections. *J. Clin. Microbiol.* **31,** 2679–2682.
211. Eyster, M. E., Alter, H. J., Aledort, L. M., Quam, S., Hatzakin, A., and Goedert, J. J. (1991) Heterosexual co-transmission of hepatitis C virus (HCV) and human immunodeficiency virus (HIV). *Ann. Int. Med.* **115,** 764–768.
212. Zeuzem, S., Schmidt, J. M., Lee, J.-H., Ruster, B., and Roth, W. K. (1996) Effect of interferon alpha on the dynamics of hepatitis C virus turnover *in vivo*. *Hepatology* **23,** 366–371.
213. Shindo, M., Di Bisceglie, A. M., Cheung, L., Shih, W.-K., Cristiano, K., Feinstone, S. M., and Hoofnagle, J. H. (1991) Decrease in serum hepatitis C viral RNA during alpha-interferon therapy for chronic hepatitis C. *Ann. Int. Med.* **115,** 700–704.
214. Davis, G. L., Balart, L. A., Schiff, E. R., Lindsay, K., Bodenheimer, H. C., Perrillo, R. P., Carey, W., Jacobson, I. M., Payne, J., Dienstag, J. L., Van Thiel, D. H., Tamburro, C., Lefkowitch, J., Albrecht, J., Meschievitz, C., Ortego, T. J., Givbas, A., and The Hepatitis Interventional Group (1989) Treatment of chronic hepatitis C with recombinant interferon alpha. A multicenter randomized controlled trial. *N. Engl. J. Med.* **321,** 1501–1505.
215. Magrin, S., Craxi, A., Fabiano, C., Simonetti, R. G., Fiorentino, G., Marino, L., Diquattro, O., DiMarco, V., Loiacono, O., Volpes, R., Almasio, P., Urdea, M. S., Neuwald, P., Sanchez-Pescador, R., Detmer, J., Wilber, J. C., and Pagliaro, L. (1993) Hepatitis C viremia in chronic liver disease: relationship to interferon-a or corticosteroid treatment. *Hepatology* **19,** 273–279.
216. Lau, J. Y. N., Davis, G. L., Kniffen, J., Qian, K. P., Urdea, M. S., Chan, C. S., Mizokami, M., Neuwald, P. D., and Wilber, J. C. (1993) Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet* **341,** 1501–1504.
217. Orito, E., Mizokami, M., Nakano, T., Terashima, H., Nojiri, O., Sakakibara, K., Mizuno, M., Ogino, M., Nakamura, M., Matsumoto, Y., Miyata, K.-I., and Lau, J. Y. N. (1994) Serum hepatitis C virus RNA level as a predictor of subsequent response

to interferon-alpha therapy in Japanese patients with chronic hepatitis. *C. J. Med. Virol.* **44,** 410–414.

218. Davis, G., Lau, J., Urdea, M., Neuwald, P. D., Wilber, J. C., Lindsay, K., Perrillo, R. P., and Albrecht, J. (1994) Quantitative detection of hepatitis C virus RNA with a solid-phase signal amplification method: Definition of optimal conditions for specimen collection and clinical application in interferon-treated patients. *Hepatology* **19,** 1337–1341.
219. Wright, T. L., Combs, C., Kim, M., Ferrell, L., Bacchetti, P., Ascher, N., Roberts, J., Wilber, J., Sheridan, P., and Urdea, M. (1994) Interferon-α therapy for hepatitis C virus infection after liver transplantation. *Hepatology* **20,** 773–779.
220. Yun, Z. B., Reichard, O., Chen, M., Lundeberg, J., Norkrans, G., Fryden, A., Sonnerborg, A., and Weiland, O. (1994) Serum hepatitis C virus RNA levels in chronic hepatitis C: importance for outcome of interferon alfa–2b treatment. *Scand. J. Infect. Dis.* **26,** 263–270.
221. Martinot-Peignoux, M., Marcellin, P., Pouteau, M., Castelnau, C., Boyer, N., Poliquin, M., Degott, C., Descombes, I., LeBreton, V., Milotova, V., Benhamou, J. P., and Erlinger, S. (1995) Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alpha therapy in chronic hepatitis C. *Hepatology* **22,** 1050–1056.
222. Davis, G. L. (1996) Development of treatment strategies for chronic hepatitis C. *Antiviral Therapy* **1,** 90–93.
223. Terrault, N. A., Dailey, P. J., Ferrell, L., Collins, M. L., Wilber, J. C., Urdea, M. S., Bhandari, B. N., and Wright, T. L. (1997) Hepatitis C virus: quantitation and distribution in liver. *J. Med. Virol.* **51,** 217–224.
224. Li, X., de Medina, M., LaRue, S., Shao, L., and Schiff, E. R. (1993) Comparison of assays for HCV RNA. *Lancet* **342,** 1174–1175.
225. Bresters, D., Cuypers, H. T. M., Reesink, H. W., Mauser-Bunschoten, E. P., van den Berg, H. M., Schaasberg, W. P., Wilber, J. C., Urdea, M. S., Neuwald, P., and Lelie, P. N. (1994) Comparison of quantitative cDNA-PCR with the branched DNA hybridization assay for monitoring plasma hepatitis C virus RNA levels in hemophilia patients participating in a controlled interferon trial. *J. Med. Virol.* **43,** 262–268.
226. Gretch, D. R., dela Rosa, C., Carithers Jr., R. L., Wilson, R. A., Williams, B., and Corey, L. (1995) Assessment of hepatitis C viremia using molecular amplification technologies: correlations and clinical implications. *Ann. Int. Med.* **123,** 321–329.
227. Mayerat, C., Burgisser, P., Lavanchy, D., Mantegani, A., and Frei, P. C. (1996) Comparison of a competitive combined reverse transcription-PCR assay with a branched-DNA assay for hepatitis C virus RNA quantitation. *J. Clin. Microbiol.* **34,** 2702–2706.
228. Roth, W. K., Lee, J.-H., Ruster, B., and Zeuzem, S. (1996) Comparison of two quantitative hepatitis C virus reverse transcriptase PCR assays. *J. Clin. Microbiol.* **34,** 261–264.
229. Hadziyannis, E., Fried, M. W., and Nolte, F. S. (1997) Evaluation of two methods for quantitation of hepatitis C virus RNA. *Mol. Diag.* **2,** 39–46.
230. Hawkins, A., Davidson, F., and Simmonds, P. (1997) Comparison of plasma virus loads among individuals infected with hepatitis C virus (HCV) genotypes 1, 2, and 3 by quantiplex HCV RNA assay versions 1 and 2, Roche monitor assay, and an in-house limiting dilution method. *J. Clin. Microbiol.* **35,** 187–192.

231. Jacob, S., Baudy, D., Jones, E., Xu, L., Mason, A., Regenstein, F., and Perrillo, R. P. (1997) Comparison of quantitative HCV RNA assays in chronic hepatitis C. *Amer. J. Clin. Pathol.* **107,** 362–367.
232. Pawlotsky, J.-M. (1997) Measuring hepatitis C viremia in clinical samples: Can we trust the assays? *Hepatology* **26,** 1–4.
233. Shimizu, Y. K., Iwamoto, A., Hijikata, M., Purcell, R. H., and Yoshikura, H. (1992) Evidence for in vitro replication of hepatitis C virus genome in a human t cell line. *Proc. Natl. Acad. Sci. USA* **89,** 5477–5481.
234. Shimizu, Y. K., Purcell, R. H., and Yoshikura, H. (1993) Correlation between the infectivity of hepatitis C virus *in vivo* and its infectivity *in vitro*. *Proc. Natl. Acad. Sci. USA* **90,** 6037–6041.
235. Iacovacci, S., Sargiacomo, M., Parolini, I., Ponzetto, A., Peschle, C., and Carloni, G. (1993) Replication and multiplication of hepatitis C virus genome in human foetal liver cells. *Res. Virol.* **144,** 275–279.
236. Lanford, R. E., Sureau, C., Jacob, J. R., White, R., and Fuerst, T. R. (1994) Demonstration of *in vitro* infection of chimpanzees hepatocytes with hepatitis C virus using strand-specific RT-PCR. *Virology* **202,** 606–614.
237. Bertolini, L., Iacovacci, S., Ponzetto, A., Gorini, G., Battaglia, M., and Carloni, G. (1993) The human bone-marrow-derived B cell line CE, susceptible to hepatitis C virus infection. *Res. Virol.* **144,** 281–285.
238. Yoo, B. J., Selby, M. J., Choe, J., Suh, B. S., Choi, S. H., Joh, J. S., Nuovo, G. J., Lee, H. S., Houghton, M., and Han, J. H. (1995) Transfection of a differentiated human hepatoma cell line (Huh7) with in vitro-transcribed hepatitis C virus (HCV) RNA and establishment of a long-term culture persistently infected with HCV. *J. Virol.* **69,** 32–38.
239. Krawczynski, K., Beach, M., Bradley, D. W., Kuo, G., Di Bisceglie, A. M., Houghton, M., Reyes, G. R., Kim, J. P., Choo, Q.-L., and Alter, M. J. (1992) Hepatitis C virus antigen in hepatocytes: immunomorphologic detection and identification. *Gastroenterology* **103,** 622–629.
240. Blight, K., Lesniewski, R. R., LaBrooy, J. T., and Gowans, E. J. (1994) Detection and distribution of hepatitis C-specific antigens in naturally infected liver. *Hepatology* **20,** 553–557.
241. Lamas, E., Baccarini, P., Housset, C., Kremsdorf, D., and Brechot, C. (1992) Detection of hepatitis C virus (HCV) RNA sequences in liver tissue by in situ hybridization. *J. Hepatol.* **16,** 219–223.
242. Negro, F., Pacchioni, D., Shimizu, Y., Miller, R. H., Bussolati, G., Purcell, R. H., and Bonino, F. (1992) Detection of intrahepatic replication of hepatitis C virus RNA by in situ hybridization and comparison with histopathology. *Proc. Natl. Acad. Sci. USA* **89,** 2247–2251.
243. Simmonds, P. (1995) Variability of hepatitis C virus. *Hepatology* **21,** 570–583.
244. Brown, J. L., Carman, W. F., and Thomas, H. (1992) The clinical significance of molecular variation within the hepatitis B virus geneome. *Hepatology* **15,** 144–148.
245. Smith, D. B., Davidson, F., and Simmonds, P. (1995) Hepatitis C virus variants and the role of genotyping, *J. Hepatol.* **23**(Suppl. 2), 26–31.
246. Zein, N. N. and Persing, D. H. (1996a) Hepatitis C genotypes: Current trends and future implications. *Mayo Clin. Proc.* **71,** 458–462.
247. Nousbaum, J. B., Pol, S., Nalpas, B., Landais, P., Berthelot, P. and Brechot, C. (1995) Hepatitis C virus type 1b (II) infection in France and Italy. *Ann. Int. Med.* **122,** 161–168.

248. Zein, N. N., Poterucha, J. J., Gross, J. B. Jr., Weisner, R. H., Therneau, T. M., Gossard, A. A., Wendt, N. K., Mitchell, P. S., Germer, J. J., and Persing, D. H. (1996b) Increased risk of hepatocellular carcinoma in patients infected with hepatitis C genotype 1b. *Amer. J. Gastroenterol.* **91,** 2560–2562.
249. Gordon, F. D., Poterucha, J. J., Germer, J., Zein, N. N., Batts, K. P., Gross, J. B., Jr., Wiesner, R., and Persing, D. (1997) Relationship between hepatitis C genotype and severity of recurrent hepatitis C after liver transplantation. *Transplantation* **63,** 1419–1423.
250. McOmish, F., Chan, S.-W., Dow, B. C., Gillon, J., Frame, W. D., Crawford, R. J., Yap, P. L., Follett, E. A. C., and Simmonds, P. (1993) Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serological reactivity and rate of alanine aminotransferase abnormalities. *Transfusion* **33,** 7–13.
251. Zein, N. N., Rakela, J., and Persing, D. H. (1995) Genotype-dependent serologic reactivities in patients infected with hepatitis C virus in the United States. *Mayo Clin. Proc.* **70,** 449–452.
252. Dhaliwal, S. K., Prescott, L. E., Dow, B. C., Davidson, F., Brown, H., Yap, P. L., Follett, E. A., and Simmonds, P. (1996) Influence of viraemia and genotype upon serological reactivity in screening assays for antibody to hepatitis C virus. *J. Med. Virol.* **48,** 184–190.
253. Neville, J. A., Prescott, L. E., Bhattacherjee, V., Adams, N., Pike, I., Rodgers, B., El-Zayadi, A., Hamid, S., Dusheiko, G. M., Saeed, A. A., Haydon, G. H., and Simmonds, P. (1997) Antigenic variation of core, NS**3**, and NS5 proteins among genotypes of hepatitis C virus. *J. Clin. Microbiol.* **35,** 3062–3070.
254. Tsubota, A., Chayama, K., Arase, Y., Koida, I., Saitoh, S., Ikeda, K., Iwasaki, S., Matsumoto, T., Kobayashi, M., and Kumada, H. (1993) Factors useful in predicting the response to interferon therapy in chronic hepatitis C. *J. Gastroenterol. Hepatol.* **8,** 535–539.
255. Hino, K., Sainokami, S., Shimoda, K., Iino, S., Wang, Y., Okamoto, H., Miyakawa, Y., and Mayumi, M. (1994) Genotypes and titers of hepatitis C virus for predicting response to interferon in patients with chronic hepatitis C. *J. Med. Virol.* **42,** 299–305.
256. Kohara, M., Tanaka, T., Tsukiyama-Kohara, K., Tanaka, S., Mizokami, M., Lau, J. Y., and Hattori, N. (1995) Hepatitis C virus genotypes 1 and 2 respond to interferon-alpha with different virologic kinetics. *J. Infect. Dis.* **172,** 934–938.
257. Zein, N. N., Rakela, J., Krawitt, E. L., Reddy, K. R., Tominaga, T., and Persing, D. H. (1996c) Hepatitis C virus genotypes in the United States: epidemiology, pathogenicity, and response to interferon therapy. Collaborative Study Group. *Ann. Int. Med.* **125,** 634–639.
258. Cha, T.-A., Beall, E., Irvine, B., Kolberg, J., Chien, D., Kuo, G., and Urdea, M. S. (1992) At least five related, but distinct, hepatitis C viral genotypes exist. *Proc. Natl. Acad. Sci. USA* **89,** 7144–7148.
259. Nakao, T., Enomoto, N., Takada, N., Takada, A., and Date, T. (1991) Typing of hepatitis C virus genomes by restriction fragment length polymorphism. *J. Gen. Virol.* **72,** 2105–2112.
260. Davidson, F., Simmonds, P., Ferguson, J. C., Jarvis, L. M., Dow, B. C., Follett, E. A. C., Seed, C. R. G., Krusius, T., Lin, C., Medgyesi, G. A., Kiyokawa, H., Olim, G., Duraisamy, G., Cuypers, T., Saeed, A. A., Teo, D., Conradie, J., Kew, M. C., Lin, M., Nuchaprayoon, C., Ndimbie, O. K., and Yap, P. L. (1995) Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5' non-coding region. *J. Gen. Virol.* **76,** 1197–1204.

261. Okamoto, H., Sugiyama, Y., Okada, S., Kurai, K., Akahane, Y., Sugai, Y., Tanaka, T., Sato, K., Tsuda, F., Miyakawa, Y., and Mayumi, M. (1992) Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J. Gen. Virol.* **73,** 673–679.
262. Ohno, T., Mizokami, M., Wu, R.-R., Saleh, M. G., Ohba, K.-I., Orito, E., Mukaide, M., Williams, R., and Lau, J. Y. N. (1997) New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J. Clin. Microbiol.* **35,** 201–207.
263. Andonov, A. and Chaudhary, R. K. (1995) Subtyping of hepatitis C virus isolates by a line probe assay using hybridization. *J. Clin. Microbiol.* **33,** 254–256.
264. Stuyver, L., Wyseur, A., van Arnhem, W., Hernandez, F., and Maertens, G. (1996) Second-generation line probe assay for hepatitis C virus genotyping. *J. Clin. Microbiol.* **34,** 2259–2266.
265. Marshall, D. J., Heisler, L. M., Lyamichev, V., Murvine, C., Olive, D. M., Ehrlich, G. D., Neri, B. P., and de Arruda, M. (1997) Determination of hepatitis C virus genotypes in the United States by cleavase fragment length polymorphism analysis. *J. Clin. Microbiol.* **35,** 3156–3162.
266. Calvo, P. L., Kansopon, J., Sra, K., Quan, S., DiNello, R., Guaschino, R., Calabrese, G., Danielle, F., Brunetto, M. R., Bonino, F., Massaro, A. L., Polito, A., Houghton, M., and Weiner, A. J. (1998) Hepatitis C virus heteroduplex tracking assay for genotype determination reveals diverging genotype 2 isolates in Italian hemodialysis patients. *J. Clin. Microbiol.* **36,** 227–233.
267. Simmonds, P., Rose, K. A., Graham, S., Chan, S. W., McOmish, F., Dow, B. C., Follett, E. A., Yap, P. L., and Marsden, H. (1993) Mapping of serotype-specific, immunodominant epitopes in the NS–4 region of hepatitis C virus (HCV): use of type-specific peptides to serologically differentiate infections with HCV types 1, 2, and 3. *J. Clin. Microbiol.* **31,** 1493–1503.
268. Bhattacherjee, V., Prescott, L. E., Pike, I., Rodgers, B., Bell, H., El-Zayadi, A. R., Kew, M. C., Conradie, J., Lin, C. K., Marsden, H., Saeed, A. A., Parker, D., Yap, P. L., and Simmonds, P. (1995) Use of NS–4 peptides to identify type-specific antibody to hepatitis C virus genotypes 1, 2, 3, 4, 5 and 6. *J. Gen. Virol.* **76,** 1737–1748.
269. van Doorn, L.-J., Kleter, B., Pike, I., and Quint, W. (1996) Analysis of hepatitis C virus isolates by serotyping and genotyping. *J. Clin. Microbiol.* **34,** 1784–1787.
270. Pawlotsky, J.-M., Prescott, L., Simmonds, P., Pellet, C., Laurent-Puig, P., Labonne, C., Darthuy, F., Remire, J., Duval, J., Buffet, C., Etienne, J.-P., Dhumeaux, D., and Dussaix, E. (1997) Serological determination of hepatitis C virus genotype: comparison with a standardized genotyping assay. *J. Clin. Microbiol.* **35,** 1734–1739.
271. Machida, A., Ohnuma, H., Tsuda, F., Munekata, E., Tanaka, T., Akahane, Y., Okamoto, H., and Mishiro, S. (1992) Two distinct subtypes of hepatitis C virus defined by antibodies directed to the putative core protein. *Hepatology* **16,** 886–891.
272. Tanaka, S., Hasegawa, A., Ohta, Y., Hattori, N., and Kohara, M. (1994) Significance of specific antibody assay for genotyping of hepatitis C virus. *Hepatology* **19,** 1347–1353.
273. Dixit, V., Quan, S., Martin, P., Larson, D., Brezina, M., DiNello, R., Sra, K., Lau, J. Y. N., Chien, D., Kolberg, J., Tagger, A., Davis, G., Polito, A., and Gitnick, G. (1995) Evaluation of a novel serotyping system for hepatitis C virus: strong correlation with standard genotyping methodologies. *J. Clin. Microbiol.* **33,** 2978–2983.

274. Cerino, A., Cividini, A., Asti, M., Lanza, A., Silini, E., and Mondelli, M. U. (1996) Comparative evaluation of two serologic typing methods for hepatitis C virus. *J. Clin. Microbiol.* **34,** 714–716.
275. Lau, J. Y. N., Mizokami, M., Kolberg, J. A., Davis, G. L., Prescott, L. E., Ohno, T., Perrillo, R. P., Lindsay, K. L., Gish, R. G., Qian, K.-P., Kohara, M., Simmonds, P., and Urdea, M. S. (1995) Application of six hepatitis C virus genotyping systems to sera from chronic hepatitis C patients in the United States. *J. Infect. Dis.* **171,** 281–289.
276. Forns, X., Maluenda, M. D., Lopez-Labrador, F. X., Ampurdanes, S., Olmedo, E., Costa, J., Simmonds, P., Sanchez-Tapias, J. M., Jimenez de Anta, M. T., and Rodes, J. (1996) Comparative study of three methods for genotyping hepatitis C virus strains in samples from Spanish patients. *J. Clin. Microbiol.* **34,** 2516–2521.
277. Navas, S., Castillo, I., Martin, J., Quiroga, J. A., Bartolome, J., and Carreno, V. (1997) Concordance of hepatitis C virus typing methods based on restriction fragment length polymorphism analysis in 5' noncoding region and NS4 serotyping, but not in core PCR or a line probe assay. *J. Clin. Microbiol.* **35,** 317–321.
278. Rizzetto, M. (1983) The delta agent. *Hepatology* **3,** 729–737.
279. Hoofnagle, J. H. (1989) Type D (delta) hepatitis. *JAMA* **261,** 1321–1325.
280. Taylor, J., Negro, F., and Rizzetto, M. (1992) Hepatitis delta virus: from structure to disease expression. *Rev. Med. Virol.* **2,** 161–167.
281. Polish, L. B., Gallagher, M., Fields, H. A., and Hadler, S. C. (1993) Delta hepatitis: molecular biology and clinical and epidemiological features. *Clin. Microbiol. Rev.* **6,** 211–229.
282. Smedile, A., Lavarini, C., Crivelli, O., Raimondo, G., Fasone, M., and Rizzetto, M. (1982) Radioimmunoassay detection of IgM antibodies to the HBV-associated delta antigen: clinical significance in infection. *J. Med. Virol.* **9,** 131–138.
283. Dimitrakakis, M., Waters, M. J., Wootton, A., and Gust, I. (1986) Detection of IgM antibodies to delta antigen after coinfection and superinfection with the delta virus. *J. Med. Virol.* **20,** 305–311.
284. Farci, P., Gerin, J. L., Aragona, M., Lindsey, I., Crivelli, O., Balestrieri, A., Smedile, A., Thomas, H. C., and Rizzetto, M. (1986) Diagnostic and prognostic significance of the IgM antibody to the hepatitis delta virus. *JAMA* **255,** 1443–1446.
285. Shattock, A. G., Morris, M., and Kinane, K. (1989) The serology of delta hepatitis and the detection of IgM anti-HD by EIA using serum derived delta antigen. *J. Virol. Meth.* **23,** 233–240.
286. Rizzetto, M., Shih, J. W.-K., and Gerin, J. L. (1980) The hepatitis B virus-associated delta antigen: isolation from liver, development of solid-phase radioimmunoassays for delta antigen and anti-delta and partial characterization of delta antigen. *J. Immunol.* **125,** 318–324.
287. Crivelli, O., Rizzetto, M., Lavarini, C., Smedile, A., and Gerin, J. L. (1981) Enzyme-linked immunosorbent assay for detection of antibody to the hepatitis B surface antigen-associated delta antigen. *J. Clin. Microbiol.* **14,** 173–177.
288. Fields, H. A., Govindarajan, S., Margolis, H. S., Schable, C. D., Ebert, J. W., and Maynard, J. E. (1986) Experimental transmission of the delta virus to a hepatitis B chronic carrier chimpanzee with the development of persistent delta carriage. *Amer. J. Pathol.* **122,** 308–314.
289. Puig, J. and Fields, H. A. (1989) Development of an enzyme immunoassay using recombinant expressed antigen to detect hepatitis delta virus antibodies. *J. Clin. Microbiol.* **27,** 2222–2225.

290. Gowans, E. J., Macnaughton, T. B., Mickan, L., Jilbert, A. R., and Burrell, C. J. (1990) Use of recombinant hepatitis delta antigen in diagnostic assays for HDV antibody. *J. Virol. Meth.* **27,** 69–78.
291. Sumathy, S., Thyagarajan, S. P., Latif, R., Madanagopalan, N., Raguram, K., Rajasambandam, P., and Gowans, E. (1992) A dipstick immunobinding enzyme-linked immunosorbent assay for serodiagnosis of hepatitis B and delta virus infections. *J. Virol. Meth.* **38,** 145–152.
292. Shattock, A. G. and Morgan, B. M. (1984) Sensitive enzyme immunoassay for the detection of delta antigen and anti-delta, using serum as the delta antigen source. *J. Med. Virol.* **13,** 73–82.
293. Bonino, F., Heermann, K. H., Rizzetto, M., and Gerlich, W. H. (1986) Hepatitis delta virus: protein composition of delta antigen and its hepatitis B virus-derived envelope. *J. Virol.* **58,** 945–950.
294. Bergmann, K. F. and Gerin, J. L. (1986) Antigens of hepatitis delta virus in the liver and serum of humans and animals. *J. Infect. Dis.* **514,** 702–705.
295. Buti, M., Esteban, R., Jardi, R., Rodriguez-Frias, F., Casacuberta, J., Esteban, J. I., Allende, E., and Guardia, J. (1989) Chronic delta hepatitis: detection of hepatitis delta virus antigen in serum by immunoblot and correlation with other markers of delta viral replication. *Hepatology* **10,** 907–910.
296. Dubois, F. and Goudeau, A. (1988) Kinetics of delta antigen and delta antibody in acute delta hepatitis: evaluation with different enzyme immunoassays. *J. Clin. Microbiol.* **26,** 1339–1342.
297. Bezeaud, A., Rosenswajg, M., and Guillin, M. C. (1989) Evaluation of five hepatitis delta virus marker assays for detection of antigen and antibody. *J. Clin. Microbiol.* **27,** 2880.
298. Shattock, A. G. and Morris, M. C. (1991) Evaluation of commercial enzyme immunoassays for detection of hepatitis delta antigen and anti-hepatitis delta virus (HDV) and immunoglobulin M anti-HDV antibodies. *J. Clin. Microbiol.* **29,** 1873–1876.
299. Govindarajan, S., Valinluck, B., and Lake-Bakkar, G. (1991) Evaluation of a commercial anti-delta EIA kit for detection of antibodies to hepatitis delta virus. *Amer. J. Clin. Pathol.* **95,** 240–241.
300. Smedile, A., Rizzetto, M., Denniston, K., Bonino, F., Wells, F., Verme, G., Consolo, F., Hoyer, B., Purcell, R. H., and Gerin, J. L. (1986) Type D hepatitis: the clinical significance of hepatitis D virus RNA in serum as detected by a hybridization-based assay. *Hepatology* **6,** 1297–1302.
301. Buti, M., Esteban, R., Roggendorf, M., Fernandez, J., Jardi, R., Rashofer, R., Allende, H., Genesca, J., Esteban, J. I., and Guardia, J. (1988) Hepatitis D virus RNA in acute delta infection: serological profile and correlation with other markers of hepatitis D virus infection. *Hepatology* **8,** 1125–1129.
302. Rasshofer, R., Buti, M., Esteban, R., Jardi, R., and Roggendorf, M. (1988) Demonstration of hepatitis D virus RNA in patients with chronic hepatitis. *J. Infect. Dis.* **157,** 191–195.
303. Gupta, S., Valinluck, B., and Govindarajan, S. (1989) Detection of hepatitis delta virus in serum and liver tissue by molecular hybridization. Validation of a rapid spot-hybridization technique. *Amer. J. Clin. Pathol.* **92,** 218–221.
304. Smedile, A., Bergmann, K. F., Baroudy, B. M., Wells, F. V., Purcell, R. H., Bonino, F., Rizzetto, M., and Gerin, J. L. (1990) Riboprobe assay for HDV RNA: a sensitive

method for the detection of the HDV genome in clinical serum samples. *J. Med. Virol.* **30,** 20–24.
305. Gallagher, M., Morris, T. T., and Fields, H. A. (1994) Chemiluminescent hybridization assay for hepatitis delta virus RNA in serum. *J. Clin. Microbiol.* **32,** 1112–1114.
306. Zinegro, A. L., Deny, P., Feray, C., Ponzetto, A., Gentilini, P., Tiollais, P., and Brechot, C. (1990) Amplification of hepatitis delta virus RNA sequences by polymerase chain reaction: a tool for viral detection and cloning. *Mol. Cell. Probes* **4,** 43–51.
307. Simpson, L. H., Battegay, M., Hoofnagle, J. H., Waggoner, J. G., and Di Bisceglie, A. M. (1994) Hepatitis delta virus RNA in serum of patients with chronic delta hepatitis. *Dig. Dis. Sci.* **39,** 2650–2655.
308. Jardi, R., Buti, M., Cotrina, M., Rodriguez, F., Allende, H., Esteban, R., and Guardia, J. (1995) Determination of hepatitis delta virus RNA by polymerase chain reaction in acute and chronic delta infection. *Hepatology* **21,** 25–29.
309. Sakugawa, H., Nakasone, H., Kawakami, Y., Adaniya, H., Mizushima, T., Nakayoshi, T., Kinjo, F., Saito, A., Zukeran, H., Miyagi, Y., Yakabi, S., Taira, M., Kinoshita, M., and Yamakawa, Y. (1997) Determination of hepatitis delta virus (HDV)-RNA in asymptomatic cases of HDV infection. *Amer. J. Gastroenterol.* **92,** 2232–2236.
310. Cariani, E., Ravaggi, A., Puoti, M., Mantero, G., Albertini, A., and Primi, D. (1992) Evaluation of hepatitis delta virus RNA levels during interferon therapy by analysis of polymerase chain reaction products with a nonradioisotopic hybridization assay. *Hepatology* **15,** 685–689.
311. Madejon, A., Cotonat, T., Bartolome, J., Castillo, I., and Carreno, V. (1994) Treatment of chronic hepatitis D virus infection with low and high doses of interferon-alpha 2a: utility of polymerase chain reaction in monitoring antiviral response. *Hepatology* **19,** 1331–1336.
312. Tang, J. R., Cova, L., Lamelin, J. P., Baginski, I., Vitvitski, L., Gaudin, J. L., Hantz, O., and Trepo, C. (1994) Clinical relevance of the detection of hepatitis delta virus RNA in serum by RNA hybridization and polymerase chain reaction. *J. Hepatol.* **21,** 953–960.
313. Jardi, R., Buti, M., Rodriguez, F., Cotrina, M., Allende, H., Esteban, R., and Guardia, J. (1994) Comparative analysis of serological markers of chronic delta infection: HDV-RNA, serum HDAg and anti-HD IgM. *J. Virol. Meth.* **50,** 59–66.
314. Rizzetto, M., Canese, M. G., Arico, S., Crivelli, O., Trepo, C., Bonino, F., and Verme, G. (1977) Immunofluorescence detection of new antigen-antibody system (delta/anti-delta) associated to hepatitis B virus in liver and serum of HbsAg carriers. *Gut* **18,** 997–1003.
315. Recchia, S., Rizzi, R., Acquaviva, F., Rizzetto, M., Tison, V., Bonino, F., and Verme, G. (1981) Immunoperoxidase staining of the HBV-associated delta antigen in paraffinated liver specimens. *Pathologica* **73,** 773–777.
316. Stocklin, E., Gudat, F., Krey, G., Durmuller, U., Gasser, M., Schmid, M., Stalder, G., and Bianchi, L. (1981) Delta antigen in hepatitis B: immunohistology of frozen and paraffin embedded liver biopsies and relation to HBV infection. *Hepatology* **1,** 238–242.
317. Negro, F., Bonino, F., Di Bisceglie, A., Hoofnagle, J., and Gerin, J. L. (1989) Intrahepatic markers of hepatitis delta virus infection. A study by in situ hybridization. *Hepatology* **10,** 916–920.
318. Pacchioni, D., Negro, F., Chiaberge, E., Rizzetto, M., Bonino, F., and Bussolati, G. (1992) Detection of hepatitis delta virus RNA by a non-radioactive in situ hybridization procedure. *Human Pathol.* **23,** 557–561.

319. Lopez-Talavera, J. C., Buti, M., Casacuberta, J., Allende, H., Jardi, R., Esteban, R., and Guardia, J. (1993) Detection of hepatitis delta virus RNA in human liver tissue by non-radioactive in situ hybridization. *J. Hepatol.* **17,** 199–203.
320. Taylor, J., Mason, W., Summers, J., Goldberg, J., Aldrich, C., Coates, L., Gerin, J., and Gowans, E. (1987) Replication of human hepatitis delta virus in primary cultures of woodchuck hepatocytes. *J. Virol.* **61,** 2891–2895.
321. Sureau, C., Jacob, J. R., Eichberg, J. W. and Lanford, R. E. (1991) Tissue culture system for infection with human hepatitis delta virus. *J. Virol.* **65,** 3443–3450.
322. Buti, M., Esteban, R., Jardi, R., Esteban, J. I., and Guardia, J. (1986) Serological diagnosis of acute delta hepatitis. *J. Med. Virol.* **18,** 81–85.
323. Buti, M., Esteban, R., Jardi, R., Allende, H., Esteban, J. I., Genesca, J., and Guardia, J. (1987) Clinical and serological outcome of acute delta infection. *J. Hepatol.* **5,** 59–64.
324. Aragona, M., Macagno, S., Caredda, F., Crivelli, O., Lavarini, C., Maran, E., Farci, P., Purcell, R. H., and Rizzetto, M. (1987) Serological response to the hepatitis delta virus in hepatitis D. *Lancet* **1,** 478–480.
325. Grippon, P., Ribiere, O., Cadranel, J. F., Pelletier, S., Pillot, B., Emerit, J., and Opolon, P. (1987) Long-term delta antigenemia without appearance of delta antibody in two immunodeficiency patients. *Lancet* **1,** 1031.
326. Di Bisceglie, A. M. and Negro, F. (1989) Diagnosis of hepatitis delta virus infection. *Hepatol.* **10,** 1014–1016.
327. Salassa, B., Daziano, E., Bonino, F., Lavarini, C., Smedile, A., Chiaberge, E., Rosina, F., Brunetto, M. R., Pessione, E., Spezia, C., Bramato, C., and Soranzo, L. (1991) Serological diagnosis of hepatitis B and delta virus (HBV/HDV) coinfection. *J. Hepatol.* **12,** 10–13.
328. Buti, M., Esteban, R., Esteban, J. I., Allende, H., Jardi, R., and Guardia, J. (1987) Anti-HD IgM as a marker of chronic deta infection. *J. Hepatol.* **4,** 62–65.
329. Wu, J. C., Lee, S. D., Govindarajan, S., Kung, T. W., Tsai, Y. T., Lo, K. J., and Ting, L. P. (1990) Correlation of serum delta RNA with clinical course of acute hepatitis delta virus superinfection in Taiwan: a longitudinal study. *J. Infect. Dis.* **161,** 1116–1120.
330. Balayan, M. S., Andzhaparidze, A. G., Savinskaya, S. S., Ketiladze, E. S., Braginsky, D. M., Savinov, A. P., and Poleschuk, V. F. (1983) Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. *Intervirology* **20,** 23–31.
331. Chauhan, A., Jameel, S., Dilawari, J. B., Chawla, Y. K., Kaur, U., and Ganguly, N. K. (1993) Hepatitis E virus transmission to a volunteer. *Lancet* **341,** 149–150.
332. Ticehurst, J., Popkin, T. J., Bryan, J. P., Innis, B. L., Duncan, J. F., Ahmed, A., Iqbal, M., Malik, I., Kapikian, A. Z., Legters, L. J., and Purcell, R. H. (1992) Association of hepatitis E virus with an outbreak of hepatitis in Pakistan: serologic responses and pattern of virus excretion. *J. Med. Virol.* **36,** 84–92.
333. Humphrey, C. D., Cook, Jr., E. H., and Bradley, D. W. (1990) Identification of enterically transmitted hepatitis virus particles by solid phase immune electron microscopy. *J. Virol. Meth.* **29,** 177–188.
334. Krawczynski, K. and Bradley, D. W. (1989) Enterically transmitted non-A, non-B hepatitis: identification of virus-associated antigen in experimentally infected cynomolgus macaques. *J. Infect. Dis.* **159,** 1042–1049.
335. Longer, C. F., Denny, S. L., Caudill, J. D., Miele, T. A., Asher, L. V. S., Myint, K. S. A., Huang, C.-C., Engler, W. F., LeDuc, J. W., Binn, L. N., and Ticehurst, J. R.

(1993) Experimental hepatitis E: pathogenesis in cynomolgus macaques *(Macaca fascicularis). J. Infect. Dis.* **168,** 602–609.
336. Pillot, J., Sharma, M. D., Lazizi, Y., Budkowska, A., Dauguet, C., Galimand, M., and Sarthou, J. L. (1987) Immunological characterization of a viral agent involved in epidemic and sporadic non A non B hepatitis. *Ann. Inst. Pasteur/Virologie* **138,** 145–158.
337. Huang, R. T., Li, D. R., Wei, J., Huang, X. R., Yuan, X. T., Tian, X. (1992) Isolation and identification of hepatitis E virus in Xinjiang, China. *J. Gen. Virol.* **73,** 1143–1148.
338. Huang, R., Nakazono, N., Ishii, K., Li, D., Kawamata, O., Kawaguchi, R., Tsukada, Y. (1995) Hepatitis E virus (87A strain) propagated in A549 cells. *J. Med. Virol.* **47,** 299–302.
339. Kazachkov, Y. A., Balayan, M. S., Ivannikova, T. A., Panina, L. I., Orlova, T. M., Zamyatina, N. A., and Kusov, Y. Y. (1992) Hepatitis E virus in cultivated cells. *Aech. Virol.* **127**, 399–402.
340. Tam, A. W., White, R., Reed, E., Short, M., Zhang, Y., Fuerst, T. R., and Lanford, R. E. (1996) In vitro propagation and production of hepatitis E virus from in vivo-infected primary macaque hepatocytes. *Virology* **215,** 1–9.
341. Tam, A. W., White, R., Yarbough, P. O., Murphy, B. J., McAtee, C. P., Lanford, R. E., and Fuerst, T. R. (1997) In vitro infection and replication of hepatitis E virus in primary cynomolgus macaque hepatocytes. *Virology* **238,** 94–102.
342. McCaustland, K. A., Bi, S., Purdy, M. A., and Bradley, D. W. (1991) Application of two RNA extraction methods prior to amplification of hepatitis E virus nucleic acid by the polymerase chain reaction. *J. Virol. Meth.* **35,** 331–342.
343. Jameel, S., Durgapal, H., Habibullah, C. M., Khuroo, M. S., and Panda, S. K. (1992) Enteric non-A, non-B hepatitis: epidemics, animal transmission, and hepatitis E virus detection by the polymerase chain reaction. *J. Med. Virol.* **37,** 263–270.
344. Tsarev, S. A., Emerson, S. U., Reyes, G. R., Tsareva, T. S., Legters, L. J., Malik, I. A., Iqbai, M., and Purcell, R. H. (1992) Characterization of a prototype strain of hepatitis E virus. *Proc. Natl. Acad. Sci. USA* **89,** 559–563.
345. van Cuyck-Gandre, H., Caudill, J. D., Zhang, H. Y., Longer, C. F., Molinie, C., Roue, R., Deloince, R., Coursaget, P., Mamouth, N. N., and Buisson, Y. (1996) Short report: polymerase chain reaction detection of hepatitis E virus in north African fecal samples. *Amer. J. Trop. Med. Hyg.* **54,** 134–135.
346. Turkoglu, S., Lazizi, Y., Meng, J., Kordosi, A., Dubreuil, P., Crescenzo, B., Benjelloun, S., Nordmann, P., and Pillot, J. (1996) Detection of hepatitis E virus RNA in stools and serum by reverse transcription-PCR. *J. Clin. Microbiol.* **34,** 1568–1571.
347. Chobe, L. P., Chadha, M. S., Banerjee, K., and Arankalle, V. A. (1997) Detection of HEV RNA in faeces, by RT-PCR during the epidemics of hepatitis E in India (1976–1995) *J. Viral Hep.* **4,** 129–133.
348. Jothikumar, N., Aparna, K., Kamatchiammal, S., Paulmurugan, R., Saravanadevi, S., and Khanna, P. (1993) Detection of hepatitis E virus in raw and treated wastewater with the polymerase chain reaction. *Appl. Environ. Microbiol.* **59,** 2558–2562.
349. Clayson, E. T., Myint, K. S., Snitbhan, R., Vaughn, D. W., Innis, B. L., Chan, L., Cheung, P., and Shrestha, M. P. (1995) Viremia, fecal shedding, and IgM and IgG responses in patients with hepatitis E. *J. Infect. Dis.* **172,** 927–933.
350. Reyes, G. R., Purdy, M. A., Kim, J. P., Luk, K.-C., Young, L. M., Fry, K. E., and Bradley, D. W. (1990) Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science* **247,** 1335–1339.

351. Yarbough, P. O., Tam, A. W., Fry, K. E., Krawcznski, K., McCaustland, K. A., Bradley, D. W., and Reyes, G. R. (1991) Hepatitis E virus: identification of type-common epitopes. *J. Virol.* **65,** 5790–5797.
352. Dawson, G. J., Chau, K. H., and Cabal, C. M. (1992) Solid-phase enzyme-linked immunosorbent assay for hepatitis E virus IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides. *J. Virol. Meth.* **38,** 175–186.
353. Favorov, M. O., Fields, H. A., Purdy, M. A., Yashina, T. L., Aleksandrov, A. G., Alter, M. J., Yarasheva, D. M., Bradley, D. W., and Margolis, H. S. (1992) Serologic identification of hepatitis E virus infections in epidemic and endemic settings. *J. Med. Virol.* **36,** 246–250.
354. Coursaget, P., Buisson, Y., Depril, N., Le Cann, P., Chabaud, M., Molinie, C., and Roue, R. (1993) Mapping of linear B cell epitopes on open reading frames 2– and 3–encoded proteins of hepatitis E virus using synthetic peptides. *FEMS Microbiol. Lett.* **109,** 251–255.
355. Favorov, M. O., Khudyakov, Y. E., Fields, H. A., Khudyakova, N. S., Padhye, N., Alter, M. J., Mast, E., Polish, L., Yashina, T. L., Yarasheva, D. M., Onischenko, G. G., and Margolis, H. S. (1994) Enzyme immunoassay for the detection of antibody to hepatitis E virus (anti-HEV) based on synthetic peptides. *J. Virol. Meth.* **46,** 237–250.
356. Purdy, M. A., McCaustland, K. A., Krawczynski, K., Tam, A., Beach, M. J., Tassopoulos, N. C., Reyes, G. R., and Bradley, D. W. (1992) Expression of a hepatitis E virus (HEV)-*trpE* fusion protein containing epitopes recognized by antibodies in sera from human cases and experimentally infected primates. *Arch. Virol.* **123,** 335–349.
357. Paul, D. A., Knigge, M. F., Ritter, A., Guitierrez, R., Pilot-Matias, T., Chau, K. H., and Dawson, G. J. (1994) Determination of hepatitis E virus seroprevalence by using recombinant fusion proteins and synthetic peptides. *J. Infect. Dis.* **169,** 801–806.
358. Tsarev, S. A., Tsareva, T. S., Emerson, S. U., Kapikian, A. Z., Ticehurst, J., London, W., and Purcell, R. H. (1993) ELISA for antibody to hepatitis E virus (HEV) based on complete open-reading frame–2 protein expressed in insect cells: identification of HEV infection in primates. *J. Infect. Dis.* **168,** 369–378.
359. He, J., Tam, A. W., Yarbough, P. O., Reyes, G. R., and Carl, M. (1993) Expression and diagnostic utility of hepatitis E virus putative structural proteins expressed in insect cells. *J. Clin. Microbiol.* **31,** 2167–2173.
360. Hyams, K. C., Purdy, M. A., Kaur, M., McCarthy, M. C., Hussain, M. A., El-Tigani, A., Krawczynski, K., Bradley, D. W., and Carl, M. (1992) Acute sporadic hepatitis E in Sudanese children: analysis based on a new Western blot assay. *J. Infect. Dis.* **165,** 1001–1005.
361. Li, F., Zhuang, H., Kolivas, S., Locarnini, S. A., and Anderson, D. A. (1994) Persistent and transient antibody responses to hepatitis E virus detected by western immunoblot using open reading frame 2 and 3 and glutathione *S*-transferase fusion proteins. *J. Clin. Microbiol.* **32,** 2060–2066.
362. Lok, A. S., Kwan, W. K., Moeckli, R., Yarbough, P. O., Chan, R. T., Reyes, G. R., Lai, C. L., Chung, H. T., and Lai, T. S. (1992) Seroepidemiological survey of hepatitis E in Hong Kong by recombinant-based enzyme immunoassays. *Lancet* **340,** 1205–1208.
363. Mushahwar, I. K., Dawson, G. J., Bile, K. M., and Magnius, L. O. (1993) Serological studies of an enterically transmitted non-A, non-B hepatitis in Somalia. *J. Med. Virol.* **40,** 218–221.

364. Arankalle, V. A., Chadha, M. S., Tsarev, S. A., Emerson, S. U., Risbud, A. R., Banerjee, K., and Purcell, R. H. (1994) Seroepidemiology of water-borne hepatitis in India and evidence for a third enterically-transmitted hepatitis agent. *Proc. Natl. Acad. Sci. USA* **91,** 3428–3432.
365. Bryan, J. P., Tsarev, S. A., Iqbal, M., Ticehurst, J., Emerson, S., Ahmed, A., Duncan, J., Rafiqui, A. R., Malik, I. A., Purcell, R. H., and Legters, L. J. (1994) Epidemic hepatitis E in Pakistan: patterns of serologic response and evidence that antibody to hepatitis E virus protects against disease. *J. Infect. Dis.* **170,** 517–521.
366. Goldsmith, R., Yarbough, P. O., Reyes, G. R., Fry, K. E., Gabor, K. A., Kamel, M., Zakaria, S., Amer, S., and Gaffar, Y. (1992) Enzyme-linked immunosorbent assay for diagnosis of acute sporadic hepatitis E in Egyptian children. *Lancet* **339,** 328–331.
367. Khuroo, M. S., Kamili, S., Dar, M. Y., Moecklii, R., and Jameel, S. (1993) Hepatitis E and long-term antibody status. *Lancet* **341,** 1355.
368. Chau, K. H., Dawson, G. J., Bile, K. M., Magnius, L. O., Sjogren, M. H., and Mushahwar, I. K. (1993) Detection of IgA class antibody to hepatitis E virus in serum samples from patients with hepatitis E virus infection. *J. Med. Virol.* **40,** 334–338.
369. Thomas, D. L., Yarbough, P. O., Vlahov, D., Tsarev, S. A., Nelson, K. E., Saah, A. J., and Purcell, R. H. (1997) Seroreactivity to hepatitis E virus in areas where the disease is not endemic. *J. Clin. Microbiol.* **35,** 1244–1247.
370. Deinhardt, F., Holmes, A. W., Capps, R. B., and Poper, H. (1967) Studies on the transmission of disease of human viral hepatitis to marmoset monkeys. I. Transmission of disease, serial passage and description of liver lesions. *J. Exp. Med.* **125,** 673–687.
371. Parks, W. P., Melnick, J. L., Voss, W. R., Singer, D. B., Rosenberg, H. S., Alcott, J., and Casazza, A. M. (1969) Characterization of marmoset hepatitis virus. *J. Infect. Dis.* **120,** 548–559.
372. Deinhardt, F., Peterson, D., Cross, G., Wolfe, L., and Holmes, A. W. (1975) Hepatitis in marmosets. *Amer. J. Med. Sci.* **270,** 73–80.
373. Karayiannis, P., Petrovic, L. M., Fry, M., Moore, D., Enticott, M., McGarvey, M. J., Scheuer, P. J., and Thomas, H. C. (1989) Studies of GB hepatitis agent in tamarins. *Hepatology* **9,** 186–192.
374. Simons, J. N., Pilot-Matias, T. J., Leary, T. P., Dawson, G. J., Desai, S. M., Schlauder, G. G., Muerhoff, A. S., Erker, J. C., Buijk, S. L., Chalmers, M. L., van Sant, C. L., and Mushahwar, I. K. (1995) Identification of two flavivirus-like genomes in the GB hepatitis agent. *Proc. Natl. Acad. Sci. USA* **92,** 3401–3405.
375. Muerhoff, A. S., Leary, T. P., Simons, J. N., Pilot-Matias, T. J., Dawson, G. J., Erker, J. C., Chalmers, M. L., Schlauder, G. G., Desai, S. M., and Mushahwar, I. K. (1995) Genomic organization of GB viruses A and B: two new members of the Flaviviridae associated with GB agent hepatitis. *J. Virol.* **69,** 5621–5630.
376. Schlauder, G. G., Dawson, G. J., Simons, J. N., Pilot-Matias, T. J., Gutierrez, R. A., Heynen, C. A., Knigge, M. F., Kurpiewski, G. S., Buijk, S. L., Leary, T. P., Muerhoff, A. S., Desai, S. M., and Mushahwar, I. K. (1995) Molecular and serologic analysis in the transmission of the GB hepatitis agents. *J. Med. Virol.* **46,** 81–90.
377. Simons, J. N., Leary, T. P., Dawson, G. J., Pilot-Matias, T. J., Muerhoff, A. S., Schlauder, G. G., Desai, S. M., and Mushahwar, I. K. (1995) Isolation of novel virus-like sequences associated with human hepatitis. *Nature Med.* **1,** 564–569.

378. Schlauder, G. G., Pilot-Matias, T. J., Gabriel, G. S., Simons, J. N., Muerhoff, A. S., Dawson, G. J., and Mushahwar, I. K. (1995) Origin of GB-hepatitis viruses. *Lancet* **346,** 447–448.
379. Bukh, J. and Apgar, C. L. (1997) Five new or recently discovered (GBV-A virus species are indigenous to New World monkeys and may constitute a separate genus of the Flaviviridae. *Virology* **229,** 429–436.
380. Alter, H. J. (1996) The cloning and clinical implications of HGV andHGBV-C. *N. Engl. J. Med.* **334,** 1536–1537.
381. Leary, T. P., Muerhoff, A. S., Simons, J. N., Pilot-Matias, T. J., Erker, J. C., Chalmers, M. L., Schlauder, G. G., Dawson, G. J., Desai, S. M., and Mushahwar, I. K. (1996) Sequence and genomic organization of GBV-C: a novel member of the Flaviviridae associated with human non-A-E hepatitis. *J. Med. Virol.* **48,** 60–67.
382. Feucht, H. H., Zollner, B., Polywka, S., and Laufs, R. (1996) Vertical transmission of hepatitis G. *Lancet* **347,** 615.
383. Dawson, G. J., Schlauder, G. G., Pilot-Matias, T. J., Thiele, D., Leary, T. P., Murphy, P., Rosenblatt, J. E., Simons, J. N., Martinson, F. E. A., Gutierrez, R. A., Lentino, J. R., Pachucki, C., Muerhoff, A. S., Widell, A., Tegtmeier, G., Desai, S., and Mushahwar, I. K. (1996) Prevalence studies of GB virus-C infection using reverse transcriptase-polymerase chain reaction. *J. Med. Virol.* **50,** 97–103.
384. Nubling, C. M. and Lower, J. (1996) GB-C genomes in a high-risk group, in plasma pools, and in intravenous immunoglobulin. *Lancet* **347,** 68.
385. Feucht, H. H., Zollner, B., Polywka, S., Knodler, B., Schroter, M., Nolte, H., and Laufs, R. (1997) Prevalence of hepatitis G viremia among healthy subjects, individuals with liver disease, and persons at risk for parenteral transmission. *J. Clin. Microbiol.* **35,** 767–768.
386. Lara, C., Halasz, R., Sonnerborg, A., and Sallberg, M. (1998) Detection of hepatitis G virus RNA in persons with and without known risk factors for blood-borne viral infections in Sweden and Honduras. *J. Clin. Microbiol.* **36,** 255–257.
387. Tanaka, E., Tacke, M., Kobayashi, M., Nakatsuji, Y., Kiyosawa, K., Schmolke, S., Engel, A. M., Hess, G., and Alter, H. J. (1998) Past and present hepatitis G virus infections in areas where hepatitis C is highly endemic and those where it is not endemic. *J. Clin. Microbiol.* **36,** 110–114.
388. Yoshiba, M., Okamoto, H., and Mishiro, S. (1995) Detection of the GBV-C hepatitis virus genome in serum from patients with fulminant hepatitis of unknown aetiology. *Lancet* **346,** 1131–1132.
389. Heringlake, S., Osterkamp, S., Trautwein, C., Tillmann, H. L., Boker, K., Muerhoff, S., Mushahwar, I. K., Hunsmann, G., and Manns, M. P. (1996) Association between fulminant hepatic failure and a strain of GBV virus C. *Lancet* **348,** 1626–1629.
390. Kanda, T., Yokosuka, O., Ehata, T., Maru, Y., Imazeki, F., Saisho, H., Shiratori, Y., and Omata, M. (1997) Detection of GBV-C RNA in patients with non-A-E fulminant hepatitis by reverse-transcription polymerase chain reaction. *Hepatology* **25,** 1261–1265.
391. Byrnes, J. J., Banks, A. T., Piatack, M., and Kim, J. P. (1996) Hepatitis G-associated aplastic anaemia. *Lancet* **348,** 472.
392. Kao, J. H., Chen, J., Lai, M. Y., and Chen, D. S. (1996) GBV-C/HGV infection and aplastic anaemia. *Lancet* **348,** 1032–1033.
393. Zaidi, Y., Chapman, C. S., and Myint, S. (1996) Aplastic anaemia after HGV infection. *Lancet* **348,** 471–472.

394. Alter, H. J., Nakatsuji, Y., Melpolder, J., Wages, J., Wesley, R., Shih, J. W. K., and Kim, J. P. (1997) The incidence of transfusion-associated hepatitis G virus infection and its relation to liver disease. *N. Engl. J. Med.* **336,** 741–746.
395. Tanaka, E., Alter, H. J., Nakatsuji, Y., Shih, J. W. K., Kim, J. P., Matsumoto, A., Kobayashi, M., and Kiyosawa, K. (1996) Effect of hepatitis G virus co-infection on patients with chronic hepatitis C. *Ann. Int. Med.* **125,** 740–743.
396. Kao, J. H., Chen, P. J., Lai, M. Y., Chen, W., Liu, D. P., Wang, J. T., Shen, M. C., and Chen, D. S. (1997) GB virus-C/hepatitis G virus infection in an area endemic for viral hepatitis, chronic liver disease, and liver cancer. *Gastroenterology* **112,** 1265–1270.
397. Muerhoff, A. S., Simons, J. N., Erker, J. C., Desai, S. M., and Mushahwar, I. K. (1996) Identification of conserved nucleotide sequences within the GB virus C 5'-untranslated region: design of PCR primers for detection of viral RNA. *J. Virol. Meth.* **62,** 55–62.
398. Schlueter, V., Schmolke, S., Stark, K., Hess, G., Ofenloch-Haehnle, B., and Engel, A. M. (1996) Reverse transcription-PCR detection of hepatitis G virus. *J. Clin. Microbiol.* **34,** 2660–2664.
399. Cantaloube, J. F., Charrel, R. N., Attoui, H., Biagini, P., DeMicco, P., and DeLamballarie, X. (1997) Evaluation of four PCR systems amplifying different genomic regions for molecular diagnosis of GB virus C infections. *J. Virol. Meth.* **64,** 131–135.
400. Bhardwaj, B., Qian, K., Detmer, J., Mizokami, M., Kolberg, J. A., Urdea, M. S., Schlauder, G., Linnen, J. M., Kim, J. P., Davis, G. L., and Lau, J. Y. (1997) Detection of GB virus-C/hepatitis G virus RNA in serum by reverse transcription polymerase chain reaction. *J. Med. Virol.* **52,** 92–96.
401. Andonov, A., Sauder, C., Jacobsen, H., and Chaudhary, R. (1998) Comparison of six sets of PCR primers from two different genomic regions for amplification of GB virus C/hepatitis G virus RNA. *J. Clin. Microbiol.* **36,** 286–289.
402. Forns, X., Tan, D., Alter, H. J., Purcell, R. H., and Bukh, J. (1997) Evaluation of commercially available and in-house reverse transcription-PCR assays for detection of hepatitis G virus or GB virus C. *J. Clin. Microbiol.* **35,** 2698–2702.
403. Madejon, A., Fogeda, M., Bartolome, J., Pardo, M., Gonzalez, C., Cotonat, T., and Carreno, V. (1997) GB virus C RNA in serum, liver, and peripheral blood mononuclear cells from patients with chronic hepatitis B, C., and D. *Gastroenterology* **113,** 573–578.
404. Lopez-Alcorocho, J. M., Millan, A., Garcia-Trevijano, E. R., Bartolome, J., Ruiz-Moreno, M., Otero, M., and Carreno, V. (1997) Detection of hepatitis GB virus type C RNA in serum and liver from children with chronic viral hepatitis B and C. *Hepatology* **25,** 1258–1260.
405. Sheng, L., Soumillion, A., Peerlinck, K., Verslype, C., Lin, L., van Pelt, J., Hess, G., Vermylen, J., and Yap, S. H. (1997) Hepatitis G viral RNA in serum and in peripheral blood mononuclear cells and its relation to HCV-RNA in patients with clotting disorders. *Thromb. Haemost.* **77,** 868–872.
406. Chen, M., Sonnerborg, A., Johansson, B., and Sallberg, M. (1997) Detection of hepatitis G virus (GB virus C) RNA in human saliva. *J. Clin. Microbiol.* **35,** 973–975.
407. Dille, B. J., Surowy, T. K., Gutierrez, R. A., Coleman, P. F., Knigge, M. F., Carrick, R. J., Aach, R. D., Hollinger, F. B., Stevens, C. E., Barbosa, L. H., Nemo, G. J., Mosley, J. W., Dawson, G. J., and Mushahwar, I. K. (1997) An ELISA for detection of antibodies to the E2 protein of GB virus C. *J. Infect. Dis.* **175,** 458–461.

408. Pilot-Matias, T. J., Carrick, R. J., Coleman, P. F., Leary, T. P., Surowy, T. K., Simons, J. N., Muerhoff, A. S., Buijk, S. L., Chalmers, M. L., Dawson, G. J., Desai, S. M., and Mushahwar, I. K. (1996) Expression of the GB virus C E2 glycoprotein using the Semliki Forest virus vector system and its utility as a serologic marker. *Virology* **225,** 282–292.
409. Tacke, M., Kiyosawa, K., Stark, K., Schlueter, V., Ofenloch-Haehnle, B., Hess, G., and Engel, A. M. (1997) Detection of antibodies to a putative hepatitis G virus envelope protein. *Lancet* **349,** 318–320.
410. Surowy, T. K., Leary, T. P., Carrick, R. J., Knigge, M. F., Pilot-Matias, T. J., Heynen, C., Gutierrez, R. A., Desai, S. M., Dawson, G. J., and Mushahwar, I. K. (1997) GB virus C E2 glycoprotein: expression in CHO cells, purification and characterization. *J. Gen. Virol.* **78,** 1851–1859.
411. Lou, S., Qiu, X., Tegmeier, G., Leitza, S., Brackett, J., Cousineau, K., Varma, A., Seballos, H., Kundu, S., Kuemmerle, S., and Hunt, J. C. (1997) Immunoassays to study prevalence of antibody against GB virus C in blood donors. *J. Virol. Meth.* **68,** 45–55.
412. Ikeda, M., Sugiyama, K., Mizutani, T., Tanaka, T., Tanaka, K., Shimotohno, K., and Kato, N. (1997) Hepatitis G virus replication in human cultured cells displaying susceptibility to hepatitis C virus infection. *Biochem. Biophys. Res. Commun.* **235,** 505–508.
413. Lefrere, J. J., Loiseau, P., Maury, J., Lasserre, J., Mariotti, M., Ravera, N., Lerable, J., Lefevre, G., Morand-Joubert, L., and Girot, R. (1997) Natural history of GBV-C/hepatitis G virus infection through the follow-up of GBV-C/hepatitis G virus-infected blood donors and recipients studied by RNA polymerase chain reaction and anti-E2 serology. *Blood* **90,** 3776–3780.
414. Gutierrez, R. A., Dawson, G. J., Knigge, M. F., Melvin, S. L., Heynen, C. A., Kyrk, C. R., Young, C. E., Carrick, R. J., Schlauder, G. G., Surowy, T. K., Dille, B. J., Coleman, P. F., Thiele, D. L., Lentino, J. R., Pachucki, C., and Mushahwar, I. K. (1997) Seroprevalence of GB virus C and persistence of RNA and antibody. *J. Med. Virol.* **53,** 167–173.
415. Hassoba, H. M., Terrault, N. A., el-Gohary, A. M., Scheffel, C., Jou, C., Brackett, J., Hunt, J., Lou, S. C., and Wright, T. L. (1997) Antibody to GBV-C second envelope glycoprotein (anti-GBV-C E2): is it a marker for immunity? *J. Med. Virol.* **53,** 354–360.
416. Tacke, M., Schmolke, S., Schlueter, V., Sauleda, S., Esteban, J. I., Tanaka, E., Kiyosawa, K., Alter, H. J., Schmitt, U., Hess, G., Ofenloch-Haehnle, B., and Engel, A. M. (1997) Humoral immune response to the E2 protein of hepatitis G virus is associated with long-term recovery from infection and reveals a high frequency of hepatitis G virus exposure among healthy blood donors. *Hepatology* **26,** 1626–1633.

9

Antiviral Chemotherapy for Viral Hepatitis

Christopher O'Brien and Dilip Moonka

1. Hepatitis B

1.1. Introduction

Chronic hepatitis B viral infection remains one of the major medical problems in the world and is a leading cause of morbidity and mortality. Advances in the treatment and prevention of hepatitis B virus (HBV) infection represent among the most dramatic achievements of modern medicine and have resulted from fully exploiting discoveries in molecular immunology and virology as well as rational drug design. The first major breakthrough in the therapy of HBV was the development of regimens of interferon-alfa that give 30–40% long term remission rates in chronically infected patients. Despite this advance, the glass has also remained half empty. The more recent development of nucleoside analog agents with potent antiviral activity have now provided additional armamentarium in the care of HBV infected patients. The initial part of this chapter will address, in a practical manner, the therapy of appropriate patients with interferon and the anticipated effects and benefits of this therapy. The chapter will then discuss the evolving role of nucleoside analogs and even more experimental agents in the management of patients who are either refractory to or are poor candidates for interferon-alfa therapy.

1.2. Initial Evaluation

The long-term goal of therapy for chronic HBV infection is the elimination of virus from the body with a resultant improvement in morbidity and mortality. The short-term goal of therapy is to lower the burden of replicat-

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

ing virus in the body. Hepatitis B infections are not homogeneous from patient to patient. They vary widely in terms of the host immune response, the level of replicating virus, and the amount of hepatic inflammation. Initial evaluation of the patient should be directed at determining the nature of the hepatitis B infection and the extent of liver disease.

Patients referred for evaluation and therapy of hepatitis B typically have a positive hepatitis B surface antigen (HBsAg) and abnormal serum aminotransferases. In standard fashion, all patients should have a full evaluation for other causes of liver disease, especially associated coinfection with the hepatitis C virus (HCV) or the hepatitis D virus (HDV). We typically obtain HIV tests as well. Patients should then be evaluated for replicating forms of the virus, which are typically determined by the serum HBV DNA level and hepatitis B "e" antigen (HBeAg) level.

The serum HBV DNA and HBeAg serve two functions; they help identify those patients most appropriate for therapy and provide the most practical markers for determining response to therapy. Individuals with abnormal serum aminotransferases, positive markers of viral replication and no evidence of other causes of liver disease or decompensated liver disease are potential candidates for interferon therapy. Patients who are HBsAg positive, but with absent serum HBV DNA and HBeAg and normal serum aminotransferases, are termed "healthy carriers" *(1)*. Although the designation of "healthy" to these patients could be considered argumentative, these patients have very poor responses to therapy with interferon-alfa and typically are not treated *(2)*.

As standard evaluation, liver biopsy should be obtained from all patients when possible prior to therapy. The biopsy provides information on baseline histology and prognosis. It can also provide information useful to guiding therapy. A patient with marked cirrhosis, who is experiencing significant adverse reactions from interferon-alfa, might best be served with discontinuation of this particular therapy.

1.3. Mechanism of Viral Clearance

1.3.1. Normal Immune Response

The normal immune response to a viral hepatitis consists of both a humoral defense, a cell-mediated defense and the actions of cytokines and intracellular enzymatic cascades. During the humoral response, B cells produce nonneutralizing antibodies. The cell-mediated immune defense consists of natural killer (NK) cells and cytotoxic T cells.

The host immune system plays an active and usually an effective role in the control of hepatitis B. The large majority of patients infected with HBV spontaneously resolve their infection. Both the cellular and humoral

arms of the immune system appear to play a critical role in this process. Patients who spontaneously clear their infections mount a strong, polyclonal CD4-positive *(3)* and cytotoxic T cell *(4)* response to multiple HBV proteins. These cellular immune responses are significantly weaker in chronically infected patients. Antibody directed against HBsAg (HBsAb) is protective and usually heralds resolution of the infection. In fact, the ability of the human immune system to recognize hepatitis B proteins as foreign antigens has allowed the development of successful HBV vaccines.

1.3.2. Interferon: Immune Activation vs Direct Antiviral Effect

The interferons (IFN) consist of a family of species-specific proteins that are secreted in response to a virus. Besides their antiviral actions, the IFN also have antiproliferative and immunomodulatory effects.

IFN are divided into three major groups: alpha (α), beta (β), and gamma (γ). Greek letters: α, β, and γ are, by agreement, used to describe IFN derived from natural sources. The terms alpha and beta, on the other hand, designate those IFN that have been produced by recombinant technology. Many companies spell alpha as alfa to further emphasize that their IFN has been produced by recombinant technology.

Of the three interferons, IFNα has shown the most activity against HBV and HCV. IFNα is produced by monocytes and transformed B cells *(5)*. There are two subfamilies of genes located on chromosome 9 that produce approx 22 functional subtypes of IFNα. Thus, the IFNα released during the normal human immune response to a viral infection is, in essence, a "witches brew" of all 22 subtypes of IFNα. The recombinant forms of interferon differ in that they represent a single IFN species.

The actions of IFN are myriad, but in their activity against viral hepatitis, they can be thought of has having direct immunomodulatory effects and direct antiviral effects. A large number of immune cells have IFN receptors, and IFN mediate a large number of immune effects. They activate monocytes, macrophages, NK cells, and cytotoxic T cells *(6)*. They increase expression of both class I and class II major histocompatibility complex (MHC) molecules on cell surfaces and they increase production of additional cytokines by immune cells. As a general rule, IFNγ is more effective than IFNα in mediating immune effects.

The interferons also mediate specific antiviral effects independent of their immune effects. There appear to be three major antiviral mechanisms. Both IFNα and IFNβ use the same cell surface receptor, whereas IFNγ uses a distinct receptor *(7)*. Binding of the IFN species to its receptor induces activation of Jak, a tyrosine kinase. Activation of Jak causes the phosphorylation of signal-transducing transcriptional activators (STATs) protein. This

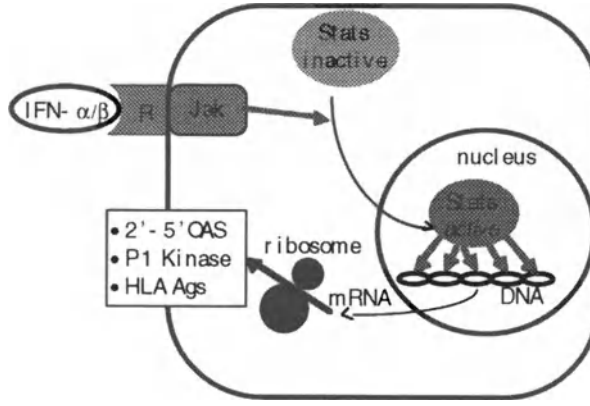


Fig. 1. Binding of IFNα/β to cell receptors.

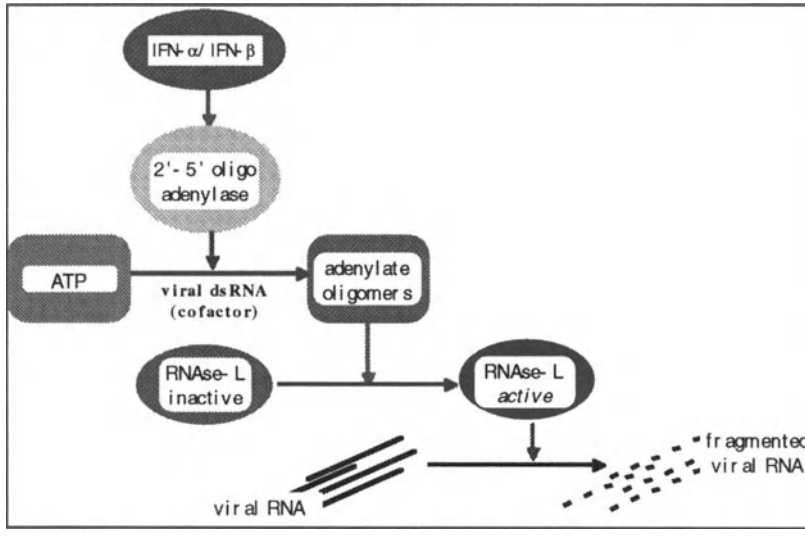


Fig. 2. Interferon activation of the intracellular 2'–5' OAS Cascade.

phosphorylated STATs protein binds to a number of gene promoter sequences within the nucleus of the host cell. The downstream genes are then transcribed into various mRNAs encoding for a series of antiviral proteins (*see* Fig. 1).

These antiviral proteins promote the activation of a number of intracellular enzymatic cascades. The intracellular enzymatic cascade that is best

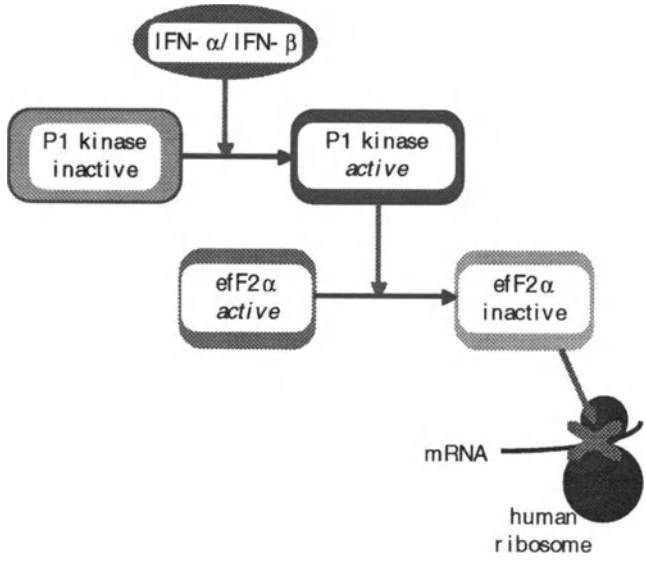


Fig. 3. Interferon activation of the serine/threonine P1 kinase cascade.

characterized is the 2'-5' oligoadenylate synthetase (2'-5' OAS) pathway *(8)*. IFNα and IFNβ with viral double-stranded RNA (dsRNA) as a cofactor activate the 2'-5' OAS. The activated 2'-5' OAS converts ATP into a group of 2'-5' linked oligomers. These adenylate oligomers proceed to activate a RNAse-L (an endoribonuclease) that has the ability to cleave viral RNA (*see* Fig. 2).

The second intracellular enzymatic cascade causes the formation of a 65 kDa RNA-activated serine/threonine (P1) kinase. This P1 kinase protein inactivates the protein synthesis initiation factor elF-2 by phosphorylation. The normal function of the host cells elF-2 is to cause the translocation of the mRNA through the ribosome. The viral mRNA cannot be translated by the human ribosome without the proper function of elF-2 (*see* Fig. 3).

The phosphorylated STATs protein also binds to other gene promoters sequences causing the synthesis and ultimate increased expression of the human leukocyte antigen (HLA) class I antigens on the host cell surface. The increase in the HLA class I expression on uninfected host cells protects them from attack by NK cells. Transporter associated with antigen processing (TAP) transporter proteins and the LMP2, LMP7, and MECL-1 components of the proteasome also increase in relative numbers. These three subunits of the proteasome (LMP2, LMP7, MECL-1) displace three other

key subunits from the proteasome. This changes the catalytic activity of the proteasome, a multicatalytic protease complex, to produce the necessary antigen-peptide ligands for the HLA class I molecules. The TAP transporter delivers the viral peptides from the cytosol to the inside of the endoplasmic reticulum where it binds to the HLA class I molecules and completes the folding of the HLA class I molecule. The viral peptide complexed to the HLA class I antigen travels through the Golgi to the cell surface where it is recognized by the CD8(+) cytotoxic T-cell.

Finally, the third mechanism of antiviral activity is through the generation of Mx proteins. These proteins, produced only by IFNα and IFNβ, appear to inhibit production of viral RNA *(9)*.

1.3.3. Clearance of Hepatitis B Virus

The exact mechanisms by which IFN exerts its antiviral effect in patients with HBV is not clear at this time but several observations point to the importance of immunomodulatory effects. Patients with hepatitis B who respond to IFN with remission appear to mount a strong proliferative and cytotoxic T cell response to HBV proteins *(4)*. In addition, multiple studies have demonstrated that those patients most likely to respond to IFN have evidence of a preexisting immune response to HBV as suggested by low serum HBV DNA (less than 100 pg/mL) and high serum aminotransferases (ALT >100 IU/L) *(10,11)*. Patients with high serum HBV DNA (greater than 100 pg/mL) and little evidence of hepatic inflammation (ALT <100 IU/L) are least likely to respond. Finally, about two thirds of responders have a distinct "acute hepatitislike flare" approximately 2 mo into therapy followed by disappearance of markers of viral replication *(2)*. This flare is thought to represent an immune mediated attack on hepatocytes infected with virus. These observations by no means exclude direct antiviral activity by IFN in therapy for HBV. In fact, IFNγ, which is generally felt to exhibit more potent immunomodulatory effects than IFNα appears less effective in the therapy of HBV than IFNα (*see* Subheading 1.11.2.2.) *(6)*. In addition, some investigators have argued that responses to IFN correlate with high levels of 2,5' OAS activity in peripheral blood lymphocytes *(12)*.

1.4. Standard Therapy Protocols

The standard regimen of IFNα for treatment of chronic hepatitis B is 5 million International Units (MIU) administered subcutaneously each day for 4 mo. International Units are determined by the comparison of the antiviral activity of the test IFN with the activity of an international reference human leukocyte interferon established by the World Health Organization (WHO). An alternative regimen of 10 MIU each Monday, Wednesday, and

Friday is also frequently used and gives comparable results *(13)*. Our center typically uses the former regimen because we feel that it is better tolerated despite the daily dosing. Patients are instructed to rotate sites of injection between the upper arms, abdomen, and thighs, although, in practice, many patients avoid the abdomen and have difficulty self-administering drug to the upper arms. Both recombinant IFN alfa-2b (Intron A: Schering-Plough Corp., Kenilworth, NJ) and recombinant IFN alfa-2a, (Roferon-A: Hoffmann-Roche, Summit, NJ) are available in single-use vials. In our experience, formulations that are already reconstituted in fluid markedly simplify use for the patients. Blood work, including a CBC profile and serum aminotransferases, is routinely obtained 1, 2, and 4 wk after starting therapy and then on a monthly basis. We typically reevaluate the serum HBV DNA and HBeAg at the end of therapy. Additional monitoring is done as the clinical course dictates.

1.5. Common Side Effects of Interferon

Interferon alfa therapy is unusual in that, while the majority of patients develop significant adverse symptoms while on drug, few experience potentially life-threatening complications that necessitate discontinuing treatment. In addition, most of the adverse effects of IFN are dose dependent and reversible. They are less common with the doses used for viral hepatitis and the side effects usually respond, if necessary, to dose modification. Only four of 126 treated patients in the U.S. multicenter trial discontinued therapy prior to completion *(11)*.

Almost all patients initially experience the classic "flu-like symptoms" of IFNα, consisting of fatigue, fever, chills, myalgia, and headache. The symptoms are typically severe the first week of therapy and then improve as therapy is continued. Patients are well advised to allow for a light schedule during this first week. These symptoms can be treated with acetaminophen at the time of injection or nonsteroidal antiinflammatory drugs (NSAID) throughout therapy. In addition, many of our patients have found that administering the drug at night allows them to sleep through some of the side effects.

Additional side effects that are relatively common that usually do not require significant dosage modification include alopecia, anorexia, nausea, crampy abdominal pain, diarrhea and weight loss. Psychiatric side effects are relatively common, occurring in about 15% of patients *(14,15)*. These range from anxiety and irritability to paranoia, acute psychosis, and depression. The depression can frequently be controlled successfully especially with the use of newer antidepressant medications that include fluoxetine hydrochloride(Prozac) and sertraline hydrochloride (Zoloft). Acute psycho-

sis and delirium were more common in patients with prior evidence of organic brain dysfunction *(15)*. Severe or unremitting psychiatric complications should lead to discontinuation of drug.

Interferon is myelosuppressive, which typically manifests in decreases in either the platelet or white cell count. Thrombocytopenia or leukopenia were the most common cause for dose adjustment in the U.S. multicenter trial, affecting approx 10% of patients *(11)*. On average in this trial, the platelet count dropped 30–50%, the white count 20–40%, and the hemoglobin 3–5% *(11)*. The possibility of an immune thrombocytopenia should always be considered in patients with especially marked or problematic drops in platelet count *(16,17)*. Bacterial infections are also more common in patients receiving IFNα, possibly as a result of neutropenia. This is especially true of patients with a predisposition to develop bacterial infections *(14)*. Both myelosuppressive effects and bacterial infections are a more significant concern in patients with portal hypertension with potential hypersplenism (*see* Subheading 1.9.1.).

Patients can experience worsening of serum aminotransferases during therapy because of a variety of mechanisms. Elevations in aminotransferases are commonly found 4–8 wk after starting IFNα *(2)*. But, an IFNα-induced flare in the serum transaminases can also cause frank hepatic dysfunction in patients with advanced liver disease (*see* Subheading 1.9.1.). Interferon can also, in rare circumstances, exacerbate an underlying autoimmune hepatitis *(18,19)*.

Interferon has also been associated with a variety of other autoimmune conditions, though cause and effect are not always clear. The development of autoantibodies during IFN use is common *(20)*. The most frequent autoimmune manifestation is thyroiditis which can present as either hypo- or hyperthyroidism. The condition is seen in up to 5% of patients with hepatitis C and in 2% of patients with hepatitis B who are treated with IFNα *(21)*. Other autoimmune conditions seen rarely include type 1 *(22,23)* and type 2 diabetes *(24)*, immune thrombocytopenia *(16,17)*, hemolytic anemia *(25,26)*, and autoimmune hepatitis (discussed previoiusly). Interestingly, IFNα has been used and tolerated in patients with known autoimmune conditions *(27)*.

There are additional, unusual side effects due to IFNα of which the practitioner should be aware. These include reports of retinopathy *(28)*, renal toxicity *(29)*, and hearing loss *(30)*.

1.6. Response to Therapy

As discussed, the end points used to determine "response" to therapy are the loss of serum HBV DNA and HBeAg. These are the commonly used markers of active viral replication. Extensive experience now demonstrates

that between 30 and 40% of patients with infected HBV treated with an appropriate course of IFN a will respond to therapy with a loss of these markers as compared with less than 10% of untreated patients. Approximately one third of the responders appear to become true "cures" on the basis of the loss of detectable HBsAg. Abundant long-term data now suggest that in the vast majority of responders, loss of viral replication is indefinite and that with time, an increasing number of responders will lose HBsAg. Emerging evidence suggests that this serologic response is accompanied by improvement in liver histology and in clinical outcome.

1.6.1. Short-Term Response

The benchmark study for the short-term response to IFN alfa-2b in patients with chronic hepatitis B remains the U.S. multicenter trial *(11)*. The trial is unparalleled in terms of the number of patients enrolled, the clearly stated and pursued objectives, and the availability of paired liver biopsies in the majority of treated patients and controls. Moreover, probably as a result of these factors, the results of the trial have withstood the test of time in a highly reproducible manner.

In this trial, of the 85 patients treated with 5 MIU of IFN alfa-2b daily for 16 wk, 31 patients *(37%)* had a sustained loss of HBV DNA and seroconversion of HBeAg vs 3 of 43 untreated controls (7%). Of the 31 responders, 10 patients also had loss of HBsAg during a 1-yr follow-up period, and one patient had a relapse of viral replication. Eighty-seven percent of the responders had normal serum aminotransferases at the time of last observation. Patients who responded had an improvement in histology, whereas nonresponders had worsening of histology though the differences did not reach statistical significance.

This study made additional interesting observations. As noted later by Dr. Perrillo in a review article, approximately two-thirds of responders and less then one-third of nonresponders a transient but pronounced rise in aminotransferases can be seen approximately 4–8 wk after initiation of therapy *(2)*. This increase is considered favorable, is usually well tolerated, and coincides with a decline in viral replication (*see* Fig. 4). In addition, seven of the patients treated with 5 MIU of IFNα lost HBV DNA, but not the HBeAg. These patients were termed "indeterminate responders," but had significant improvements in their liver histology. Though most responders lost HBV DNA within 2 mo of starting IFNα, patients continued to lose HBV DNA throughout the therapy period and even after completion of therapy. Loss of HBeAg lagged behind loss of HBV DNA by one to several months. Finally, the response rate in patients treated with 1 MIU daily was not statistically different than untreated controls (17 vs 7%).

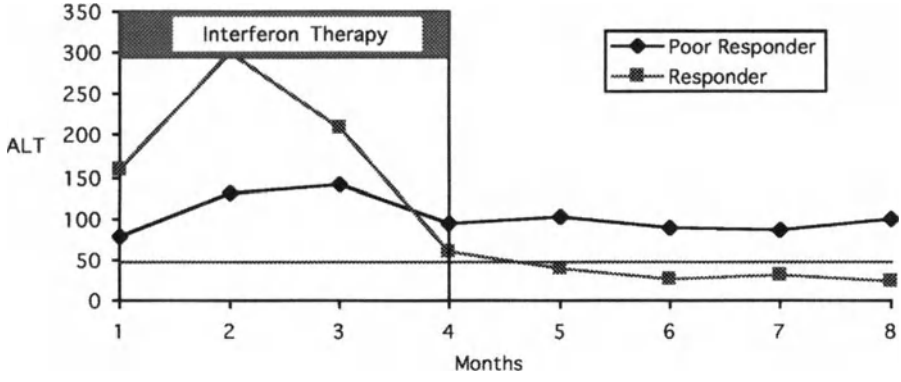


Fig. 4. Hepatitis B: ALT response patterns to IFNα therapy.

A meta-analysis performed in 1993 confirms many of the observations made by the U.S. multicenter trial *(31)*. The analysis looked at 15 randomized, controlled trials conducted between 1987 and 1992. It found that patients with chronic hepatitis B treated with IFNα, when compared with untreated controls, were statistically more likely to lose HBV DNA (37 vs 17%), HBeAg (33 vs 12%) and HBsAg (8 vs 2%). These cumulative results were obtained despite different patient populations representing five continents and differences in doses and formulations of IFNα. Overall 5% of patients were unable to tolerate a complete course of drug.

1.6.2. Long Term Response

It should be noted that no study has yet demonstrated that therapy with IFNα in chronic hepatitis B patients affects their rate of development of cirrhosis or hepatocellular carcinoma. This is, in part, because of the chronic nature of the HBV infection, and because of the ethical concerns of withholding IFNα therapy from control patients indefinitely. However, emerging data suggest that response to IFNα is generally durable and associated with both improvement in liver histology and in clinical outcome.

Studies of long-term outcome of IFNα responders have shown relapse rates among responders of between 0 and 15% *(32–35)*. Of the responders, between 19 and 57% will lose HBsAg. The long-term National Institutes of Health (NIH) experience included 23 patients who were followed for periods between 3 to 7 yr after therapy *(34)*. Three patients relapsed and 13 eventually lost HBsAg (57%). Long-term liver biopsy data were available for patients who cleared their HBsAg in response to therapy *(36,37)*. Sustained loss of HBsAg was associated with an improvement in histology that

Table 1
Hepatitis B: Predictors of Success After Therapy with Interferon—Multivariate Regression Analysis

Patient	Laboratory parameters
Adult acquisition of HBV	HBV DNA < 100 pg/mL
Non-Asian	ALT > 100 IU/L
Normal immune system	

paralleled the duration of nondetectable HBsAg. Patients who had loss of detectable HBsAg for less than 2 yr had a 50% improvement, whereas those patients who lost detectable HBsAg greater than 2 yr had an 83% improvement in histology *(37)*. Liver biopsies of patients with HBsAg negative serum were invariably negative on special stains for HBsAg and HBcAg. Little histologic data are available for patients who are without markers of viral replication but who are still HBsAg positive.

Niederau et al. looked at long-term clinical outcomes of patients treated with IFN alfa-2b *(33)*. They compared 53 patients who responded to IFNα with 50 patients who did not respond and 53 patients who were not treated. Patients were followed for a median of 50 mo. No responders relapsed and 10 eventually became seronegative for HBsAg *(19%)*. None of the 53 untreated controls lost HBsAg. Of the 50 nonresponders, eight either died or required liver transplantation. Another eight developed clinical progression to cirrhosis. Of the 53 untreated patients, five patients died or required transplantation and another eight developed complications of cirrhosis. Among the responders, no patients died, but one patient developed ascites. This paper is unique in that no patients were lost to follow-up.

1.7. Determinants of Response

Because IFNα therapy is expensive, has significant side effects, and fails in a majority of hepatitis B patients, attempts have been made to identify subsets of patients that are more likely to respond to therapy (*see* Table 1).

Determinants that appear most consistent in identifying individuals' good responses are low levels of serum HBV DNA and high levels of aminotransferases *(10,11,38)*. The U.S. multicenter trial identified four variables that were associated with response to therapy: a low HBV DNA, high aspartate aminotransferase, a short duration of hepatitis, and heterosexual orientation *(11)*. However, the strongest independent predictor of response was the level of HBV DNA. Patients with HBV DNA levels of less than 100 pg/mL had a response rate of 50% and those with levels of greater than 200 pg/mL had a response rate of 7%, which was comparable with untreated controls.

Factors consistently associated with a less favorable response in multiple trials include infection in childhood *(39,40)* and membership in an ethnic group where vertical transmission of virus is common *(40)* as is the case in many Asian populations. Other negative factors include significant immunocompromise. Poor response rates are seen in patients with advanced human immunodeficiency viral (HIV) infection *(31,38)* and in patients receiving immunosuppressive therapy after organ transplantation *(41,42)*. Of note, in contrast to IFNα therapy for HCV, the presence of compensated cirrhosis does not appear to be a negative prognostic factor. In the U.S. multicenter trial, two thirds of patients had evidence of cirrhosis on liver biopsy and cirrhotic patients responded with the same frequency as noncirrhotics *(11)*.

The prognostic factors as a group point to the importance of an ongoing immune response to hepatitis B in determining response to therapy. Patients with high transaminases, active inflammation on liver biopsy, and low HBV DNA are most likely to respond to IFNα in part because these factors imply that the immune system recognizes HBV antigens as foreign and is actively engaging the virus. In contrast, when the immune system is exposed to HBV while still immature, as is the case in patients exposed at birth, there is presumably some propensity for the immune system to see the virus as a form of self and to develop a "tolerant" response. These patients are less likely to have a successful outcome.

1.8. Corticosteroid Withdrawal and IFNa

Multiple trials have consistently demonstrated that patients with relatively low elevations of serum aminotransaminases respond less well to IFNα *(11,38)*. One approach to increasing response in these patients is to use a course of corticosteroids prior to initiation of IFNα. This strategy, also termed "steroid" or "prednisone priming," is based on the observation that the withdrawal of corticosteroids from patients with HBV often results in an "acute hepatitis-like flare." This flair evolves in a characteristic fashion with an initial increase in HBV DNA levels, which is then followed by an elevation of serum aminotransferases with a corresponding decline in HBV DNA. The mechanism for this phenomenon is unclear. Serum HBV DNA levels may rise secondary to a glucocorticoid-response element in the HBV genome *(43)* or secondary to a steroid induced inhibition of endogenous IFNα and 2'-5' OAS activity *(44)*. The rationale behind this prednisone pretreatment is that patients with hepatitis B with an unfavorable biochemical and immunological profile can be changed to the more favorable profile of a high serum aminotransferase and a low serum HBV DNA.

Trials that have compared the combination of prednisone followed by IFNα head to head with IFNα alone have failed to demonstrate a benefit from steroid priming *(11,45,46)*. However, several studies have suggested that the subgroup of patients with compensated liver disease and a low elevation of serum aminotransferases may in fact benefit from this strategy *(11,45,47)*. In the U.S. multicenter trial, one treatment group received prednisone in decreasing daily doses of 60, 40, and 20 mg for 2 wk each, followed by a 2-wk rest, then 16 wk of IFNα. In those patients with a low alanine aminotransferase level (less than 100 U/L) at entry, there was a higher response rate in patients who received prednisone with IFNα (8 out of 18) than those who received IFNα alone (2 out of 12). Six of the 8 patients who responded to the combination of prednisone and IFNα had at least a twofold increase in alanine aminotransferase levels during the rest period between prednisone and IFNα with a corresponding decline in HBV DNA. It should be noted that a prednisone taper can cause marked hepatic deterioration in patients who have marginal or decompensated hepatic function and should not be used in this setting *(11,48)*.

1.9. Special Treatment Situations

The following section discusses subsets of hepatitis B patients with special considerations.

1.9.1. Patients with Atypical Serological Patterns

The discussion has focused on patients who have both detectable serum HBV DNA and HBeAg; however, many patients have atypical serological patterns. Some patients present with HBsAg and HBV DNA but no detectable HBeAg. Many of these patients have a particular "pre-core" mutation which introduces a "stop" codon into the HBV nucleotide sequence that prevents production of the HBeAg. This particular form of HBV infection, described first in the Mediterranean countries, also occurs throughout Europe and the United States. Multiple studies suggest these patients with this particular form of HBV disease can respond to IFNα *(49,50)*, but that they respond less well than the wild-type HBeAg positive patients and also have high rates of relapse post therapy *(49)*. Some of these patients may be candidates for alternative therapy, including nucleoside analog agents. At least one study found that Thymosin-a1, a "thymic gland-derived, immunomodulatory agent" may be effective in these patients *(51)*.

Some patients will present with HBsAg and elevated aminotransferases, but undetectable HBV DNA and HBeAg. In these patients, especially, it is critical to rule out other causes of liver disease. It is likely that some of these patients do have serum HBV DNA but that the level is below

that detectable with current commercial HBV DNA assays. Newer quantitative PCR assays currently being developed with higher sensitivity may identify HBV DNA in some of these patients. Therapy for these patients is not clearly defined, but a course of IFNα in these patients may be reasonable if the liver biopsy is consistent with viral hepatitis and staining for HBcAg is positive.

1.9.2. Asian Patients

Multiple trials of primarily Chinese patients suggest that, even though these patients may benefit from IFNα as compared with untreated controls *(31)*, they do not appear to respond as well as non-Asian patients *(47,52–54)*. A large percentage of Asian hepatitis B patients, both in the United States or in their native countries, acquire infection either at birth or in the first few years of life from either household or child-to-child transmission *(54)*. As discussed in Subheading 1.7., the exposure to virus at a young age may result in some level of immune tolerance to it. Presumably as a result of this, many patients have minimal elevations of serum aminotransferases and a poor response to IFNα.

Lok et al. stratified Asian patients on the basis of their baseline alanine aminotransferase levels and found that patients with elevated transaminases had good response rates to IFNα comparable with non-Asian patients, whereas those with normal transaminase levels had no response *(52)*. The role of corticosteroids prior to IFNα is not clear in Asian patients with normal or minimally elevated aminotransferases. One study from Taiwan found marked benefit from this approach *(47)* while Lok's trial in Hong Kong found no benefit *(52)*. Asian patients who are inappropriate for or who do not respond to IFNα may be appropriate candidates for the newer nucleoside analog agents.

1.9.3. Pediatric Patients

Results of trials of IFNα in children with HBV infection suggest that many of the same variables that predict a poor response in adults also predict a poor response in children *(54)*. These include high HBV DNA *(55)* and low aminotransferases *(56–58)* pretreatment, and exposure to virus at birth *(56)*. Presumably as a result of these determinants, trials from Europe typically report good rates of response that are comparable with adults *(57–60)*. In contrast, in Asia, where vertical transmission is common and aminotransferase elevations are often low, trials more typically note poor results *(39,40)*. Brugera et al. specifically found children infected at birth had a 7% response rate compared to a 72% response for those infected after birth *(56)*.

The low response to chronic hepatitis B patients exposed at birth emphasizes the importance of immunoprophylaxis and vaccination strate-

gies in this setting. In the United States, the Centers for Disease Control and Prevention recommends screening all pregnant women for HBsAg *(61)*. Children born to women who are HBsAg positive should receive hepatitis B immune globulin (HBIG) and initiation of the HBV vaccine series within 12–24 h of birth *(54)*. Use of just such a strategy in chronically infected Asian American women using the recombinant HBV vaccine lowered the incidence of HBV infection in the infants to 4.8%, compared with an expected incidence of chronic infection of 70–80% in untreated infants *(62)*.

1.9.4. Immunocompromised Patients

Patients with hepatitis B who are immunocompromised are less likely to respond to IFNα therapy. These include patients receiving immunosuppressive medication and those individuals infected with HIV. Patients who undergo liver transplantation for end-stage liver disease secondary to HBV frequently develop recurrent hepatitis B infection in the transplanted organ. This recurrent infection often presents as a rapidly progressive, fibrosing cholestasis ending in hepatic failure. These patients almost never experience a complete response to IFNα and treatment is wrought with difficulties in this setting *(42)*. Recurrent infection in these patients is now often well controlled with monthly infusions of HBIG. In addition, newer nucleoside analog agents will undoubtedly also have some role in this setting (*see* Subheading 1.11.6.3.).

Numerous studies have shown that patients infected with HIV are less likely to respond to IFN *(31,38,63,64)*. However, it is important to note that HIV-infected patients can be capable of a complete response to IFN and should be considered for therapy *(65)*. This is especially true of patients early in their HIV infection with good CD4 counts and less immunocompromise. Effective treatment of HBV disease in HIV-infected patients will presumably become more important given the recent improved prognosis of these patients with more effective, multidrug regimens for the underlying HIV infection.

1.9.5. Patients with Decompensated Liver Disease

Interferon therapy for hepatitis B patients with decompensated liver disease is fraught with risk. The decision to treat these patients with IFNα should be made with caution, especially in light of the development of new agents, such as nucleoside analogs, which can significantly lower HBV DNA levels with potentially less toxicity. However, evidence suggests that patients with mildly decompensated liver disease can respond to and benefit from modified regimens of IFNα therapy *(66–68)*. In general, patients with markedly decompensated liver disease, especially those in Child's class C are at

especially high risk of complications, are unlikely to respond and should not be treated with IFN *(48)*.

A NIH study treated 18 patients with "advanced cirrhosis"; six experienced a complete response with marked clinical improvement *(66)*. This group of investigators found side effects to be frequent and often severe. Only six patients tolerated a full course and 9 patients had to terminate therapy early. Five patients developed serious bacterial infections of which one patient had a fatal outcome. Three patients developed marked psychiatric side effects. All six patients who responded had either Child's Class A or B, and four of these six patients also cleared HBsAg. All six patients continued to do well from 2–4 yr after therapy *(69)*. A multicenter trial using titrated, low doses of interferon demonstrated a 50% response rate in patients with Child's Class A or B and no response in Child's Class C patients *(67)*. The trial confirmed the poor tolerance to therapy and the high rate of serious side effects and complications.

Patients with decompensated liver disease resulting from HBV are better treated at present as candidates for liver transplantation and benefit from referral to a liver transplant unit. It is preferable to make this referral well before a patient is in the terminal stages of their disease to provide adequate time for the transplant evaluation and to obtain a suitable donor liver.

1.9.6. Interferon Nonresponders

Unfortunately, the majority of patients do not respond to an appropriate course of IFNα with a durable response. The management of these patients remains an important problem. If the original course of therapy was adequate dose and duration, retreatment of these patients also will not be successful *(10)*. A physician could consider repeat IFNα therapy if a patient who initially had an unfavorable biochemical profile spontaneously develops a low HBV DNA and high serum aminotransferases. Alternatively, one might also consider "prednisone priming" in a patient with an unfavorable biochemical profile who did not receive corticosteroids prior to the initial IFNα therapy. Otherwise, these patients are typically candidates for alternative therapy, including nucleoside analog agents.

1.9.7. Acute or Fulminant Hepatitis B

The vast majority of patients with acute icteric hepatitis B will recover from the acute phase of infection with supportive therapy alone, and no benefit has been demonstrated for IFNα in this setting. A prospective, randomized trial in Greece of 100 patients failed to demonstrate benefit from IFNα as compared with placebo. In fact, all patients recovered and none developed chronic hepatitis *(10,70)*. In an uncontrolled study, 12 patients with fulminant hepatitis B were treated with IFNα and 83% died, a number con-

sistent with historical controls *(71)*. At present, there are no reports of the use of nucleoside analog agents for either acute or fulminant hepatitis B.

1.9.8. Coinfection with Hepatitis B and Hepatitis C

Approximately 10% of patients with HBV are also coinfected with HCV, although this number varies depending on the patient population *(72)*. The treatment of this group of patients is complicated by the observations that these patients tend to have more severe disease than seen with either virus alone *(72,73)*, and that the viruses appear to "interfere" with or "suppress" the replication of each other *(72,74)*. It is tempting to suggest that patients with combined infection should be given IFNα because it is the treatment for both viruses and that the dose and duration of IFNα could be chosen on the basis of which virus is determined to be most active *(72,75)*. However, response to IFNα therapy in patients with combined HBV and HCV appears to be poor *(72,73)*, and elimination of one virus can actually result in activation of the remaining virus *(72)*. In addition, the role, if any, of nucleoside analog agents is unclear. At this time, optimal therapy for these patients is far from clear, although a course of IFNα might be considered.

1.9.9. Patients with Extrahepatic Manifestations of Hepatitis B Infection

HBV infection is associated with numerous extrahepatic manifestations that are relatively uncommon, but well documented. These include glomerulonephritis *(76–78)* polyarthritis *(79)*, cryoglobulinemia *(80)*, and vasculitis *(81–83)*. Available evidence suggests that the extrahepatic manifestations will often remit if the underlying viral infection responds to IFN.

The most extensively studied extrahepatic manifestation is HBV related glomerulonephritis, which is associated with immune complex deposition in glomerular capillaries *(76)*. This is seen most commonly in children in whom it often spontaneously resolves *(84)*. In adults, however, spontaneous remission is unusual and about one third demonstrate a "relentlessly progressive" course *(76)*. At the NIH, 15 adult patients with chronic hepatitis B, glomerulonephritis, and marked proteinuria were treated with IFNα between 1985 and 1993 *(77)*. Eight patients responded to therapy with sustained loss of HBV DNA and HBeAg and of these, five also lost HBsAg. Seven of the 8 patients had resolution of their nephrotic syndrome. The remaining patient had persistent nephrotic range-proteinuria, but also had a history of poorly controlled diabetes and diabetic nephropathy that predated the IFNα therapy. The seven nonresponders continued to have significant proteinuria with little change in biochemical indices between pretreatment values and values when last seen. Patients who responded continued to demonstrate improvement in both liver and renal function years after therapy *(69,77)*.

Case reports of individual patients also suggest that hepatitis B-related polyarthritis *(79)*, type II essential, mixed cryoglobulinemia *(80)* and polyarteritis nodosa *(83)* can respond to IFNα therapy. In all cases, the underlying HBV infection also responded to therapy.

1.10. Economics of Therapy for Hepatitis B

The economic analysis of the treatment of any disease is a complicated undertaking. Any analysis must consider the uncertainties in the long-term course of a disease with and without therapy and the difficulties in estimating both the costs of therapy and the costs associated with the disease itself. In addition, there are variations between patients and communities that can be almost impossible to take into account in broad economic models. For instance, the cost of developing end-stage liver disease may be different depending on the availability of liver transplantation. Nevertheless, it is worth considering the economic implications of the therapy of hepatitis B because of the growing importance of economic considerations in health care decisions and because meaningful data are available about chronic HBV infection and the effects of therapy.

At least two thorough examinations of the economics of IFNα therapy for HBV have been performed *(85,86)*, which provide a range of costs using a range of estimates of the effectiveness of IFNα therapy. Whereas a detailed discussion of these appraisals are beyond the scope of this chapter, both analyses suggest that the cost associated with IFNα per quality-adjusted life year gained (QALYQ) compares favorably with many commonly accepted therapies for other conditions *(85,86)*. At least one analysis suggests that interferon therapy actually saves money, because fewer patients progress to advanced disease, which has its own inherent costs *(85)*. Any analysis of this scope must make estimates and projections, and these are open to criticism *(87,88)*. The interested practitioner is invited to make an independent assessment of the validity and applicability of these economic models as they apply to his/her patient population.

1.11. Alternative Therapies

Despite the successes seen with IFNα therapy for chronic hepatitis B, most patients do not respond, and there remains a pressing need for additional agents. The most promising new drugs currently being used for hepatitis B are the nucleoside analog compounds. This section will include a discussion of newer, often experimental drugs that have been used alone or in combination with IFNα. There will be an emphasis on the development of nucleoside analog compounds and the growing data indicating their clinical efficacy. The section will conclude with a review of even more experimental

Table 2
Hepatits B: Alternative and Experimental Therapies

Interferons	New species of interferons IFNβ IFNγ Pegylated interferons
Cytokines	Granulocyte colony stimulatory factor
Interleukins	Interleukin-2 Interleukin-12
Immune modulators	Thymosin Poly-I:poly-$C_{12}U$ Levamisole
Herbal therapy	Phyllantus amaurus
Nucleoside analogs	Adenosine arabinoside Lamuvidine Famcyclovir
Therapeutic vaccines	CY-1899
Novel molecular approaches	Antisense Ribozymes External guide sequence

modalities still in development (*see* Table 2 for a list of alternative and experimental therapies).

1.11.1. Interferon Alpha

Until recently, IFN alfa-2b (Intron A: Schering-Plough Corporation, Kenilworth, NJ) was the only recombinant IFNα approved for used in the United States. More recently, IFN alfa-2a (Roferon-A: Hoffmann-La Roche, Basal, Switzerland) and "consensus IFN" (Infergen: Amgen, Thousand Oaks, CA) have been approved by the FDA for HCV.

Interferon alfa-2a is approved in Europe, where it has been used for HBV and HCV for many years. It differs from IFN alfa-2b in that it has a lysine at amino acid position 23 instead of an arginine *(89)*. Treatment may result in a higher rate of anti-IFN antibodies than IFN alfa-2b, and there are theoretical concerns that this could effect efficacy *(90)*. To date, however, it does not appear to differ markedly from IFN alfa-2b in efficacy in the treatment of HBV *(35,64)*.

Infergen is a synthetic IFNα that was generated by identifying the most common amino acid at each position in approx 11 naturally occurring IFNα subtypes. To date, it has been studied primarily in patients with hepatitis C and a large phase III, multicenter trial has recently been completed. It is not clear at this time whether Infergen will present any advantage of efficacy over IFN alfa-2b in the treatment of patients with chronic HBV (Fig. 4).

1.11.2. Pegylated Interferon

Pegylated IFN (Hoffmann-La Roche) is a form of IFN alfa-2a that is chemically conjugated to polyethylene glycol (PEG). Modification of proteins with PEG theoretically can increase their serum half-life and decrease immunogenicity. In the therapy of viral hepatitis, pegylated IFN has the potential to either increase efficacy or to provide the same efficacy with less frequent administration. However, most experience with the pegylated IFNs to date has been primarily in the therapy of hepatitis C. In this setting, phase II clinical trials of the first-generation pegylated IFN alfa-2a given once per week found less efficacy than with standard IFN administered three times a week. However, a second-generation pegylated IFN with even more favorable animal pharmacokinetic data, and in vitro antiviral efficacy has been generated and is approaching clinical trials in the U.S..

1.11.3. Interferon Beta and Gamma

As discussed in Subheading 1.3.2., there are three known naturally occurring forms of IFN: alpha, beta, and gamma. While IFNα has an established role in the therapy of both hepatitis B and C, the other two forms also have antiviral and immunomodulatory properties.

1.11.3.1. Interferon Beta

Interferon beta is a naturally occurring IFN produced by fibroblasts that binds to the same receptor as IFNα. Pilot studies in both Italy *(91)* and Japan *(92)* have demonstrated significant activity of IFNβ against hepatitis B. The Japanese study administered IFNβ (Feron; Toray Industries, Tokyo, Japan) intravenously, once weekly for 6 mo to ten patients and found five of ten patients had seroconversion of from HBeAg positive to HBeAb positive *(92)*. Four of these five patients had normalization of serum alanine aminotransferase. However, no subsequent controlled trials have been reported with either naturally occurring or recombinant IFNβ, and it is not anticipated that this drug will play an active role in the management of chronic hepatitis B.

1.11.3.2. Interferon Gamma

Interferon-γ is secreted by activated T cells. It uses a distinct receptor from IFNα and IFNβ. It is said to have the most potent immunostimulatory

effects of the IFNs *(93)*. IFNγ has been used in multiple trials either alone *(93–96)* or in combination with IFNα *(94,96)* in the treatment of hepatitis B. Trials involving this compound in both children *(94)* and adults *(93,96)* were unsuccessful. Interferon gamma also appeared to have no added benefit even when used in combination with IFNα *(94,96)*. Although ineffective, it is generally well tolerated. At present IFNγ has no clear role in the therapy of hepatitis B.

1.11.4. Cytokines

Interferons are members of a broader group of hormone proteins termed cytokines that are defined by their ability to regulate the immune system. Because of their immune-enhancing effects, several additional cytokines have properties that theoretically make them appealing candidates for antiviral therapy.

1.11.4.1. Granulocyte-Macrophage Colony Stimulating Factor

In addition to its ability to induce neutrophil production, granulocyte-macrophage colony stimulating factor (GM-CSF) is also capable of stimulating the antigen-specific immune system. GM-CSF is a potent immunomodulatory cytokine when used in conjunction with tumor vaccines in mice *(97)*. This immunomodulatory effect is, in part, caused by the ability of GM-CSF to induce the maturation and activation of dendritic cells that present antigen to T lymphocytes with a high degree of efficiency. GM-CSF alone, when administered to patients with chronic hepatitis B was able to reduce levels of serum HBV DNA by 30–50%, but not eliminate HBV DNA *(98)*. High doses resulted in excessive leukocytosis requiring dosage modification. An uncontrolled pilot study demonstrated some efficacy with the combination of GM-CSF plus IFN in patients who had previously failed IFN alone *(99)*. It remains to be seen if the combination of GM-CSF and IFN will be effective in the treatment of hepatitis B in future, well-controlled trials. GM-CSF is currently under investigation in phase II trials in patients with hepatitis C.

1.11.4.2. Interleukin 2

Interleukin-2 (IL-2) is one of the critical cytokines in the growth and proliferation of T cells and NK cells. The quantity of IL-2 produced by activated CD4+ T cells is an important determinant of the magnitude of an immune response.*(100)* Despite this, results of trials using recombinant IL-2 for hepatitis B have generally been disappointing. Two uncontrolled pilot studies found IL-2 exhibited mild antiviral activity *(101,102)*. A controlled trial that compared the combination of IL-2 and IFN alfa-2b to IFNα alone found that the combination therapy had no efficacy advantage over IFNα, but resulted in a marked increase in side effects *(103)*. A third trial found

antiviral effects only with high doses of IL-2 that resulted in significant side effects *(104)*. High doses of IL-2 used for other indications have had serious adverse reactions, including capillary leak syndromes, which limits the amount of drug that can be used.

1.11.4.3. Interleukin 12

Interleukin-12 (IL–12) is a potent immunostimulatory cytokine with a multitude of activities that has only recently made its way into human trials. Previously known as natural killer cell stimulatory factor (NKSF) and cytotoxic lymphocyte maturation factor (CLMF), it is produced by B cells, monocytes, and other antigen-presenting cells. Interleukin 12 has been shown to increase production of interferon-γ, enhance cytotoxic T cell and NK cell activity and induce differentiation of CD4 positive T helper cells into T helper type 1 cells (TH1) *(105)*. TH1 cells stimulate cell-mediated immunity and produce IL-2 and interferon gamma. IL-12 has demonstrated impressive antitumor and antiviral activity in a variety of animal models. Like IL-2, IL-12 has the potential for serious side effects, and for this reason trials have proceeded cautiously in the United States, with most of the experience being in cancer and HIV-infected patients. IL-12 with its strong immunostimulatory properties remains a potential candidate for therapy of hepatitis B. Roche has initiated trials of IL-12 for chronic HBV infection.

1.11.5. Immunomodulatory Molecules

A variety of other molecules with effects on the immune system that are not as well understood as with cytokines and IFN have been used in the therapy of hepatitis B.

1.11.5.1. Thymosin

Various crude thymic extracts have been used in the therapy of viral hepatitis, and the active agents have not always been well defined. More recently, Thymosin-α_1 (Zadaxin, SciClone Pharmaceuticals, San Mateo, CA), a synthetic polypeptide of thymic origin with some sequence homology to IFNα, has been studied in hepatitis B patients. Thymosin-α_1 has been shown to promote endogenous cytokine production and stimulate NK cell activity *(51)*. In a small pilot study, Thymosin-α_1 in combination with low-dose IFN gave a 55% response rate in patients who had previously failed IFNα therapy *(106)*. Recently an Italian study compared Thymosin-α_1 with IFN alfa-2b in the therapy of hepatitis B patients negative for HBeAg but positive for HBV DNA *(51)*. These patients are typically less responsive to IFN than HBeAg positive patients. Patients were treated for 6 mo then followed for 6 mo. At the end of therapy, 7 out of 16 patients (44%) treated with Thymosin-α_1 were negative for HBV DNA as compared with 5 out of 17

patients (29%) treated with IFN. Thymosin-α_1, which is administered parenterally exhibited almost no side effects in either trial. While current data appear promising, the efficacy of this agent will need to be reproduced in larger trials which are under way.

1.11.5.2. Poly-I:poly-C_{12}U

Poly-I:poly-C_{12}U (Ampligen; HEM Pharmaceuticals, Philadelphia, PA) is a double stranded RNA molecule that has also been shown to demonstrate immunostimulatory and antiviral activity in vitro. The molecule increases induction of endogenous cytokines and augments NK and macrophage activity *(107)*. We performed a pilot study in conjunction with Stanford Medical Center in which eight patients were treated with intravenous Ampligen for 24 wk *(108)*. Four of the patients lost HBV DNA and three lost HBeAg. Two of these four patients that originally responded later relapsed. This appears to be higher relapse rate than occurs with IFNα. Overall, poly-I:poly-C_{12}U was well tolerated with milder flu-like side effects than IFNα. However, at this time, HEM is not pursuing additional trials of Ampligen for hepatitis B.

1.11.5.3. Levamisole

Levamisole has been shown to enhance both T-cell and macrophage activity *(109)* and to restore cutaneous delayed hypersensitivity to anergic cancer patients *(110)*. Levamisole has been used in combination with IFN in patients with hepatitis B *(111–113)*. In one trial in children, the combination was no more effective than IFN alone *(111)* and in two trials in adults the combination gave lower response rates than IFN alone *(112,113)*. In two of the studies *(111,113)* significantly more adverse events were seen with the combination than with IFN alone. Levamisole does not currently have a role in the therapy of hepatitis B.

1.11.6. Herbal Therapy

A variety of plant extracts are used in the treatment of chronic hepatitis B primarily in Asia. The objective assessment of these therapies is hindered in part by the poorly defined nature of some of the extracts and in part because trials reporting efficacy are often uncontrolled. The most extensively studied "herbal therapy" is desiccated extract from the Phyllantus amaurus plant. A trial from India reported that 59% of patients treated for 1 mo with this extract lost HBsAg as opposed to 4% of controls *(114)*. However, a subsequent trial by the same group *(115)* found loss of HBsAg in only 20% of patients and other groups have failed to demonstrate any response to Phyllantus amaurus extract *(116–119)*. Studies in the duck hepatitis B virus (DHBV) model failed to show any effect of Phyllantus amaurus

extract on DHBV replication and only a modest effect on DHBsAg production *(120)*. A variety of other "herbal" or plant-extract-based regimens have been described, but it is difficult to assess them without controlled trials that follow markers of viral replication *(107)*.

1.11.7. Nucleoside Analogues

The development of nucleoside analog compounds with strong antiviral activity represents one of the more interesting and clinically important developments in the therapy of hepatitis B. The development of these drugs has benefited from cell culture systems of HBV replication that allow screening of large numbers of compounds for antiviral activity. Research has progressed from initial agents with weak antiviral activity or marked toxicity to the recent development of lamivudine (2',3'-dideoxy-3'-thiacytidine, Epivir, Glaxo Wellcome) and famciclovir, drugs with good oral bioavailability, strong antiviral activity and low toxicity. Additional nucleoside analog agents are at more preliminary stages of drug development. This section will conclude with a practical discussion of the role of newer nucleoside analogs in current medical management.

Adenosine arabinoside was the first nucleoside analog agent used in the therapy of hepatitis B. While it appeared to exhibit antiviral activity, significant adverse reactions including marked neuromuscular toxicity precluded its extensive use. Additional nucleoside analogs were used in the ensuing years with disappointing results. These included ribavirin *(121,122)*, a guanosine-like nucleoside analog, acyclovir *(123,124)*, a thymidine kinase inhibitor, zidovudine (AZT) *(125)* a reverse transcriptase inhibitor, and ganciclovir, a guanine-like nucleoside analog. Ribavirin is approved therapy for respiratory syncytial virus in the U.S. Its major side effect is a dose related hemolytic anemia Results of trials with ribavirin and acyclovir give similar results. As single agents, both ribavirin *(122)* and acyclovir *(124)* give moderate decreases of HBV DNA but no significant clinical response. In combination with IFN, neither drug was more effective than IFN alone *(121,123)*. Zidovudine in combination with IFN resulted in increased toxicity, but no improvement in outcome *(125)*. Ribavirin, acyclovir and zidovudine are all administered orally. Ganciclovir (Syntex, Palo Alto, CA) appears to give a somewhat greater suppression of viral replication, but the response is not complete and ganciclovir has the additional disadvantage that it has poor oral bioavailability and must be administered intravenously *(126,127)*.

1.11.7.1. Fialuridine

The experience with fialuridine represented an unfortunate turn in the development of nucleoside analog agents. Fialuridine was among a second generation of nucleoside analogs identified that have good oral bioavail-

ability and marked activity against HBV replication in both in vitro and in vivo models *(128)*. In a phase II trial of fialuridine, 5 out of 15 patients died of hepatic failure and progressive lactic acidosis. Two other patients survived after liver transplantation. The toxicity manifested during the 13th week of therapy and was most likely secondary to the effects of fialuridine on mitochondrial DNA *(128)*. This toxicity was not seen in extended trials in mice, rats, dogs, and monkeys but was seen subsequently in woodchucks *(129)*. The toxicity also was not apparent in shorter trials in humans. In shorter trials and prior to toxicity in the longer trial, fialuridine did demonstrate marked suppression of HBV DNA levels *(130)*.

1.11.7.2. Lamivudine

The second generation of nucleoside analogs also include lamivudine and famciclovir, and these agents appear to date to provide potent antiviral activity with few side effects. Lamivudine (Glaxo Wellcome, Research Triangle Park, NC) is an oral 2',3'-dideoxy-3'-thiacytidine (also known as 3TC) that inhibits the HBV RNA-dependent polymerase. Lamivudine has been studied in a randomized, double-blind, multicenter trial *(131)*. Patients received drug orally once daily for 12 wk and were then followed for an additional 24 wk. About half the patients previously had failed IFNα therapy. HBV DNA became undetectable in all 22 patients who received either the 100 mg or 300 mg doses. In the 300 mg dose group, 50% of patients had undetectable HBV DNA by 2 wk and all patients had undetectable HBV DNA by 6 wk. HBV DNA typically recurs after drug is stopped. However, 6 of the 22 patients had sustained suppression of HBV DNA after the end of the treatment period and four patients cleared HBeAg. The drug was well tolerated and therapy was stopped in only one patient receiving the 25-mg dose because of a marked rise in alanine aminotransferase levels.

Despite the fact that lamivudine is not thought to work through an immune mechanism, sustained responders were more likely to have low HBV DNA and high aminotransferases at baseline. Although the reported experience is less extensive, lamivudine appears to be very active in Chinese patients as well *(132)*. Finally lamivudine has been used by several groups to successfully suppress persistent HBV infection after liver transplantation *(133–135)*. In this setting, the drug can be started either prior to *(133,135)* or after transplantation *(134)*.

Of increasing concern with lamivudine therapy is the emergence of drug-resistant mutants. It is typical for patients to have reappearance of HBV DNA after lamivudine is stopped, but there are now multiple reports of the recurrence of viremia in patients still receiving the drug. In three patients described to date, the recurrent viremia appears to result from a mutation in

the HBV RNA-dependent polymerase *(136,137)*. The mutation in all three was in a highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif. In each case, the methionine was replaced with either a valine or isoleucine. This motif is thought to represent part of the active site of the enzyme and is the same motif that is mutated in HIV reverse transcriptase, which is resistant to lamivudine *(138)*. It appears that the best strategy in the future will be the simultaneous use of multiple antiviral agents in order to both improve response and to prevent emergence of resistant HBV mutations in a manner analogous to the use of multiple agents for HIV infection. Potential candidate drugs that could be used in combination therapy include both IFNα and famciclovir. A trial of lamivudine in combination with IFNα is already in progress.

1.11.7.3. Famciclovir

Famciclovir (SmithKline Beecham, Philadelphia, PA), like ganciclovir, is a guanine-like nucleoside analog. Famciclovir is an oral agent that has been proven safe in thousands of patients who have taken it for herpes simplex virus infection. Although there has not been as much clinical experience in the treatment of hepatitis B with famciclovir as with lamivudine, famciclovir also appears to cause marked suppression of HBV replication in some patients *(139,140)*. In a placebo-controlled trial, 6 of 11 patients had a fall in HBV DNA titer of greater than 90% during a 10-d course of famciclovir *(141)*. In two of the patients, however, HBV DNA returned to pretreatment levels during the 10-d treatment period. Famciclovir and its active metabolite penciclovir also suppress viral replication by inhibiting the HBV RNA-dependent polymerase *(142)*. Since famciclovir and lamuvidine interact with different regions of the HBV polymerase, it may be possible to use both agents in combination to provide extremely potent suppression of viral replication that is less vulnerable to drug-resistant mutations.

1.11.7.4. The Role of Nucleoside Analog Agents in Current Therapy

The role of lamivudine and famciclovir in the therapy of hepatitis B is not clearly established and will depend to some degree on the results of trials currently in progress. Lamivudine has not to date been as effective as IFN in establishing a permanent remission. Between 80 and 90% of patients receiving lamivudine will have recurrent viremia once drug is stopped. In addition, the generation of resistant viral mutations remains a concern with long-term, single-drug use. While lamivudine may be more effective if given longer or in combination with other drugs, this remains to be established.

Overall, the best approach at the present time is still to encourage the use of IFN in patients who are appropriate candidates, especially those with

favorable biochemical profiles. At the other extreme, patients who have failed IFN, patients who are very unlikely to respond to IFN, and patients with persistent hepatitis B after liver transplantation who no longer respond to HBIG may all be appropriate candidates for lamivudine. The ideal treatment for other patients is evolving, and these patients may benefit from referral to specialized liver units with ongoing clinical trials.

1.11.8. Therapeutic Vaccines

CY-1899 (Cytel Corp., La Jolla, CA) is a lipopeptide designed for use as a therapeutic vaccine for HBV infection. The vaccine is designed to generate a T cell response and consists of three parts that are covalently linked. There is a peptide that serves as an epitope for cytotoxic T cells, there is a helper T cell epitope, and the tail of the construct ends in two palmitic acid molecules *(143)*. The T cell epitopes are specific only for patients with human leukocyte antigen (HLA) 2.1 which is present in approx 40% of the U.S. population. Investigational use of the CY-1899 vaccine is restricted to those patients with this HLA type. In a phase-I trial, the drug was well tolerated, and a cytotoxic T cell response specific to HBV was generated. However, no sustained clinical response to CY-1899 has been reported to date. The agent is currently being tested in a multicenter trial in the United States.

1.11.9. Other Experimental Approaches

A number of extremely novel approaches to hepatitis B therapy are currently under development. Many of these approaches are based on the most recent advances in molecular biology. Two approaches discussed here include the use of antisense oligonucleotides and ribozymes.

Antisense oligonucleotides being developed for hepatitis B therapy are short pieces of DNA that are complementary to HBV RNA *(144,145)*. They exert an antiviral effect by binding to HBV RNA in a highly specific manner and inhibiting translation of the RNA. While there is intense interest in antisense oligonucleotides for a variety of biologic purposes, a persistent problem in their use is the inability of these molecules to enter into cells. One potential solution to this problem is the use of gene therapy to deliver the molecules through viral vectors *(144)*. Readers interested in this rapidly evolving technology are referred to an excellent review by Nunes and Raper *(146)*.

Hammerhead ribozymes are short RNA molecules that possess endoribonuclease activity capable of degrading target RNA. The target RNA is defined by designing flanking sequences on the ribozyme that are complementary to the target RNA. Ribozymes that are specific for HBV RNA have been designed and have been shown to exhibit antiviral activity in vitro, but it is relatively early in this technology's development.

1.12. Conclusion

The current era represents an exciting and rapidly evolving time in the therapy of hepatitis B. The availability of effective vaccines should markedly reduce the incidence of new hepatitis B infections in the years to come. The use of IFNα has resulted in sustained remission in 30–40% of patients with chronic infections. The availability of multiple nucleoside analog agents with potent, nontoxic antiviral activity offers the potential for effective therapy for the remainder of patients. Finally, even newer agents and technologies, either in clinical trials or basic drug development, provide further promise down the road.

2. Hepatitis C

2.1. Introduction

The hepatitis C virus (HCV) has risen out of obscurity to become recognized as a major health problem of worldwide concern. Originally termed non-A non-B (NANB) hepatitis, the HCV was cloned and named by Choo of the Chiron Corporation in 1989 *(147)*. The WHO estimates that there are more than 200 million people chronically infected with HCV in the world, with 700,000 new cases occurring annually. Approximately 3.5–3.9 million people in the United States are chronically infected with the HCV, and the CDC in Atlanta calculates the incidence to be 150,000–170,000 newly diagnosed cases each year *(148)*.

From posttransfusion studies, Dr. Harvey Alter and others report that approx 90% of patients are unable to clear the virus, and they develop chronic hepatitis *(149)*. The use of ALT as a surrogate marker for the presence of the virus originally resulted in the underestimation of the chronicity rate after posttransfusion acquisition of the virus. It is now appreciated that many, if not most, patients infected with HCV will have normal liver function tests despite the continued presence of the virus. Only the advent of the polymerase chain reaction (PCR) test for HCV has allowed the true incidence and prevalence to become appreciated.

2.2. Testing Technologies and Serologies

The serological testing methods as well as the direct viral detection diagnostic assays are still undergoing evolution. This has resulted in a proliferation of methods to predict outcomes after antiviral treatment with IFN. The various serological assays and direct viral detection methods are discussed in chapters 3 and 7.

2.3. Mechanism of Viral Clearance

The normal immune response to HCV consists of both a humoral defense, a cell-mediated defense, and the actions of cytokines and intracel-

lular enzymatic cascades. During the humoral response, B cells produce nonneutralizing antibodies. The cell-mediated immune defense consists of NK cells and cytotoxic T cells. The interferons consist of a family of species specific proteins, that are secreted in response to a virus (*see* Subheadings 1.3.1. and 1.3.2. for additional discussion about mechanisms of antiviral clearance).

2.4. Standard Treatment Protocols

The ultimate goal of antiviral therapy is to eradicate virus from the system and do as little damage as possible to the organ(s) infected by the virus. Presently, it is clear that those patients who have progressed to a complication of their chronic liver disease, that is, ascites, portal hypertension and/or encephalopathy, are better served by liver transplantation than by treatment with therapy as it now exists. There appears to be a point where the hemodynamic changes of cirrhosis will result in progressive liver failure despite the reduction or elimination of the virus from the liver.

In 1986, Hoofnagle et al. published the first report of successful IFNα therapy of chronic hepatitis C in an uncontrolled trial *(150)*. Ten patients with chronic hepatitis C received subcutaneous injections of IFN for up to 1 yr. Six patients still had normal liver function tests (LFTs) and undetectable HCV RNA by RT-PCR 7 yr later *(151)*.

Despite the species of IFN (or similar antiviral compound) used to treat the disease, certain treatment patterns and responses to therapy are the same. The period of initial therapy with the IFN is known as the *induction period.*

In the treatment of patients with IFN and IFN derivatives, the anticipated response is a gradual reduction in serum transaminases without the flair in ALT and AST that occurs with hepatitis B patients that receive IFN therapy. If a patient never normalizes the serum liver function tests while on therapy, they are termed a *nonresponder.* When the transaminases become normal, the patient is considered to have a *complete response* to therapy. The ideal pattern is for the LFTs to remain normal until the end of the treatment period. A *breakthrough* occurs if the transaminases rise again after they had initially normalized on therapy. If the transaminases normalize on therapy and remain normal once the treatment is withdrawn, the patient is said to have a *sustained response* to treatment. The patient who has normal liver function tests after the end of the treatment period, but whose ALT and AST become abnormal during the follow-up period, is said to have *relapsed* (*see* Fig. 5 for the typical response patterns of patients on interferon therapy).

2.4.1. Interferon alfa-2b

In the United States (U.S.), the first Food and Drug Administration (FDA) approved protocol for treatment of chronic hepatitis C was IFN alfa-

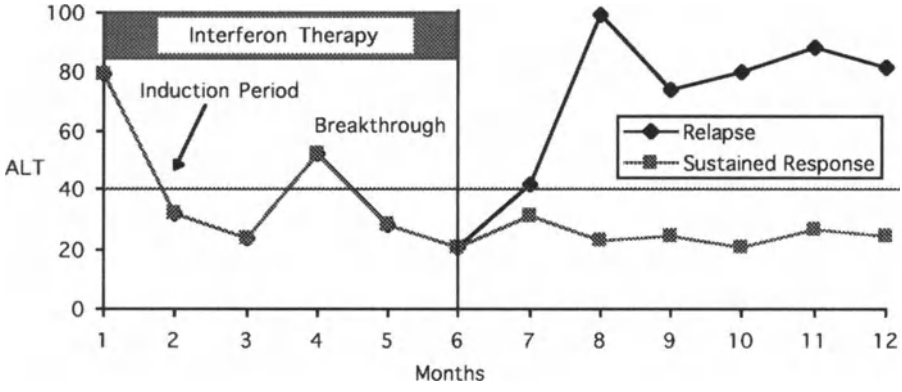


Fig. 5. Hepatitis C: ALT response patterns on IFNα.

2b (INTRON© A; Schering-Plough Corp.). Interferon alfa-2b is produced by *E. coli*, which contains a plasmid with an human leukocyte IFN alfa-2b gene. The FDA first approved treatment regime was a dose of 3 MIU given subcutaneously (SQ.) for 6 mo. It is apparent from clinical experience that those patients that do not normalize their ALT in the first 3 mo of therapy will most likely never do so, despite prolongation of dosing past that point in time. Originally, a normal ALT value was thought to be a surrogate marker for the absense of HCV. As a result, control of virus while on therapy was thought to be approx 45%, with an ultimate "cure" rate of 21% *(152)*. A patient who is a long-term responder as defined by the ALT criteria usually does not have detectable HCV RNA (by PCR) in their serum, liver tissue, or peripheral blood mononuclear cells *(153)*. The unfortunate fact is that this simply is not true for all patients that maintain long-term normalization of their ALT *(154–156)*. Overall, absence of detectable HCV posttherapy is estimated to be approx 12–20% depending on the HCV RNA titer, genotype distribution, and percentage of cirrhosis in the target population.

2.4.2. Interferon alfa-2a

The next IFN treatment to achieve approval in the United States was IFN alfa-2a (Roferon-A, Hoffmann-La Roche, Nutley, NJ). Although the approved dose remains the same, the treatment interval had been lengthened to 12 mo as opposed to original 6 mo. As a result, the complete ALT success rate is 23%. Normalization of ALT generally occurs within a few weeks in those patients responding to treatment. Similar to Intron-A, about 90% of the patients who respond to Roferon-A will do so within the first 3 mo. Approximately 50% of patients who have a complete response at the end of the 12-mo treatment subsequently relapsed (recurrence of abnormal ALT)

Table 3
Interferon Side Effects and Their Management

	Side effects	Management
Acute	Flu-like symptoms	Nocturnal administration NSAIDs, fluids, and rest
	Retinopathy	Stop therapy if grade III
Chronic	Anorexia, weight loss	High caloric diets
	Fatigue, depression	Antidepressants, counseling
	Alopecia	Stop therapy, topical minixidil

following the end of the treatment period. "In studies with long-term follow-up, 91% (39 of 43) of patients with normal ALT 6 mo after discontinuation of therapy had persistently normal ALT during continuous follow-up of up to 4 yr." Despite this FDA sanctioned claim of increased efficacy, the improved sustained response is most likely the result of a longer treatment interval and not of any benefit of one IFN species over another. The adverse effect profile of the two forms (Roferon-A and Intron-A) of IFNα are similar and are listed in the next section.

2.5. *Side Effects of Interferon Therapy and Their Management*

2.5.1. Symptoms

Therapy with IFNα produces side effects that can be divided into an acute variety and chronic (*see* Table 3 for a tabulation of typical effects and their management).

2.5.1.1. Flu-Like Side Effects

Flu-like side effects are very common and occur in nearly 100% of patients after starting IFN therapy. The most common symptoms include fever, chills, myalgias, arthralgias, headache, fatigue, and diarrhea. However, differentiating these complaints from a viral upper respiratory tract infection is that patients do not note any pharyngitis or complaints of lymphadenopathy. This constellation of symptoms responds to traditional therapy of fluids, rest, and the judicious use of an NSAID or acetaminophen. Many patients prefer self-administration of the IFNα before bedtime. This nocturnal time of administration allows them to sleep through most side effects. The intensity of the flu-like side effects is the most severe with the first dose of IFNα and tends to gradually diminish by the end of the first week (or two) of therapy. Most patients will develop a tolerance to IFNα, but a small percentage will not be able to endure the treatment.

2.5.1.2. Depression

Although more often occurring in patients with a history of emotional disturbances, this medication can cause *de novo* depression in patients with no previous history *(157)*. This can result in limitation of the optimal treatment duration. General support measures such as more frequent office visits supplemented with reassurance is frequently all that is necessary. Sometimes, administration of antidepressants and formal psychiatric intervention may be mandated *(158)*. Serious depression is an indication for complete withdrawal of IFNα and close observation until the patient returns to normal functioning.

2.5.1.3. Retinopathy

This usually occurs 1–3 mo after initiation of IFNα therapy. The retina on ophthalmologic examination displays cotton-wool spots (ischemic lesions) and occasional superficial hemorrhage near the optic disc *(159)*. Most commonly, the patient is asymptomatic, but some occasionally note impairment of visual acuity or "floaters." This retinopathy usually improves without any specific therapy, and IFNα treatment can continue. Since most patients are asymptomatic, the incidence depends on how carefully it is sought. Soushi and colleagues found that IFNα therapy resulted in retinopathy in almost 86% of their patients, a percentage that is unprecedented in other studies *(160)*. They prospectively followed 50 patients during therapy with IFN and noted that an early onset of retinopathy, presence of diabetes and hypertension, and longer duration or higher doses of IFN all correlated with more serious grades of retinal pathology. Two other Japanese studies also found retinal involvement in 57% of their patients *(161)* and in 46% of their patients *(162)*, respectively. Both of these later groups noted that diabetics were at especially high risk. However, Abe and colleges discovered that there was a significant incidence of idiopathic retinopathy and episcleritis in the patients with chronic hepatitis C that have never received IFN *(163)*.

2.5.1.4. Anorexia and Weight Loss

While common during the treatment period, this is usually mild and confined to less than 5–7 % of total body weight.

2.5.1.5. Alopecia

This can be a troubling side effect, found in 16% of patients in clinical trials *(164)*. Although more common in men experiencing male pattern baldness, occasionally women can sometimes experience significant hair loss. This tends to be reversible in those patients who have no other reason for hair loss. Topical application of minoxidil can help improve the situation in those patients who have an inherited tendency to hair loss.

2.5.2. Laboratory Abnormalities

2.5.2.1. Pancytopenia

Leukopenia and thrombocytopenia in response to IFN therapy are relatively common. Poynard found that approx 20% of his patients developed neutropenia (less than 900 leukocytes per cubic millimeter), and 10% developed significant thrombocytopenia (less than 49,000 platelets per cubic millimeter) *(164)*. Neutropenia and thrombocytopenia are especially common in those patients with subclinical portal hypertension and hypersplenism.

2.5.2.1. Exacerbation of Autoimmune Phenomena

Autoimmune markers such as rheumatoid factor (RF), antinuclear antibody (ANA), antideoxyribonucleic acid antibody (DNA), antithyroglobulin antibody, antithyroid peroxidase antibody, cryoglobulins (a mixed type II cryoglobulinemia of monoclonal IgM-kappa paraprotein and polyclonal IgG), and antiphospholipid antibodies are frequently found in patients with chronic hepatitis C who have never been treated with IFN. Matsuda studied 56 of his patients and found 13% positive for the anticardiolipin antibody, 21% (+) antiphosphatidylserine antibody, and 23% (+) antiphosphatidic acid antibody. Although 34% of the patients developed a new antiphospholipid antibody after therapy, almost 61% of patients originally (+) also lost their antiphospholipid antibody after IFN *(165)*.

2.5.2.2. Thyroid Disease

Originally, IFN therapy was thought to cause abnormal thyroid function in up to 12% of patients with chronic hepatitis C. Both thyroid microsomal and thyroglobulin antibodies can occur during the treatment period. As a result, hyperthyroidism as well as hypothyroidism can be a post-therapy sequel *(166)*. Some investigators feel, however, that those patients with positive tests for thyroid autoantibodies prior to treatment are at particular risk. Nagayama found that IFN therapy caused clinically significant thyroid dysfunction only in those patients with positive tests for thyroid autoantibodies prior to treatment but did not result in thyroid disease in thyroid autoantibody-negative patients *(167)*. Watanabe studied 139 patients who had received IFN therapy. Through the use of multivariate regression analysis, he discovered that a positive microsomal antibody at the beginning of therapy was a significant risk factor for the development of a clinical thyroid disease after IFN *(168)*. Sixty percent of his patients with a positive microsomal antibody developed either hyperthyroidism or hypothyroidism post-IFN, compared to 3% of patients with a negative microsomal antibody.

Table 4
Hepatitis C: Predictors of Response to THerapy with Interferon—
Multivariate Regression Analysis

Patient	Histology	Laboratory parameters
Age	Cirrhosis/fibrosis	Common
Sex	Hepatic iron content	HCV RNA titer
Body weight		HCV genotype
		Esoteric
		2–5' OAS level
		HCV cor Ab (IgM)

2.6. *Predictors of Response*

Interferon therapy is not particularly pleasant, must be given by subcutaneous injection, has a large number of side effects, and has a less than complete success rate. As a result, a number of factors have been studied to attempt to identify the best candidate for therapy. Based on multivariate regression analysis of a number of trials, the ideal patient to respond to therapy with IFN would be a thin, young, female, with a very low titer of HCV, genotype 2 or 3, and a liver biopsy without evidence of fibrosis or cirrhosis *(169)*. Unfortunately, this patient profile does not fit the majority of patients that present with chronic HCV infection (*see* Table 4 for parameters that predict successful therapeutic outcome after therapy with IFNα).

2.6.1. Histology

2.6.1.1. Histology

Other predictors of response include fibrosis and cirrhosis on liver biopsy. In most every study published to date, those patients with more severe scarring of the liver do not respond as well as those patients without significant fibrosis. Lin found that 42% of his patients with hepatitis only had a sustained response (as measured by ALT) as compared with 0% of his patients with cirrhosis *(170)*. In a similar fashion, Pagliaro noted that 25% of his patients with inflammation only on biopsy had a sustained response, but that only 7% of his patients with cirrhosis or severe fibrosis had a similar outcome *(171)*. Jouet *(172)* and Pagliaro, both reporting results during 1994, had very similar findings with 27%/25% of their noncirrhotic patients and 9%/7% of their cirrhotic patients normalizing their ALTs respectively. (Figure 6 shows four studies that illustrate the effect of histology on the sustained ALT response after therapy with IFNα.)

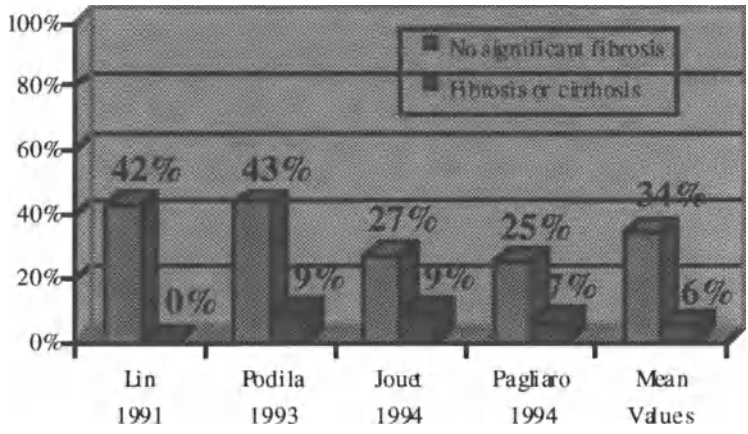


Fig. 6. Liver histology and the sustained ALT response to IFN.

2.6.2. HCV RNA Titer

The level of virus prior to therapy is an independent predictor of response to treatment. Yuki studied the effect of the pretreatment viral load on the response rate to IFNα therapy in patients with chronic hepatitis C. Chronic hepatitis C patients were treated with either 28 wk ($n = 45$) or 52 wk ($n = 43$) of therapy. The responses were correlated with pretreatment HCV RNA levels assessed by a branched DNA (bDNA) assay and genotypes. After the 28 wk of IFNα, sustained aminotransferase normalization occurred in 78% (7/9) of the HCV (bDNA assay) RNA (–) patients, but only 22% (8/36) of the HCV RNA (+) patients ($p < 0.01$). Treatment with 52 wk of IFNα resulted in a sustained response in 75% (6/8) of the HCV RNA (–) patients and 49% (17/35) of the HCV RNA (+) patients *(173)*.

Multivariate regression analysis has repetitively demonstrated that patients with low levels of virus in their serum (less than 1×10^6) are more likely to clear HCV RNA with IFNα therapy *(174)*. At one point in time, it appeared that the various genotypes tended to have different levels of HCV RNA (with type 1 having the highest). This now appears to have been an artifact of the HCV RNA testing methods.

2.6.3. Hepatitis C Genotypes

Presently, HCV can be divided into a total of 10 major genotypes, each containing a number of closely related subtypes *(175)*. Genotypes 1, 2, and 3 are ubiquitously spread around the world. Genotypes 4, 5, and 6 seem to be more limited in their distribution and tend to be located in Africa, South

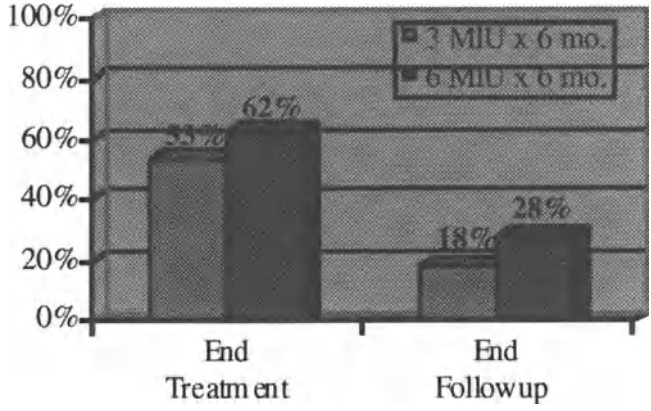


Fig. 7. Effect of higher dose IFN on ALT response.

Africa, and Hong Kong, respectively. Genotypes 7, 8, and 9 have even more recently been isolated from Vietnamese patients *(175)*.

There appear to be differences between the major HCV genotypes in terms of their response to IFN with response rates of 12–20% in type 1, 40–50% with type 2, and 40–70% with type 3 *(176)*. The use of genotypes has been also proposed as a means to predict outcome after therapy with IFN. It now appears that HCV genotype 1 (both subtypes a and b) show a relative resistance to successful IFN therapy, with an overall viral clearance of 6–30% compared with a 33–50% clearance rate in the patients with genotypes 2 and 3 *(176,177)*.

2.7. Methods to Improve Response to Interferon

2.7.1. Higher Interferon Doses

Higher IFN doses have been utilized in an attempt to improve permanent clearance of virus. Response to IFN appears to be biphasic. The first portion of the treatment period separates out those patients that will normalize their transaminases and are considered to be responders. The second portion is the consolidation phase where those patients who initially normalize there LFTs and lose detectable virus as measured by PCR are given the opportunity to permanently clear the virus. Higher doses of IFN (5 MIU and 10 MIU) do not increase the number of patients with a normal ALT after 6 mo of therapy. The higher dose will cause an ALT normalization in 12–21% of patients who initially did not respond to the 3 MIU dose *(178)*. (Fig. 7 illustrates the effect of higher dose compared with the standard 3 MIU dose of IFN on the ALT response.).

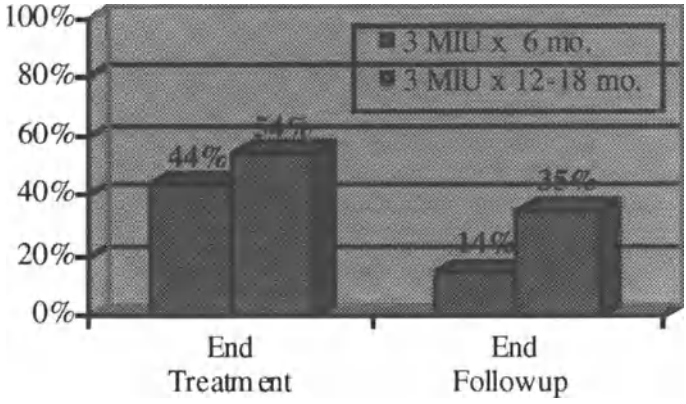


Fig. 8. Effect of longer duration IFN therapy on ALT response.

2.7.2. Longer Treatment Duration

Meta-analysis of randomized clinical controlled trials published to date demonstrate a definite effect of prolonged treatment on the sustained response rates. Poynard's trial showed a marked improvement in the long-term outcome in the 18-mo treatment group compared to the standard 6 mo of IFN therapy. Although the longer treatment was successful in improving the sustained response to therapy, it did not increase the number of patients that initially normalized on therapy.

Poynard performed an excellent meta-analysis of the effect of longer treatment duration compared to the standard 6-mo treatment. Forty-three papers were analyzed and seventeen qualified for inclusion into the core group of the analysis. Three MIU IFNα TIW for 12 mo had a superior sustained (ALT response) of 35% vs 14% for the same treatment for 6 mo *(164)* (*see* Fig. 8).

2.8. Special Treatment Situations

2.8.1. Treatment of Breakthroughs During Therapy

A breakthrough occurs when patients who originally show normalization of their transaminases in response to IFNα therapy again develop abnormal transaminases while still in the treatment period. One group of investigators reported 21 out of 71 patients (30%) treated with IFNα who had breakthrough events while on therapy *(179)*. The breakthrough events were equally divided between the first 6-mo and second 6-mo periods of the 12-mo treatment interval. Six patients' (29%) transaminases again returned to normal and persisted at that level until the end of the treatment period. Two of these patients maintained normal LFTs (9%) during the follow-up

period. In comparison, 9 of the 22 (41%) patients who had a complete response without breakthrough during the treatment with IFN had sustained normal LFTs during the follow-up period. The same dose of IFNα was used to manage 16 breakthrough events with return to normal ALT occurring in 7 (44%) patients. Twelve other patients were given an increased dose of IFNα, and six of these patients (50%) again developed a normal ALT.

Other authors have also found breakthrough on therapy to be a common event that may occur at any point during IFNα therapy of hepatitis C *(180)*. After a breakthrough, a normal ALT can be reestablished in approx 50% of cases, although the chance of a sustained normal ALT after withdrawal of therapy is about 10%. Increasing the dose of IFNα dose after a breakthrough event did not appear to improve the chance that the patient would again normalize their ALT.

Multiple reasons have been proposed to explain the breakthrough phenomena Emergence of resistant HCV strains has been demonstrated in some patients with return of HCV RNA positivity by RT-PCR *(181)*. Sequencing of the quasispecies demonstrates that this is often a different strain than the original. The development of anti-IFNα antibodies directed against a specific brand or type of IFN appears to explain those patients where the reemerging HCV quasispecies is presumably the same as the original *(182,183)*. In this particular situation, switching to a different species of IFN will frequently restore the original response. Other authors have discovered that the induction of antiliver kidney microsomal antibodies by IFN is occasionally associated with the "breakthrough" phenomenon with HCV eradication, suggesting an IFN induced autoimmune-like hepatitis that resolves with discontinuation of the IFN *(184)*.

2.8.2. Treatment of Relapsers and Long-Term Maintenance Therapy

Unfortunately, most patients will relapse once therapy has been stopped. Various studies have demonstrated that retreatment of patients with the same IFN dose and duration produces the same outcome as that of the original treatment *(185)*. In some cases, those patients that did not originally normalize their transaminases on 3 MIU of IFNα TIW do respond to higher doses (either 5 or 6 MIU). More striking is the fact that very few nonresponders to the first attempt at treatment have a response during the second treatment. Even in those patients that maintained normal transaminases during the initial exposure to IFN, the overall probability of a sustained response after a second course of therapy is still, for the most part, low.

However, there is recent evidence that long-term IFN therapy can improve not only the inflammatory activity observed on liver biopsy, but the amount of fibrosis present in the liver can actually decrease. Even though

Table 5
Sustained IFN Therapy Result in Histologic Improvement

Treatment	Knodell's Score		
	Baseline	End of treatment	Difference
3 MIU × 6 mo	8.2 ± 0.4	6.6 ± 0.4	−1.5 ± 0.1
3 MIU × 18 mo	9.0 ± 0.3	.5 ± 0.3	−3.3 ± 0.1

patients do not normalize their ALT on therapy, they can still have a significant improvement in histology. Poynard found that 61% of his patients who did not normalize their ALT at 6 mo had an improvement in their liver histology on biopsy after a total of 18 mo of therapy *(186)* (*see* Table 5).

2.8.3. Very Early Therapy for Acute Hepatitis C

Interferon therapy given soon after initial exposure appears to decrease the otherwise 75–85% anticipated chronicity rate. This frequently will be a health care worker who sustains a needle-stick or is exposed to infected blood in some other fashion. Not all exposures need or require treatment. The risk of transmission after a needle-stick injury is calculated to be 10% *(187)*. However, if an HCV RNA determination by PCR becomes positive within 2 wk after the exposure, this confirms that the patient has become viremic. Multiple studies now suggest that immediate treatment may be indicated to decrease the chance of developing chronic infection with HCV.

There have been eight trials published to date that examine this group of patients with acute HCV. Only four of these eight papers are true randomized, controlled trials *(188–191)*. Interferon-alpha was used in three trials, IFNβ in the fourth. The treatment consisted of 3 MIU SQ three times a week for 12 mo. Interferon therapy produced a sustained ALT response that persisted 12 mo in 53% of acute exposures vs 32% in the nontreated group. Even more significant was that 41% of treated patients had no detectable virus by PCR at the 12-mo mark compared to only 4% in the nontreated group.

2.8.4. Treatment of "Healthy Carriers"

Patients that have persistently normal serum alanine aminotransferase levels are a special group. Originally, this patient population was thought to be "healthy carriers" of HCV *(192)*. Several studies now show that despite normal liver function tests, histology can vary from normal to cirrhosis *(193)*. Interferon therapy in this group has not been demonstrated to be effective. These individuals do not respond to therapy, and they frequently develop evidence of worsening liver function after treatment *(194)*. Presently, treatment of patients with chronic hepatitis C and normal transaminases is not recommended.

Table 6
Extrahepatic Manifestations of Hepatitis C

Skin	Renal (glomerulonephritis)
Porphyria cutanea tarda	Membranoproliferative
Leukocytoclastic vasculitis	Membranous
Lichen planus	Focal segmental
Erythema nodosum	Neurological
Urticaria	peripheral polyneuropathy
Erythema multiforme	Immunologic
Polyarteritis nodosa	Cryoglobulinemia
Eye	Antiphospholipid syndrome
Uveitis	Endocrine
Mooren corneal ulcer	Hypothyroidism
	Hyperthyroidism
	Diabetes mellitus?

2.8.5. Treatment of Syndromes Associated with Hepatitis C Virus Infection

Sometimes, extrahepatic manifestations of HCV may be the first evidence that the patient has chronic hepatitis C. Extrahepatic manifestations associated with HCV infection are listed in Table 6.

Some investigators have found proteinuria in up to 21% of their patients with chronic hepatitis C *(195)*. The proteinuria, when present, is most often in the nephrotic range. Clinical manifestations of associated liver disease can be subtle or absent. A renal biopsy most frequently will demonstrate a membranoproliferative glomerulonephritis characterized by the deposition of IgG, IgM, and C3 in glomeruli. Almost 33% of these patients have normal serum LFTs. When there is a high index of suspicion, 100% will have HCV RNA detected in their serum and about two thirds will have detectable cryoglobulins, and are positive for rheumatoid factor. Treatment with IFN will reduce the proteinuria, but has no effect on the serum creatinine or glomerular filtration rate.

More rarely, patients have a membranous glomerulonephritis on renal biopsy. These patients, in contrast, have negative cryoglobulins, nondetectable rheumatoid factor, and normal or marginally abnormal serum complement levels *(196)*. Finally, some groups have described focal, segmental glomerulonephritis associated with HCV *(195)*. Regardless of the type of glomerulonephritis found, all patients will become viremic again posttreatment and have a return of their proteinuria to pretreatment levels *(197)*.

2.8.6. Treatment of Patients with Allografts in Place

2.8.6.1. Renal Transplant Recipients

Interferon has been used in an attempt to treat chronic HCV infection in patients that have a renal allograft in place. Possibly, because of the simultaneous immunosuppression required to keep the kidney transplant in place, IFN has not been successful and all patients relapse posttherapy *(198)*. Although the LFTs can sometime improve on therapy despite the presence of immunosuppression, rejection of the kidney is often the sequel to treatment of this kind *(199,200)*.

2.8.6.2. Liver Transplant Recipients

Feray and fellow investigators treated 14 patients with IFNα in an open label trial. They found that only one patient (7%) did not relapse after withdrawal of the treatment. Chronic rejection occurred in 5 of 14 (36%) treated patients leading to retransplantation in three of them. In contrast, chronic rejection occurred in only 1 out of 32 untreated patients in the control group during the follow-up *(201)*. Another group of investigators suggested that some patients may respond to 3 MIU of IFNα three times weekly given for at least 4 mo (by ALT criteria), but in reality no patient had sustained loss of HCV RNA after withdrawal of the IFN *(202)*.

There are two reports of experimental use of ribavirin as monotherapy for treatment of recurrent hepatitis C post liver transplant. The first report comes from Roger Williams' group that treated seven patients with ribavirin at doses ranging from 200 to 1200 mg/d in an open-label, uncontrolled trial. Four patients were able to tolerate the full dose and had normal ALT's at the end of 6 mo of treatment. Even more impressive was the decrease in inflammation on liver biopsy despite the fact that the HCV RNA remained persistently positive throughout the treatment *(203)*.

Cattral also conducted an uncontrolled pilot study of ribavirin as monotherapy in nine patients post liver transplant with evidence of liver disease caused by recurrent HCV. Patients received ribavirin at 800–1200 mg/d for 3 mo. Four patients normalized their transaminases. All patients remained positive for HCV RNA by PCR, and all LFTs again became abnormal after withdrawal of the ribavirin. In contrast with Roger Williams' group, Cattral found no histologic improvement after ribavirin therapy. Ribavirin caused reversible hemolysis in all patients. These results suggest that ribavirin may be of benefit in the treatment of HCV infection after liver transplantation. Further studies are needed to determine the optimal dosage and duration of therapy *(204)*.

Table 7
Hepatitis C: Experimental Therapies

Interferons	Others
New species of interferons	Antisense
Modified interferons	Amantadine
Combinations with ribavirin	Serine protease inhibitors
Interleukins	Ribozymes
IL-12	Therapeutic vaccines

2.9. Economics of Treatment

This is an increasingly important area of concern with managed health care and dwindling health care resources. The two major methods of analysis are that of quality years of life saved and whether the treatment of the patient saves money in comparison to the health care costs associated with the natural progression of disease *(205)*.

Important new information pertinent to this discussion is whether the treatment of patient with cirrhosis will decrease the incidence of future hepatocellular carcinoma. Nishiguchi randomized 90 patients with compensated cirrhosis due to HCV into two groups: 45 patients received IFNα 6 MIU TIW for 3–6 mo and 45 patients were the control group *(206)*. The patients were followed for 2–7 yr posttherapy. In the treatment group, seven patients developed persistently normal ALT with nondetectable HCV RNA. Hepatocellular carcinoma developed in two (4%) patients who were treated with IFNα and in 17 *(38%)* patients in the control group.

2.10. Experimental therapies

It is clear at the present time that there is a critical need for better therapy for patients with chronic hepatitis C. The newer treatments tend to fall into categories as described (*see* Table 7 for a list of potential, experimental therapies for hepatitis C).

2.10.1. Interferons as Monotherapy

2.10.1.1. Consensus Interferon

Consensus interferon (Infergen, Amgen) was developed through computer analysis of known IFN sequences. The theory behind Infergen was that common amino acid sequences in the various IFN were present because they were necessary to produce the maximum antiviral effect. Those motifs that were common among the various sequences were noted and a synthetic IFN molecule was synthesized. It has been difficult to analyze the results of various consensus IFN trials reported to date. There has been controversy con-

verting the microgram protein dose used in the Infergen trials to the MIU doses previously reported for the other interferon species. There has not been convincing evidence to date that treatment of HCV with Infergen will produce a response rate significantly better than the individual IFN species.

2.10.1.2. Lymphoblastoid Interferon

Lymphoblastoid interferon (Wellferon [Interferon Alfa-n1 {lns}], Glaxo Wellcome) This is a highly purified blend of natural human IFNα, obtained from human lymphoblastoid cells following induction with Sendai virus. Interferon Alfa-n1 consists of a mixture of natural IFNα subtypes but in different proportions than in human leukocyte IFN.

There have been multiple reasons proposed to explain the inability to clear HCV from patients undergoing IFNα therapy. One possibility would be that a particular mutation of HCV was one that was resistant to the form of IFNα that the patient was receiving. Lymphoblastoid IFNα is a mixture of all 22 different IFN normally produced by the body in response to a viral infection. This natural mixture of IFN types could, in theory, be more effective than therapy with one IFN species.

2.10.1.3. Interferon-Beta, Recombinant

Three companies are in the process of testing IFNβ in trials for the treatment of chronic hepatitis C. IFNβ (Serono Laboratories, Randolph, MA) is in phase II trials while preparations from both Biogen, Inc. (Cambridge, MA) and the Chiron Corporation (Emeryville, CA) are in phase III clinical trial testing.

There have only been a few trials reported to date in the literature. Perez did a direct comparison of IFNβ against IFN alfa-2b in the treatment of 40 patients with chronic hepatitis C *(207)*. Both groups received the initial dose of 6 MIU TIW for 2 mo (induction phase) followed by 3 MIU TIW for 4 mo (consolidation phase). The ALT response rate was 57% (12/21) on IFN alfa-2b and 5% (1/19) on IFNβ. Although both IFNβ and IFN alfa-2b induced a significant decrease in mean ALT values at the 6 MIU dose. Virtually all patients in the IFNβ group had a return of elevated ALT when the dose was reduced to 3 MIU. The 19 patients who received IFNβ had less side effects from the therapy than did patients receiving the IFN alfa-2b. Interferon beta also produced less thrombocytopenia and neutropenia than did IFNα. However, the response to interferon-beta at a dose of 3 MIU TIW clearly is inadequate, although higher doses (6 MIU) may produce similar response rates as IFNα.

Kobayashi treated 146 patients with IFNβ; 24 patients (16%) were long-term responders *(208)*. Echoing the results in the IFNα trials, their analysis also demonstrated that the pretreatment serum level of HCV RNA

was an important predictor of outcome of IFNβ therapy. Again, there has not been convincing evidence to date that the response rate will be significantly better than the IFNα species.

2.10.1.4. Natural Source Interferon

Natural source interferon (human leukocyte), Alpha-n3 (Alferon N, Interferon Sciences) Phase III.

2.10.2. Pegylated Interferons

Both Hoffmann-La Roche and Schering-Plough have studied the pegylated IFNα in an attempt to improve their respective IFN. However, these first-generation IFN never made it past the initial phase II clinical trials. Among other problems, it appeared that the grafting of the PEG molecule to the IFN molecule resulted in a decrease in antiviral activity of the IFN when calculated on an activity per weight basis.

Both Hoffmann-La Roche and Schering-Plough have been testing second-generation pegylated IFN and each has recently initiated phase-II clinical trials.

2.10.3. Combination Therapies with Interferon

2.10.3.1. Intron-A + Ribavirin

Di Bisceglie and colleagues performed an uncontrolled pilot study of ribavirin monotherapy in 13 patients with chronic hepatitis C. Four of the thirteen patients normalized their serum transaminases. No patient became HCV RNA-negative in serum either on therapy or in the follow-up period *(209)*. The same group then followed up with an randomized, double-blind, placebo-controlled study *(210)*. Twenty-nine patients with chronic hepatitis C received oral ribavirin (600 mg twice daily) and 29 controls received placebo for 12 mo. Serum aminotransferase levels normalized in 10 patients (35%) treated with ribavirin, no patients in the placebo group responded. Sustained normalization of ALT was seen in two patients (7%) after withdrawal of therapy. There was no change in the serum HCV RNA titers on therapy or following withdrawal. Liver histology improved in the ribavirin-treated patients whose aminotransferase levels became normal.

Twenty patients who had failed therapy with a natural leukocyte IFNα were treated by Brillanti with a combination of the same natural leukocyte IFNα and ribavirin or natural leukocyte IFNα alone *(211)*. Four of the ten patients that received the combination therapy for 6 mo had a sustained loss of detectable HCV RNA 6 mo after therapy was withdrawn. Schvarcz found similar results in his ten patients *(212)*.

2.10.4. Interleukin 12

Recombinant human interleukin 12 may be of benefit in the treatment of diseases that might respond to stimulation of cell-mediated immunity such

as malignancies, viral infections and intracellular pathogens (*see* Subheading 1.11.4.3.). Clinical trials with recombinant human interleukin 12 (rhIl-12) are currently underway in patients with advanced malignancies, HIV infection, chronic HBV and HCV infections. We have recently finished a safety trial of recombinant Interleukin 12 (Genetics Institute, Cambridge, MA and Wyeth-Ayerst, PA) in patients with hepatitis C and are currently proceeding with a phase-II trial of its safety in HCV infected patients.

2.10.5. Antisense

Development of effective molecular biology-based antiviral drugs, such as antisense RNAs and DNA oligonucleotides will require the ability to identify optimal target sequences that favor disruption of viral, rather than human, RNAs. An antisense RNA molecule is not technically feasable because of the rapid degradation of RNA by intrinsic enzymes that exist in the human cells. For this reason, modified oligodeoxynucleotides (ODN) form a more stable molecule where a modification of their sugar backbone enables them to resist enzymatic breakdown.

An ideal target for an antisense therapy would be the 5' noncoding region (5' NCR) of HCV *(213)*. This region is highly conserved among the viral genotypes from all areas around the world. The translation of HCV is directed by an internal ribosome entry site (IRES) located within the 5' NCR. Investigators have studied the inhibition of HCV gene expression using antisense oligonucleotides complementary to the 5' NCR, translation initiation codon, and core protein coding sequences *(214–217)*.

In theory, the complementary sequence, however, might need to be as long as 17 nucleotides in length to ensure a specific binding with the target region of the HCV RNA. This can occur with a high degree of specificity at the high temperatures in a PCR reaction (72°C), but the temperature in the human body is 37°C. Unfortunately, this lower temperature will result in a myriad of unpredictable hybridizations that can occur between partial segments of the complementary sequence and a number of bystander molecules in the human system *(218)*. Antivirals, Hybridon, Isis Pharmaceuticals, and Lynx Pharmaceuticals are all investigating this method at the present time.

2.10.6. Amantidine

Amantidine is a medication more commonly known for its use in the treatment of Parkinson's disease and drug-induced extrapyramidal reactions. But, amantadine also has antiviral activity most notably against influenza A virus isolates (subtypes H1N1, H2N2, and H3N2), but very little or no activity against influenza B virus isolates. Its antiviral effect seems to prevent the release of infectious viral nucleic acid into the host cell by interfering with the function of the transmembrane domain of the viral M2 protein. Amanta-

dine can also prevent virus assembly. The usual dose employed against the infuenza A virus is 100 mg given orally twice a day.

One investigation, reported in abstract form only, describes a small study of the combination of this agent with IFN alfa-2b *(219)*. The group that received amantadine alone, however, appeared to have the same chance of normalizing their ALT and losing detectable HCV RNA as did the patients receiving interferon alfa-2b. Confounding interpretation of the study results were patients that switched from one group to another, the study's small size and the use of the bDNA assay for the detection of the HCV RNA (with a sensitivity to detect virus only down to 200,000 copies/mL). Nevertheless, the results appear intriguing.

2.10.7. Serine Protease Inhibitors

The HCV is translated into a single polypeptide approx 3000 amino acids long. This polypeptide undergoes posttranslational modification from which 10 smaller viral proteins are produced. There are three different proteases that perform the necessary catalytic cleavages. The hepatocyte itself contains a signal peptidase that cleaves the capsid and the envelope 1 protein (C/E1), C/E1 and the envelope 2 protein (E1/E2), and the envelope 2 protein and nonstructural regions (E2/NS2). The NS2-NS3 portion of the polypeptide chain encodes a protease that is able to cleave itself at the NS2-NS3 junction.

The liberated NS3 protein encodes three regions of different activities: an N-terminal serine protease, a C-terminal RNA-stimulated NTPase, and an RNA helicase *(220)*. The cleavage of the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B regions of the HCV is performed by the serine protease activity of the N-terminal 180 amino acids of the NS3 protein *(221)*. Full protease activity can only occur after interaction of the NS3 region's N terminus with the NS4A protein *(222)*.

The interaction between the NS3/NS4A regions provides a possible ideal target for a serine protease inhibitor. A number of pharmaceutical companies are actively attempting to find a serine protease inhibitor that will specifically block this region. Both Vertex Pharmaceuticals, Agouron Pharmaceuticals, Hoffmann LaRoche (and others) are actively pursuing candidate compounds.

2.10.8. Ribozymes

Ribozymes are theoretically useful for specific cleavage of viral (hepatitis C) mRNA's. Hammerhead ribozymes are molecules that consist of a small catalytic core region plus a 5' and a 3' flanking double-helical motifs as well as a complementary sequence to the target RNA *(223,224)*. The hammerhead ribozymes directed against HCV RNA appear to specifically cleave substrate RNA and block cap-independent translation directed by the 5'-

noncoding region (5'-NCR) in vitro. Ribozymes have a potential advantages over antisense therapy because of their cleavage results in the destruction of the target RNA as well as in the reusability of the ribozyme on other target sequences. The major disadvantages would be the selection of escape variants to the complementary sequence on the ribozyme. A further stumbling block to their effective development is the difficulty with achieving the necessary degree of specificity (*see* Section 2.10.5. for additional insight into this problem).

Immune Response, Immunol, and RiboGene are all actively investigating this approach as potential therapy for HCV.

2.10.9. Therapeutic Vaccines

This area has reached a road block of sorts. The most immunogenic region of HCV is the core protein. Unfortunately, it now appears that the core protein may function as an oncogene in the human liver and could be the major means by which HCV causes liver cancer. Clearly, however, this prevents the core protein from being used in the construction of a therapeutic vaccine. The second most immunogenic region is the NS4 portion of the genome.

Apollon, Cytel (San Diego, CA), Univax Biologic, and Vical (San Diego, CA) all are in the process of developing technology in this field.

3. Hepatitis D

Hepatitis D virus (HDV) is endemic in Southern Italy, the Amazon Basin, and parts of the Middle East and Africa. HDV is unusual in the United States and is rare outside of intravenous drug users or multiply transfused individuals, such as hemophiliacs. HDV is a small, circular, defective RNA virus that requires the presence of HBV for infection and propagation *(225)*. HDV specifically uses HBV surface proteins to package or encapsulate the HDV genome *(226)*. As a result, individuals can become infected with HDV only if they were previously infected with HBV, which is termed "superinfection," or if they are coinfected with HBV at the same time, which is termed "simultaneous" infection. While patients with simultaneous infection are more likely to develop a fulminant hepatitis than patients infected only with HBV, simultaneous infection is usually self-limited *(226)*. Only a small percentage of these patients, typically less than 5%, go on to develop chronic liver disease *(227,228)*. Patients with chronic HBV infection who become superinfected with HDV, however, are at risk for developing an active, progressive hepatitis and the therapy of these patients has proven to be a difficult challenge.

3.1. Standard Therapy

Most studies have indicated that patients with chronic HDV have a more rapid progression to cirrhosis than patients with infected only with HBV *(229–231)*. Reports from Italy note that between 40 and 70% of patients with HDV infection, but no cirrhosis will progress to cirrhosis within 2–6 yr *(229)*. Disease progression was more rapid than seen in control populations infected with HBV alone. These findings emphasize the need for effective therapy for HDV.

Unfortunately, IFN therapy has been disappointing for patients with HDV infection. Patients typically demonstrate a decrease in serum HDV RNA and some improvement of serum aminotransferases while receiving IFN, but almost invariably relapse when therapy is stopped. In one randomized controlled trial, 14 patients received 9 MIU IFN alfa-2a three times a week for 48 wk *(232)*. Seven of the patients had a complete response on therapy as measured by normal levels of alanine aminotransferase and undetectable serum HDV RNA. However, all patients had recurrence of viremia after therapy was stopped. Three of the 14 patients treated with 3 MIU TIW and none of the 14 untreated controls had a complete response. Although results were generally disappointing, patients treated with 9 MIU had marked improvement in histologic findings, and several patients maintained normal aminotransferases for several years. Control patients, as a group, experienced significant histologic deterioration.

A meta-analysis of five randomized, controlled trials confirms the generally transient nature of the IFN response and does provide some general guidelines for potential IFN therapy in HDV infected patients *(233)*. The analysis confirms that therapy with higher doses of IFN is more likely to give a response than with low-dose regimens. Regimens of 5 MIU daily or 9 MIU TIW were associated with response rates of greater than 50%. Some increased efficacy is found with longer courses of 1-yr duration *(233)*. In addition, in many trials, a durable or permanent response is seen in about 10% of patients treated with IFN *(233–236)*. Neither the meta-analysis nor individual trials have been able to determine factors that predict a favorable response to therapy.

It appears that patients with HDV infection can be offered a course of therapy with IFNα after carefully explaining the low likelihood of a permanent response and the adverse reactions associated with IFN therapy. The higher 5-MIU daily dose appears to provide the best chance of a response, and we attempt to give at least a 1-yr course. It is preferable to follow both serum aminotransferases and HDV RNA, but HDV RNA assays are not readily available. We are inclined to terminate therapy in patients who do

not have significant improvement in aminotransferases within 4 mo or in patients who initially have improvement, but then have an apparent relapse based on LFT results. It is possible that even a transient response to IFN therapy may favorably alter the rate of progression to cirrhosis, but this has not been demonstrated.

3.2. Experimental Therapies

To date, no additional agents have demonstrate marked antiviral activity to HDV. To some degree, alternative therapies tried for HDV follow from those tried for HBV. This is in part because the greater number of HBV patients facilitates the performance of clinical trials.

Although many trials with alternative agents have been small or uncontrolled, no efficacy has been demonstrated in trials with adenine arabinoside, levamisole *(237)*, prednisone and azathioprine *(229)*, ribavirin *(238)*, acyclovir *(239)*, and thymosin derivatives *(240)*. It is not clear at this time if newer nucleoside analog agents such as lamivudine or famciclovir will be effective for patients with HDV infection.

3.3 Liver Transplantation

Patients with end-stage liver disease from HDV are appropriate candidates for liver transplantation and should be considered for referral to a liver transplant unit. A 5-yr actuarial survival of 88% has been reported following liver transplantation *(241)*. The use of anti-HBs immunoprophylaxis is critical in preventing recurrent viral hepatitis *(241)*. While increasing vaccination rates for HBV should, with time, decrease the incidence of HDV infection, existing therapy for patients with HDV clearly leaves room for improvement.

4. Hepatitis G

In 1995, three novel, hepatotrophic viruses of the *Flaviviridae* family were identified through molecular techniques by Abbott Laboratories. Two of the viruses, GBV-A and GBV-B, were isolated from the serum of a primate (tamarin) by representational difference analysis (a subtractive polymerase chain reaction [PCR] technique) *(242,243)*. Evidence now suggests that GBV-A is only a primate virus, and it is also not clear yet whether GBV-B can infect humans *(244)*. A third novel virus, GBV-C, was isolated by reverse-transcription PCR (RT-PCR) utilizing degenerate, oligonucleotide primers to the helicase gene segment of the HCV *(245)*. Sequence analysis of the GBV-C isolate has shown that it is closely related to another, independently cloned *Flaviviridae* virus named hepatitis G virus (HGV) by Genelabs (Redwood City, CA) *(246)*.

While it was clear what place of importance the HCV occupied, the same cannot be said for GBV-C/HGV. Even the name of this virus is mired in confusion and controversy. Similar to HCV, most if not almost all patients with GBV-C/HGV virus will have normal LFTs despite the continued presence of the virus. Only the advent of PCR for GBV-C/HGV virus will demonstrate its true incidence, prevalence, and significance. Harvey Alter of the National Institutes of Health recently presented evidence at the American Association of Study of Liver Disease Conference (1996) that it actually may not be a cause of liver damage in humans.

4.1. Testing Technologies and Serologies

The serological testing methods as well as the direct viral detection methods are still undergoing evolution. The various serological assays and direct viral detection methods are discussed in chapters 7 and 8.

4.2. Standard Therapy

None.

4.3. Experimental Therapy

All treatment for HGV must be considered experimental at this time. One group, however, studied 95 patients positive for the presence of GBV-C/HGV by RT-PCR *(247)*. Forty-one patients were coinfected with either HBV (14 patients) or HCV (27 patients). Fifty-four patients had GBV-C/HGV alone. Those patients that were coinfected had received IFNα for their primary infection (either HBV or HCV). All patients with GBV-C/HGV had return of this virus after withdrawal of the IFN therapy. However, whether treatment is even necessary is unclear at this time.

References

1. Hoofnagle, J. H., Shafritz, D. A., and Popper, H. (1987) Chronic type B hepatitis and the "healthy" HBsAg carrier state (review). *Hepatology* **7(4),** 758–763.
2. Perrillo, R. P. (1994) The management of chronic hepatitis B (review). *Amer. J. Med.* **96(1A),** 34S–39S.
3. Lohr, H. F., Weber, W., Schlaak, J., Goergen, B., Meyer zum Buschenfelde, K. H., and Gerken, G. (1995) Proliferative response of CD4+ T cells and hepatitis B virus clearance in chronic hepatitis with or without hepatitis B e-minus hepatitis B virus mutants. *Hepatology* **22(1),** 61–68.
4. Rehermann, B., Lau, D., Hoofnagle, J. H., and Chisari, F. V. (1996) Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. *J. Clin. Invest.* **97(7),** 1655–1665.
5. Peters, M. (1989) Mechanisms of action of interferons (review). *Sem. Liver Dis.* **9(4),** 235–239.
6. Dorr, R. T. (1993) Interferon-alpha in malignant and viral diseases. A review. *Drugs* **45(2),** 177–211.

7. Grossberg, S. E., Taylor, J. L., and Kushnaryov, V. M. (1989) Interferon receptors and their role in interferon action (review). *Experientia* **45(6),** 508–513.
8. Solinas, A., Cossu, P., Poddighe, P., Tocco, A., Deplano, A., Garrucciu, G., and Diana, M. S. (1993) Changes of serum 2', 5'-oligoadenylate synthetase activity during interferon treatment of chronic hepatitis C. *Liver* **13(5),** 253–258.
9. Horisberger, M. A., Staeheli, P., and Haller, O. (1983) Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. *Proc.Natl. Acad. Sci. USA* **80(1),** 1910–1914.
10. Hoofnagle, J. H. (1994) Therapy of acute and chronic viral hepatitis (review). *Adv. Int. Med.* **39,** 241–275.
11. Perrillo, R. P., Schiff, E. R., Davis, G. L., Bodenheimer, H. C., Jr., Lindsay, K., Payne, J., Dienstag, J. L., O'Brien, C., Tamburro, C., Jacobson, I. M., et al. (1990) A randomized, controlled trial of interferon alpha–2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. The Hepatitis Interventional Therapy Group (see comments). *N. Engl. J. Med.* **323(5),** 295–301.
12. Furuta, M., Akashi, K., Nakamura, Y., Matsumoto, K., Yamaguchi, H., Takamatsu, S., and Shimizu, T. (1987) 2', 5'-Oligoadenylate synthetase activity in peripheral blood lymphocytes as a clinical marker in interferon therapy for chronic hepatitis B. *J. Interferon Res.* **7(1),** 111–119.
13. Perez, V., Tanno, H., Villamil, F., and Fay, O. (1990) Recombinant interferon alpha-2b following prednisone withdrawal in the treatment of chronic type B hepatitis. *J. Hepatol.* **11**(Suppl. 1), S113–S117.
14. Renault, P. F. and Hoofnagle, J. H. (1989) Side effects of alpha interferon (review). *Sem. Liver Dis.* **9(4),** 273–277.
15. Renault, P. F., Hoofnagle, J. H., Park, Y., Mullen, K. D., Peters, M., Jones, D. B., Rustgi, V., Jones, E. A. (1987) Psychiatric complications of long-term interferon alfa therapy. *Arch. Int. Med.* **147(9),** 1577–1580.
16. McLaughlin, P., Talpaz, M., Quesada, J. R., Saleem, A., Barlogie, B., Gutterman, J. U. (1985) Immune thrombocytopenia following alpha-interferon therapy in patients with cancer. *JAMA* **254(10),** 1353–1354.
17. Hoofnagle, J. H. (1991) Thrombocytopenia during interferon alfa therapy. *JAMA* **266(6),** 849.
18. Vento, S., Di Perri, G., Garofano, T., Cosco, L., Concia, E., Ferraro, T., Bassetti, D. (1989) Hazards of interferon therapy for HBV-seronegative chronic hepatitis. *Lancet* **2(8668),** 926.
19. Cianciara, J., Laskus, T. (1995) Development of transient autoimmune hepatitis during interferon treatment of chronic hepatitis B. *Dig. Dis. Sci.* **40(8),** 1842–1844.
20. Mayet, W. J., Hess, G., Gerken, G., Rossol, S., Voth, R., Manns, M., and Meyer zum Buschenfelde, K. H. (1989) Treatment of chronic type B hepatitis with recombinant alpha-interferon induces autoantibodies not specific for autoimmune chronic hepatitis. *Hepatology* **10(1),** 24–28.
21. Lisker-Melman, M., Di Bisceglie, A. M., Usala, S. J., Weintraub, B., Murray, L. M., Hoofnagle, J. H. (1992) Development of thyroid disease during therapy of chronic viral hepatitis. *Gastroenterology* **102(6),** 2155–2160.
22. Waguri, M., Hanafusa, T., Itoh, N., Imagawa, A., Miyagawa, J., Kawata, S., Kono, N., Kuwajima, M., Matsuzawa, Y. (1994) Occurrence of IDDM during interferon therapy for chronic viral hepatitis. *Diabetes Res. Clin. Practice* **23(1),** 33–36.

23. Fabris, P., Betterle, C., Floreani, A., Greggio, N. A., de Lazzari, F., Naccarato, R., Chiaramonte, M. (1992) Development of type 1 diabetes mellitus during interferon alfa therapy for chronic HCV hepatitis. *Lancet* **340(8818),** 548.
24. Lopes, E. P., Oliveira, P. M., Silva, A. E., Ferraz, M. L., Costa, C. H., Miranda, W., Dib, S. A. (1994) Exacerbation of type 2 diabetes mellitus during interferon-alfa therapy for chronic hepatitis B (letter). *Lancet* **343(8891),** 244.
25. Fornaciari, G., Bassi, C., Beltrami, M., Castagnetti, E., Maccari, S., Plancher, A. C. (1991) Hemolytic anemia secondary to interferon treatment for chronic B hepatitis (letter). *J. Clin. Gastroenterol.* **13(5),** 596–597.
26. Akard, L. P., Hoffman, R., Elias, L., Saiers, J. H. (1986) Alpha-interferon and immune hemolytic anemia (letter). *Ann. Int. Med.* **105(2),** 306.
27. Yoshida, E. M., Rock, N. R., Zeng, L., Haniak, W. A., Anderson, F. H. (1995) The use of interferon-alpha 2B in the treatment of chronic viral hepatitis in patients with preexisting idiopathic thrombocytopenic purpura (letter; comment). *Amer. J. Gastroenterol.* **90(5),** 854–855.
28. Hayasaka, S., Fujii, M., Yamamoto, Y., Noda, S., Kurome, H., and Sasaki, M. (1995) Retinopathy and subconjunctival haemorrhage in patients with chronic viral hepatitis receiving interferon alfa. *Br. J. Ophthalmol.* **79(2),** 150–152.
29. Miranda-Guardiola, F., Fernandez-Llama, P., Badia, J. R., Botey, A., Estruch, R., Darnell, A., Rozman, C., and Revert, L. (1995) Acute renal failure associated with alpha-interferon therapy for chronic hepatitis B. *Nephrol. Dial. Transplant.* **10(8),** 1441–1443.
30. Kanda, Y., Shigeno, K., Kinoshita, N., Nakao, K., Yano, M., and Matsuo, H. (1994) Sudden hearing loss associated with interferon. *Lancet* **343(8906),** 1134–1135.
31. Wong, D. K., Cheung, A. M., O'Rourke, K., Naylor, C. D., and Detsky, A. S. (1993) Effect of alpha-interferon treatment in patients with hepatitis B e. *Ann. Int. Med.* **119(4),** 312–323.
32. Carreno, V., Castillo, I., Molina, J., Porres, J. C., and Bartolome (1992) Long-term follow-up of hepatitis B chronic carriers who responded to interferon therapy. *J. Hepatol.* **15(1–2),** 102–106.
33. Niederau, C., Heintges, T., Lange, S., Goldmann, G., Niederau, C. M., Mohr, L., and Haussinger, D. (1996) Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B (see comments). *N. Engl. J. Med.* **334(22),** 1422–1427.
34. Korenman, J., Baker, B., Waggoner, J., Everhart, J. E., Di Bisceglie, A. M., and Hoofnagle, J. H. (1991) Long-term remission of chronic hepatitis B after alpha-interferon therapy. *Ann. Int. Med.* **114(8),** 629–634.
35. Hope, R. L., Weltman, M., Dingley, J., Fiatarone, J., Hope, A. H., Craig, P. I., Grierson, J. M., Bilous, M., Williams, S. J., and Farrell, G. C. (1995) Interferon alfa for chronic active hepatitis B. Long term follow-up of 62 patients: outcomes and predictors of response. *Med. J. Australia* **162(1),** 8–11.
36. Fong, T. L., Di Bisceglie, A. M., Gerber, M. A., Waggoner, J. G, Hoofnagle, J. H. (1993) Persistence of hepatitis B virus DNA in the liver after loss of HBsAg in chronic hepatitis B. *Hepatology* **18(6),** 1313–1318.
37. Perrillo, R. P. and Brunt, E. M. (1991) Hepatic histologic and immunohistochemical changes in chronic hepatitis B. *Ann. Int. Med.* **115(2),** 113–115.
38. Krogsgaard, K., Bindslev, N., Christensen, E., Craxi, A., Schlichting, P., Schalm, S., Carreno, V., Trepo, C., Gerken, G., Thomas, H. C., et al. (1994) The treatment effect

of alpha interferon in chronic hepatitis B is independent of pre-treatment variables. Results based on individual patient data from 10 clinical controlled trials. European Concerted Action on Viral Hepatitis (Eurohep). *J. Hepatol.* **21(4),** 646–655.
39. Lai, C. L., Lok, A. S., Lin, H. J., Wu, P. C., Yeoh, E. K., and Yeung, C. Y. (1987) Placebo-controlled trial of recombinant alpha 2-interferon in Chinese HBsAg-carrier children. *Lancet* **2(8564),** 877–880.
40. Lok, A. S. (1993) Antiviral therapy of the Asian patient with chronic hepatitis B (review). *Sem. Liver Dis.* **13(4),** 360–366.
41. Perrillo, R. P. (1995) Chronic hepatitis B: problem patients (including patients with decompensated disease) (review). *J. Hepatol.* **22**(Suppl. 1), 45–48.
42. Wright, H. I., Gavaler, J. S., and Van Thiel, D. H. (1992) Preliminary experience with alpha-2b-interferon therapy of viral hepatitis in liver allograft recipients. *Transplantation* **53,** 121–124.
43. Tur-Kaspa, R., Burk, R. D., Shaul, Y., Shafritz, D. A. . (1986) Hepatitis B virus DNA contains a glucocorticoid-responsive element. *Proc. Natl. Acad. Sci. USA* **83(6),** 1627–1631.
44. Hirai, N., Shimizu, M., Morioka, T., Hinoue, Y., Tanaka, N., Kobayashi, K., Hattori, N., and Hashimoto, T. (1988) Activities of the interferon system in patients with HBsAg-positive chronic hepatitis B during short-term steroid withdrawal therapy. *Liver* **8(3),** 138–145.
45. Niederau, C., Heintges, T., Niederau, M., Stremmel, W., and Strohmeyer, G. (1992) Prospective randomized controlled trial of sequential treatment with corticoids and alpha-interferon versus treatment with interferon alone in patients with chronic active hepatitis B. *Eur. J. Med.* **1(7),** 396–402.
46. Cohard, M., Poynard, T., Mathurin, P., and Zarski, J. P. (1994) Prednisone-interferon combination in the treatment of chronic hepatitis B: direct and indirect metanalysis. *Hepatology* **20(6),** 1390–1398.
47. Liaw, Y. F., Lin, S. M., Chen, T. J., Chien, R. N., Sheen, I. S., and Chu, C. M. (1994) Beneficial effect of prednisolone withdrawal followed by human lymphoblastoid interferon on the treatment of chronic type B hepatitis in Asians: a randomized controlled trial. *J. Hepatol.* **20(2),** 175–180.
48. Perrillo, R. P., and Regenstein, F. G. (1986) Corticosteroid therapy for chronic active hepatitis B: is a little to much? *Hepatology* **6(6),** 1416–1418.
49. Brunetto, M. R., Giarin, M., Oliveri, F., Saracco, G., and Barbera, C. (1991) "e" antigen defective hepatitis B virus and course of chronic infection. *J. Hepatol.* **13**(Suppl. 4), S82–86.
50. Fattovich, G., Farci, P., Rugge, M., Brollo, L., Mandas, A., Pontisso, P., Giustina, G., Lai, M. E., Belussi, F., Busatto, G., et al. (1992) A randomized controlled trial of lymphoblastoid interferon-alpha in patients with chronic hepatitis B lacking HBeAg. *Hepatology* **15(4),** 584–589.
51. Andreone, P., Cursaro, C., Gramenzi, A., Zavaglio, C., Rezakovic, I., Altomare, E., Severini, R., Franzone, J. S., Albano, O., Ideo, G., Bernardi, M., and Gasbarrini, G. (1996) A randomized controlled trial of thymosin-α1 versus interferon alfa treatment in patients with hepatitis B e antigen antibody—and hepatitis B virus DNA-positive chronic hepatitis B. *Hepatology* **24,** 774–777.
52. Lok, A. S., Wu, P. C., Lai, C. L., Lau, J. Y., Leung, E. K., Wong, L. S., Ma, O. C., Lauder, I. J., Ng, C. P., and Chung, H. T. (1992) A controlled trial of interferon with or without prednisone priming for chronic hepatitis B. *Gastroenterology* **102(6),** 2091–2097.

53. Lok, A. S., Chung, H. T., Liu, V. W., and Ma, O. C. (1993) Long-term follow-up of chronic hepatitis B patients treated with interferon alfa. *Gastroenterology* **105(6),** 1833–1838.
54. Tong, M. J., and Hwang, S. J. (1994) Hepatitis B virus infection in Asian Americans (review). *Gastroenterol. Clin. N. Amer.* **23(3),** 523–536.
55. Utili, R., Sagnelli, E., Gaeta, G. B., Galanti, B., Nardiello, S., Felaco, F. M., Pasquale, G., Marrone, A., Aprea, L., Pizzella, T., et al. (1994) Treatment of chronic hepatitis B in children with prednisone followed by alfa-interferon: a controlled randomized study. *J. Hepatol.* **20(2),** 163–167.
56. Bruguera, M., Amat, L., Garcia, O., Lambruschini, N., Carnicer, J., Bergada, A., Martin Orte, E., Costa, J., Sanchez-Tapias, J. M., and Rodes, J. (1993) Treatment of chronic hepatitis B in children with recombinant alfa interferon. Different response according to age at infection. *J. Clin. Gastroenterol.* **17(4),** 296–299.
57. Ruiz-Moreno, M., Camps, T., Jimenez, J., Lopez, R., Castillo, I., Bartolome, J., and Carreno, V. (1995) Factors predictive of response to interferon therapy in children with chronic hepatitis B. *J. Hepatol.* **22(5),** 540–544.
58. Barbera, C., Bortolotti, F., Crivellaro, C., Coscia, A., Zancan, L., Cadrobbi, P., Nebbia, G., Pillan, M. N., Lepore, L., Parrella, T., et al. (1994) Recombinant interferon-alpha 2a hastens the rate of HBeAg clearance in children with chronic hepatitis B. *Hepatology* **20(2),** 287–290.
59. Sokal, E. M., Wirth, S., Goyens, P., Depreterre, A., and Cornu, C. (1993) Interferon alpha–2b therapy in children with chronic hepatitis B. *Gut* **34**(2 Suppl), S87-S90.
60. Cullu, F., Tumay, G. T., Kutlu, T., Erkan, T., Ozbay, G., and Badur, S. (1995) (Treatment of chronic viral hepatitis B in children with moderate doses of alpha interferon). (Review) (French). *Gastroenterol. Clin. Biol.* **19(1),** 53–57.
61. Anonymous (1990) Protection against viral hepatitis. Recommendations of the Immunization Practices Advisory Committee (ACIP). *MMWR* **39(RR–2),** 1–26.
62. Stevens, C. E., Taylor, P. E., Tong, M. J., Toy, P. T., Vyas, G. N., and Nair (1987) Yeast-recombinant hepatitis B vaccine. Efficacy with hepatitis B immune globulin in prevention of perinatal hepatitis B virus transmission. *JAMA* **257(19),** 2612–2616.
63. Wong, D. K., Yim, C., Naylor, C. D., Chen, E., Sherman, M., Vas, S., Wanless, I. R., Read, S., Li, H., and Heathcote, E. J. (1995) Interferon alfa treatment of chronic hepatitis B: randomized trial in a predominantly homosexual male population. *Gastroenterology* **108(1),** 165–171.
64. Brook, M. G., McDonald, J. A., Karayiannis, P., Caruso, L., Forster, G., Harris, J. R., and Thomas, H. C. (1989) Randomised controlled trial of interferon alfa 2A (rbe) (Roferon-A) for the treatment of chronic hepatitis B virus (HBV) infection: factors that influence response. *Gut* **30(8),** 1116–1122.
65. Hoofnagle, J. H., Peters, M., Mullen, K. D., Jones, D. B., Rustgi, V., Di Bisceglie, A., Hallahan, C., Park, Y., Meschievitz, C., and Jones, E. A. (1988) Randomized, controlled trial of recombinant human alpha-interferon in patients with chronic hepatitis B. *Gastroenterology* **95(5),** 1318–1325.
66. Hoofnagle, J. H., Di Bisceglie, A. M., Waggoner, J. G., and Park, Y. (1993) Interferon alfa for patients with clinically apparent cirrhosis due to chronic hepatitis B. *Gastroenterology* **104(4),** 1116–1121.
67. Perrillo, R., Tamburro, C., Regenstein, F., Balart, L., Bodenheimer, H., Silva, M., Schiff, E., Bodicky, C., Miller, B., Denham, C., et al. (1995) Low-dose, titratable

interferon alfa in decompensated liver disease caused by chronic infection with hepatitis B virus. *Gastroenterology* **109(3),** 908–916.
68. Nevens, F., Goubau, P., Van Eyken, P., Desmyter, J., Desmet, V., and Fevery, J. (1993) Treatment of decompensated viral hepatitis B-induced cirrhosis with low doses of interferon alpha. *Liver* **13(1),** 15–19.
69. Di Bisceglie, A. M. (1995) Long-term outcome of interferon-alpha therapy for chronic hepatitis B (review). *J. Hepatol.* **22**(Suppl. 1), 65–67.
70. Tassopoulos, N. C., Hadziyannis, S. J., and Wright, G. E. (1989) Recombinant human interferon alpha-2b in the management of acute type B hepatitis. *Hepatology* **10,** 576A.
71. Sanchez-Tapias, J. M., Mas, A., and Costa, J. (1987) Recombinant alpha 2c-interferon therapy in fulminant viral hepatitis. *J. Hepatol.* **5(2),** 205–210.
72. Liaw, Y. F. (1995) Role of hepatitis C virus in dual and triple hepatitis virus infection (review). *Hepatology* **22**(4 Pt. 1), 1101–1108.
73. Weltman, M. D., Brotodihardjo, A., Crewe, E. B., Farrell, G. C., Bilous, M., Grierson, J. M., and Liddle, C. (1995) Coinfection with hepatitis B and C or B, C and delta viruses results in severe chronic liver disease and responds poorly to interferon-alpha treatment. *J. Viral Hep.* **2(1),** 39–45.
74. Poniachik, J., Reddy, R., Jeffers, L., and Bernstein, D., Jonnalagadda, S., De Medina, M., O'Sullivan, H., Li, X. M., and Schiff, E. R. (1995) Quantitative hepatitis B virus DNA and hepatitis C virus RNA levels in co-infected patients. *Hepatology* **22** (4 Part 2), 266.
75. Martin, P. (1994) Hepatitis C infection in patients with chronic hepatitis B: curiosity or cofactor? *Amer. J. Gastroenterol.* **89(8),** 1139–1141.
76. Lai, K. N., Li, P. K., Lui, S. F., Au, T. C., Tam, J. S., Tong, K. L., and Lai, F. M. (1991) Membranous nephropathy related to hepatitis B virus in adults. *N. Engl. J. Med.* **324(21),** 1457–1463.
77. Conjeevaram, H. S., Hoofnagle, J. H., Austin, H. A., Park, Y., Fried, M. W., and Di Bisceglie, A. M. (1995) Long-term outcome of hepatitis B virus-related glomerulonephritis after therapy with interferon alfa. *Gastroenterology* **109(2),** 540–546.
78. Lin, C. Y. (1995) Treatment of hepatitis B virus-associated membranous nephropathy with recombinant alpha-interferon. *Kidney Intern.* **47(1),** 225–230.
79. Scully, L. J., Karayiannis, P., and Thomas, H. C. (1992) Interferon therapy is effective in treatment of hepatitis B-induced polyarthritis. *Dig. Dis. Sci.* **37(11),** 1757–1760.
80. Lohr, H., Goergen, B., Weber, W., Godderz, W., Meyer zum Buschenfelde, K. H., and Gerken, G. (1994) Mixed cryoglobulinemia type II in chronic hepatitis B associated with HBe-minus HBV mutant: cellular immune reactions and response to interferon treatment. *J. Med. Virol.* **44(4),** 330–335.
81. Bonkovsky, H. L., Liang, T. J., Hasegawa, K., and Banner, B. (1995) Chronic leukocytoclastic vasculitis complicating HBV infection. Possible role of mutant forms of HBV in pathogenesis and persistence of disease. *J. Clin. Gastroenterol.* **21(1),** 42–47.
82. Guillevin, L., Lhote, F., Leon, A., Fauvelle, F., Vivitski, L., and Trepo, C. (1993) Treatment of polyarteritis nodosa related to hepatitis B virus with short term steroid therapy associated with antiviral agents and plasma exchanges. A prospective trial in 33 patients. *J. Rheumatol.* **20(2),** 289–298.
83. Mouthon, L., Deblois, P., Sauvaget, F., Meyrier, A., Callard, P., and Guillevin, L. (1995) Hepatitis B virus-related polyarteritis nodosa and membranous nephropathy. *Amer. J. Nephrol.* **15(3),** 266–269.

84. Wong, S. N., Yu, E. C., and Chan, K. W. (1993) Hepatitis B virus associated membranous glomerulonephritis in children—experience in Hong Kong. *Clin. Nephrol.* **40(3),** 142–147.
85. Wong, J. B., Koff, R. S., Tine, F., and Pauker, S. G. (1995) Cost-effectiveness of interferon-alpha 2b treatment for hepatitis B e antigen-positive chronic hepatitis B. (See comments) *Ann. Int. Med.* **122(9),** 664–675.
86. Dusheiko, G. M., and Roberts, J. A. (1995) Treatment of chronic type B and C hepatitis with interferon alfa: an economic appraisal. (See comments) *Hepatology* **22(6),** 1863–1873.
87. Koretz, R. L., and Ofman, J. (1996) Interferoning with natural history: dollars and sense. *Gastroenterology* **110(1),** 313–315.
88. Koff, R. S., and Seeff, L. B. (1995) Economic modeling of treatment in chronic hepatitis B and chronic hepatitis C: promises and limitations. (Editorial; comment) *Hepatology* **22(6),** 1880–1882.
89. Liao, M. J., Lee, N., Dipaola, M., Hussain, M., Brissette, R., Ni, D., Smith, T., Desai, M., Ferencz-Biro, K., and Testa, D. (1994) Distribution of interferon-alpha 2 genes in humans. *J. Interferon Res.* **14(4),** 183–185.
90. Antonelli, G., Giannelli, G., Pistello, M., Maggi, F., Vatteroni, L., Currenti, M., Del Vecchio, S., Roffi , L., Pastore, G., and Dianzani, F. (1994) Clinical significance of recombinant interferon-alpha 2 neutralizing antibodies in hepatitis patients. *J. Interferon Res.* **14(4),** 211–213.
91. Capalbo, M., Palmisano, L., Bonino, F., Pellas, C., and Maset, J. (1994) Intramuscular natural beta interferon in the treatment of chronic hepatitis B: a multicentre trial. Italian Hepatitis B Study Group. *Ital. J. Gastroenterol.* **26(5),** 238–241.
92. Kagawa, T., Morizane, T., Saito, H., Tsunematsu, S., Tada, S., Kumagai, N., Tsuchimoto, K., Sugiura, H., Mukai, M., and Tsuchiya, M. (1993) A pilot study of long-term weekly interferon-beta administration for chronic hepatitis B. (See comments) *Amer. J. Gastroenterol.* **88(2),** 212–216.
93. Lau, J. Y., Lai, C. L., Wu, P. C., Chung, H. T., Lok, A. S., and Lin, H. J. (1991) A randomised controlled trial of recombinant interferon-gamma in Chinese patients with chronic hepatitis B virus infection. *J. Med. Virol.* **34(3),** 184–187.
94. Ruiz-Moreno, M., Rua, M. J., Moraleda, G., Guardia, L., Moreno, A., and Carreno, V. (1992) Treatment with interferon gamma versus interferons alfa and gamma in children with chronic hepatitis B. *Pediatrics* **90**(2 Pt 1), 254–258.
95. Kakumu, S., Ishikawa, T., Mizokami, M., Orido, E., Yoshioka, K., Wakita, T., and Yamamoto, M. (1991) Treatment with human gamma interferon of chronic hepatitis B: comparative study with alpha interferon. *J. Med. Virol.* **35(1),** 32–37.
96. Di Bisceglie, A. M., Rustgi, V. K., Kassianides, C., Lisker-Melman, M., Park, Y., Waggoner, J. G., and Hoofnagle, J. H. (1990) Therapy of chronic hepatitis B with recombinant human alpha and gamma interferon. *Hepatology* **11(2),** 266–270.
97. Pardoll, D. M. (1995) Paracrine cytokine adjuvants in cancer immunotherapy (review). *Annu. Rev. Immunol.* **13,** 399–415.
98. Martin, J., Bosch, O., Moraleda, G., Bartolome, J., Quiroga, J. A., and Carreno, V. (1993) Pilot study of recombinant human granulocyte-macrophage colony-stimulating factor in the treatment of chronic hepatitis B. *Hepatology* **18(4),** 775–780.
99. Silva, M., Perez, V., Manero, E., Escoms, S., Tanno, H., Bariletto, S., and Albrecht, J. (1994) GM-CSF plus interferon in chronic hepatitis B patients who failed previous interferon therapy. *Hepatology* **20** (4 Part 2), 300.

100. Abbas, A. K., Lichtman, A. H., and Pober, J. S. (1991) *Cellular and Molecular Immunology*, W. B. Saunders, Philadelphia, PA.
101. Onji, M., Kondoh, H., Horiike, N., Yamaguchi, S., Ogawa, Y., Kumon, I., and Ohta, Y. (1987) Effect of recombinant interleukin 2 on hepatitis B e antigen positive chronic hepatitis. *Gut* **28(12),** 1648–1652.
102. Nishioka, M., Kagawa, H., Shirai, M., Terada, S., and Watanabe, S. (1987) Effects of human recombinant interleukin 2 in patients with chronic hepatitis B: a preliminary report. *Amer. J. Gastroenterol.* **82(5),** 438–442.
103. Bruch, H. R., Korn, A., Klein, H., Markus, R., Malmus, K., Baumgarten, R., and Muller, R. (1993) Treatment of chronic hepatitis B with interferon alpha-2b and interleukin-2. *J. Hepatol.* **17(Suppl. 3),** S52–S55.
104. Tilg, H., Vogel, W., Tratkiewicz, J., Aulitzky, W. E., Herold, M., Gruber, M., Geissler, D., Umlauft, F., Judmaier, G., Schwulera, U., et al. (1993) Pilot study of natural human interleukin-2 in patients with chronic hepatitis B. *J. Hepatol.* **19(2),** 259–267.
105. Trinchieri, G. (1995) Interleukin–12, a proinflammatory cytokine with immunoregulatory functions. *Annu. Rev. Immunol.* **13,** 251–276.
106. Rasi, G., Mutchnik, M. G., DiVirgilio, D., Sinibaldi-Vallebona, P., Pierimarchi, P., Colella, F., Favalli, C., and Garaci, E. (1996) Combination low-dose lymphoblastoid interferon and thymosin-a1 therapy in the treatment of chronic hepatitis B. *J. Viral Hepatitis* **3,** 191–196.
107. Gish, R. G., and Keeffe, E. B. (1995) Recent developments in the treatment of chronic hepatitis B virus infection. *Exp. Opin. Invest. Drugs* **4(2),** 95–115.
108. O'Brien, C. B., Garcia, G., Henzel, B., Martin, M., Ladenheim, J., Ginsberg, G., Hubbell, H., Strayer, D., and Carter, W. (1994) Ampligen treatment of chronic hepatitis B. *Gastroenterology* **106**(4 Suppl.), 952 .
109. Renoux, G. (1980) The general immunopharmacology of levamisole (review). *Drugs* **20(2),** 89–99.
110. Taki, H. N., and Schwartz, S. A. (1994) Levamisole as an immunopotentiator for T cell deficiency. *Immunopharmacol. Immunotoxicol.* **16(2),** 129–137.
111. Ruiz-Moreno, M., Garcia, R., Rua, M. J., Serrano, B., Moraleda, G., Feijoo, E., Bartolome, J., Ortiz, F., Castillo, I., and Carreno, V. (1993) Levamisole and interferon in children with chronic hepatitis B. *Hepatology* **18(2),** 264–269.
112. Fattovich, G., Giustina, G., Brollo, L., Guido, M., Pontisso, P., Noventa, F., and Alberti, A. (1992) Therapy for chronic hepatitis B with lymphoblastoid interferon-alpha and levamisole. *Hepatology* **16(5),** 1115–1119.
113. Bosch, O., Moraleda, G., Castillo, I., and Carreno, V. (1993) Treatment of chronic hepatitis B with recombinant interferon alpha versus recombinant interferon alpha plus levamisole. *J. Hepatol.* **19(3),** 437–441.
114. Thyagarajan, S. P., Subramanian, S., and Thirunalasundari, T. (1988) Effect of Phyllanthus amarus on chronic carriers of hepatitis B virus. *Lancet* **2(8614),** 764–766.
115. Thyagarajan, S. P., Jayaram, S., Valliammai, T., and Madanagopalan (1990) Phyllanthus amarus and hepatitis B. (Letter; comment) *Lancet* **336(8720),** 949–950.
116. Wang, M., Cheng, H., Li, Y., Meng, L., Zhao, G., and Mai, K. (1995) Herbs of the genus Phyllanthus in the treatment of chronic hepatitis B: observations with three preparations from different geographic sites. *J. Lab. Clin. Med.* **126(4),** 350–352.
117. Milne, A., Hopkirk, N., Lucas, C. R., Waldon, J., and Foo, Y. (1994) Failure of New Zealand hepatitis B carriers to respond to Phyllanthus amarus. *N. Zealand Med. J.* **107(980),** 243.

118. Doshi, J. C., Vaidya, A. B., Antarkar, D. S., Deolalikar, R., and Antani, D. H. (1994) A two-stage clinical trial of Phyllanthus amarus in hepatitis B carriers: failure to eradicate the surface antigen. *Indian. J. Gastroenterol.* **13(1),** 7–8.
119. Thamlikitkul, V., Wasuwat, S., and Kanchanapee, P. (1991) Efficacy of Phyllanthus amarus for eradication of hepatitis B virus in chronic carriers. *J. Med. Assoc. Thailand* **74(9),** 381–385.
120. Niu, J. Z., Wang, Y. Y., Qiao, M., Gowans, E., and Edwards, P. (1990) Effect of Phyllanthus amarus on duck hepatitis B virus replication in vivo. *J. Med. Virol.* **32(4),** 212–218.
121. Kakumu, S., Yoshioka, K., Wakita, T., Ishikawa, T., Takayanagi, M., and Higashi, Y. (1993) Pilot study of ribavirin and interferon-beta for chronic hepatitis B. *Hepatology* **18(2),** 258–263.
122. Fried, M. W., Fong, T. L., and Swain, M. G. (1992) Hepatitis B virus (HBV) DNA suppression during long-term therapy with ribavirin. *Gastroenterology* **102,** A808.
123. Berk, L., Schalm, S. W., de Man, R. A., Heytink, R. A., Berthelot, P., Brechot, C., Boboc, B., Degos, F., Marcellin, P., Benhamou, J. P., et al. (1992) Failure of acyclovir to enhance the antiviral effect of alpha lymphoblastoid interferon on HBe-seroconversion in chronic hepatitis B. A multi-centre randomized controlled trial. *J. Hepatol.* **14(2–3),** 305–309.
124. Alexander, G. J., Fagan, E. A., Hegarty, J. E., Yeo, J., and Eddleston (1987) Controlled clinical trial of acyclovir in chronic hepatitis B virus. *J. Med. Virol.* **21(1),** 81–87.
125. Janssen, H. L., Berk, L., Heijtink, R. A., ten Kate, F. J., and Schalm, S. W. (1993) Interferon-alpha and zidovudine combination therapy for chronic hepatitis B: results of a randomized, placebo-controlled trial. *Hepatology* **17(3),** 383–388.
126. Chossegros, P., Pouteil-Noble, C., Samuel, D., Causse, X., Ouxan D., Bizollon T., Touraine J. L., and Trepo C. (1993) Ganciclovir is an effective antiviral agent for post-transplant chronic HBV infections; maintenance therapy may be required in some cases. *Gastroenterology* **104**(4 Suppl.), 888.
127. De La Mata, M., Montero, J. L., Fraga, E., Costan, G., Delgado, M., Lopez, P., Gonzalez, R., Pera, C., and Mino, G. (1995) Efficacy of ganciclovir in the treatment of hepatitis B virus infection of the liver graft: Long-term results. *Hepatology* **22** (4 Part 2), 130.
128. McKenzie, R., Fried, M. W., Sallie, R., Conjeevaram, H., Di Bisceglie, A. M., Park, Y., Savarese, B., Kleiner, D., Tsokos, M., Luciano, C., et al. . (1995) Hepatic failure and lactic acidosis due to fialuridine (FIAU), an investigational nucleoside analogue for chronic hepatitis B. (See comments) *N. Engl. J. Med.* **333(17),** 1099–1105.
129. Hoofnagle, J., McKenzie, R., and Straus, S. (1995) Severe toxicity of fialuridine (FIAU) (Letter). *N. Engl. J. Med.* **334,** 1137.
130. Fried, M. W., DiBisceglie, A. M., Straus, S. E., Savarese, B., Beames, M. P., and Hoofnagle, J. H. (1992) FIAU, a new oral anti-viral agent, profoundly inhibits HBV DNA in patients with chronic hepatitis B. *Hepatology* **16,** 127A.
131. Dienstag, J. L., Perrillo, R. P., Schiff, E. R., and Bartholomew, M. (1995) A preliminary trial of lamivudine for chronic hepatitis B infection. *N. Engl. J. Med.* **333(25),** 1657–1661.
132. Lai, C. L., Ching, C. K., Tung, A., Hill, A. M., Wong, B. C. Y., Lim, S. P., Dent, J. C., and Wu, P. C. (1994) Short term lamivudine suppresses HBV DNA in Chinese HBsAg carriers. *Hepatology* **20**(4 Part 2), 298 .

133. Grellier, L., Mutimer, D., Ahmed, M., Brown, D., Burroughs, A. K., Rolles, K., McMaster, P., Beranek, P., Kennedy, F., Kibbler, H., McPhillips, P., Elias, E., and Dusheiko, G. (1996) Lamivudine prophylaxis against reinfection in liver transplantation for hepatitis B cirrhosis. *Lancet* **348,** 1212–1215.
134. Woolf, G. M., Wagner, D. A., Makowka, L., and Vierling, J. M. (1995) Quantitative liver function testing pre-and post-liver transplantation (LT). *Hepatology* **22** (4 Part 2), 510.
135. Gutfreund, K. S., Fischer, K. P., Tipples, G., Ma, M., Bain, V. G., Kneteman, N., Tyrrell, D. L. J. (1995) Lamivudine results in a complete and sustained suppression of hepatitis B virus replication in patients requiring orthotopic liver transplantation for cirrhosis secondary to hepatitis B. *Hepatology* **22** (4 Part 2), 328.
136. Ling, R., Mutimer, D., Ahmed, M., Boxall, E. H., Elias, E., Dusheiko, G. M., and Harrison, T. J. (1996) Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* **24,** 711–713.
137. Tipples, G. A., Ma, M. M., Fischer, K. P., Bain, V. G., Kneteman, N. M., and Tyrrell, D. L. (1996) Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine *in vivo*. *Hepatology* **24,** 714–717.
138. Tisdale, M., Kemp, S. D., Parry, N. R., Larder, B. A. (1993) Rapid *in vitro* selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **90(12),** 5653–5656.
139. Boker, K. H., Ringe, B., Kruger, M., Pichlmayr, R., and Manns, M. P. (1994) Prostaglandin E plus famciclovir—a new concept for the treatment of severe hepatitis B after liver transplantation. *Transplantation* **57(12),** 1706–1708.
140. Tillman, H. L., Trautwein, C. H., Bode, U., Oldhafer, K., Boker, K., Pichlmayr, R., and Manns, M. P. (1994) Treatment of hepatitis B virus reinfection after liver transplantation with famciclovir. *Hepatology* **20,** 130.
141. Main, J., Brown, J. L., Howells, C., Galassini, R., Crossey, M., Karayiannis, P., Georgiou, P., Atkinson, G., and Thomas, H. C. (1996) A double blind, placebo-controlled study to assess the effect of famciclovir on virus replication in patients with chronic hepatitis B virus infection. *J. Viral. Hep.* **3,** 211–215.
142. Zoulim, F., Dannaoui, E., and Trepo, C. (1995) Inhibitory effect of penciclovir, the active metabolite of famciclovir on the priming of hepadnavirus reverse transcriptase. *Hepatology* **22,** 329A.
143. Vitiello, A., Ishioka, G., Grey, H. M., Rose, R., Farness, P., LaFond, R., Yuan, L., Chisari, F. V., Furze, J., Bartholomeuz, R., et al. (1995) Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. I. Induction of a primary cytotoxic T lymphocyte response in humans. *J. Clin. Invest.* **95(1),** 341–349.
144. Madon, J., and Blum, H. E. (1995) Adenovirus-associated receptor-mediated delivery of hepatitis B virus DNA and oligonucleotides to liver cells. *Hepatology* **22,** 263A.
145. Nakazono, K., Wu, C. H., and Wu, G. Y. (1994) Targeted administration of antisense DNA inhibits subsequent HBV transfection in vitro. *Hepatology* **20,** 203A.
146. Nunes, F. A. and Raper, S. E. (1996) Liver-directed gene therapy (review). *Med. Clin. N. Amer.* **80(5),** 1201–1213.
147. Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244(4902),** 359–362.
148. Alter, M. J., and Sampliner, R. E. (1989) Hepatitis C: and miles to go before we sleep. (Editorial) *N. Engl. J. Med.* **321(22),** 1538–1540.

149. Zeldis, J. B., and Jensen, P. (1994) Hepatitis C virus pathogenicity: the corner pieces of the jigsaw puzzle are found. (Editorial; comment) *Gastroenterology* **106(4),** 1118–1120.
150. Hoofnagle, J. H., Mullen, K. D., Jones, D. B., Rustgi, V., Di Bisceglie, A., Peters, M., Waggoner, J. G., Park, Y., and Jones, E. A. (1986) Treatment of chronic non-A, non-B hepatitis with recombinant human alpha interferon. A preliminary report. *N. Engl. J. Med.* **315(25),** 1575–1578.
151. Shindo, M., Di Bisceglie, A. M., and Hoofnagle, J. H. (1992) Long-term follow-up of patients with chronic hepatitis C treated with alpha-interferon. *Hepatology* **15(6),** 1013–1016.
152. Tine, F., Magrin, S., Craxi, A., and Pagliaro, L. (1991) Interferon for non-A, non-B chronic hepatitis. A meta-analysis of randomised clinical trials. *J. Hepatol.* **13(2),** 192–199.
153. Romeo, R., Stanislas, P., Berthelot, P., and Brechot, C. (1994) Eradication of hepatitis C virus RNA after alpha-interferon therapy. *Ann. Int. Med.* **121,** 276–277.
154. Davis, G. L., and Lau, J. Y. (1995) Choice of appropriate end points of response to interferon therapy in chronic hepatitis C virus infection (review). *J. Hepatol.* **22**(1 Suppl), 110–114.
155. Aiyama, T., Yoshioka, K., Hirofuji, H., Kusakabe, A., Yamada, M., Tanaka, K., and Kakumu, S. (1994) Changes in serum hepatitis C virus RNA titer and response to interferon therapy in patients with chronic hepatitis C. *Dig. Dis. Sci.* **39(10),** 2244–2249.
156. Kakumu, S., Yoshika, K., Tanaka, K., Higashi, Y., Kurokawa, S., Hirofuji, H., and Kusakabe, A. (1993) Long-term carriage of hepatitis C virus with normal aminotransferase after interferon treatment in patients with chronic hepatitis C. *J. Med. Virol.* **41(1),** 65–70.
157. Matsushita, E., Unoura, M., and Kobayashi, K. (1994) Psychiatric complications of interferon therapy. *Japan J. Clin. Med.* **52(7),** 1910–1913.
158. Levenson, J. L., and Fallon, H. J. (1993) Fluoxetine treatment of depression caused by interferon-alpha. *Amer. J. Gastroenterol.* **88(5),** 760–761.
159. Okuno, H., Hirota, T., Shiozaki, Y., Inoue, K., Hagihara, M., Sugasawa, K., and Okami, T. (1994) Interferon-associated retinopathy (review). *Jpn. J. Clin. Med.* **52(7),** 1919–1923.
160. Soushi, S., Kobayashi, F., Obazawa, H., Kigasawa, K., Shiraishi, K., Itakura, M., and Matsuzaki, S. (1996) Evaluation of risk factors of interferon-associated retinopathy in patients with type C chronic active hepatitis. *Acta Societatis Ophthalmol. Jpn.* **100(1),** 69–76.
161. Kawano, T., Shigehira, M., Uto, H., Nakama, T., Kato, J., Hayashi, K., Maruyama, T., Kuribayashi, T., Chuman, T., Futami, T., and Tsubouchi, H. (1996) Retinal complications during interferon therapy for chronic hepatitis C. *Amer. J. Gastroenterol.* **91(2),** 309–913.
162. Chuman, T., Nao-i, N., Sawada, A., Kawano, T., and Shigehira, M. (1994) Interferon-induced retinal changes. (Japanese). *Acta Societatis Ophthalmol. Jpn.* **98(6),** 616–621.
163. Abe, T., Nakajima, A., Satoh, N., Koizumi, T., Sakuragi, S., Fujimori, K., Ono, T., Kuramitsu, T., Komatsu, M., and Masamune, O. (1995) Incidence of eye lesions in patients with chronic hepatitis C. *Rinsho Ganka* **49(3),** 391–394.
164. Poynard, T., Leroy, V., Cohard, M., Thevenot, T., Mathurin, P., Opolon, P., and Zarski, J. (1996) Meta-analysis of interferon randomized trials in the treatment of viral hepatitis C: effects of dose and duration. *Hepatology* **24(4),** 778–789.

165. Matsuda, J., Saitoh, N., Gotoh, M., Gohchi, K., Tsukamoto, M., Syoji, S., Miyake, K., and Yamanaka, M. (1995) High prevalence of anti-phospholipid antibodies and anti-thyroglobulin antibody in patients with hepatitis C virus infection treated with interferon-alpha. *Amer. J. Gastroenterol.* **90(7),** 1138–1141.
166. Baudin, E., Marcellin, P., Pouteau, M., Colas-Linhart, N., Le Floch, J. P., Lemmonier, C., Benhamou, J. P., and Bok, B. (1993) Reversibility of thyroid dysfunction induced by recombinant alpha interferon in chronic hepatitis C. *Clin. Endocrinol.* **39(6),** 657–661.
167. Nagayama, Y., Ohta, K., Tsuruta, M., Takeshita, A., Kimura, H., Hamasaki, K., Ashizawa, K., Nakata, K., Yokoyama, N., and Nagataki, S. (1994) Exacerbation of thyroid autoimmunity by interferon alpha treatment in patients with chronic viral hepatitis: our studies and review of the literature (review). *Endocrin. J.* **41(5),** 565–572.
168. Watanabe, U., Hashimoto, E., Hisamitsu, T., Obata, H., and Hayashi, N. (1994) The risk factor for development of thyroid disease during interferon-alpha therapy for chronic hepatitis C. (See comments) *Amer. J. Gastroenterol.* **89(3),** 399–403.
169. Fried, M. W. and Hoofnagle, J. H. (1995) Therapy of hepatitis C (review). *Sem. Liv. Dis.* **15(1),** 82–91.
170. Lin, R., Schoeman, M. N., Craig, P. I., Bilous, M., Grierson, J., McDonald, J. A., Batey, R. G., and Farrell, G. C. (1991) Can the response to interferon treatment be predicted in patients with chronic active hepatitis C? *Austral. N. Zealand J. Med.* **21(3),** 387–392.
171. Pagliaro, L., Craxi, A., Cammaa, C., Tine, F., Di Marco, V., Lo Iacono, O., and Almasio, P. (1994) Interferon-alpha for chronic hepatitis C: an analysis of pretreatment clinical predictors of response. *Hepatology* **19(4),** 820–828.
172. Jouet, P., Roudot-Thoraval, F., Dhumeaux, D., and Metreau, J. M. (1994) Comparative efficacy of interferon alfa in cirrhotic and noncirrhotic patients with non-A, non-B, C hepatitis. *Gastroenterology* **106(3),** 686–690.
173. Yuki, N., Hayashi, N., Kasahara, A., Hagiwara, H., Takehara, T., Oshita, M., Katayama, K., Fusamoto, H., and Kamada, T. (1995) Pretreatment viral load and response to prolonged interferon-alpha course for chronic hepatitis C. *J. Hepatol.* **22(4),** 457–463.
174. Lau, J. Y., Davis, G. L., Kniffen, J., Mizokami, M., Ohno, T., and Diamond, D. A. (1993) Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet* **341,** 1501–1504.
175. Stuyver, L., Wyseur, A., van Arnhem, W., Lunel, F., Laurent-Pui, P., Pawlotsky, J. M., Kleter, B., Bassit, L., Nkengasong, J., van Doorn, L. J., and Maertens, G. (1995) Hepatitis C virus genotyping by means of 5'-UR/core line probe assays and molecular analysis of untypeable samples. *Virus Res.* **(38),** 137–157.
176. Mahaney, K., Tedeschi, V., Maertens, G., Di Bisceglie, A. M., Vergalla, J., Hoofnagle, J. H., and Sallie, R. (1994) Genotypic analysis of hepatitis C virus in American patients. *Hepatology* **20(6),** 1405–1411.
177. Chemello, L., Bonetti, P., Cavalletto, L., Talato, F., Donadon, V., Casarin, P., Belussi, F., Frezza, M., Noventa, F., Pontisso, P., et al. (1995) Randomized trial comparing three different regimens of alpha-2a-interferon in chronic hepatitis C. The Triveneto Viral Hepatitis Group. *Hepatology* **22(3),** 700–706.
178. Marcellin, P., Boyer, N., Pouteau, M., Benhamou, J. P., and Erlinger, S. (1994) Retreatment with interferon-alpha of chronic hepatitis C virus infection (letter; comment). *Lancet* **344(8923),** 690–691.

179. Lebovics, E., Lantin, J., Chaurushia, G., Dworkin, B. M., Casellas, A., and Rosenthal, W. S. (1995) The breakthrough phenomenon during alpha-interferon therapy of chronic hepatitis C: incidence, management, and outcome. *Amer. J. Gastroenterol.* **90(6),** 951–954.
180. Diodati, G., Bonetti, P., Noventa, F., Casarin, C., Rugge, M., Scaccabarozzi, S., Tagger, A., Pollice, L., Tremolada, F., Davite, C., et al. (1994) Treatment of chronic hepatitis C with recombinant human interferon-alpha 2a: results of a randomized controlled clinical trial. *Hepatology* **19(1),** 1–5.
181. Finkelstein, S. D., Sayegh, R., Uchman, S., Christensen, S., and Swalsky, P. (1992) HCV undergoes extensive mutational change in NS5 region in association with relapse-breakthrough following alpha interferon therapy. *Hepatology* **16**(4 Part 2), 132A.
182. Roffi, L., Mels, G. C., Antonelli, G., Bellati, G., Panizzuti, F., Piperno, A., Pozzi, M., Ravizza, D., Angeli, G., Dianzani, F., et al. (1995) Breakthrough during recombinant interferon alfa therapy in patients with chronic hepatitis C virus infection: prevalence, etiology, and management. *Hepatology* **21(3),** 645–649.
183. Antonelli, G., Giannelli, G., Currenti, M., Simeoni, E., Del Vecchio, S., Maggi, F., Pistello, M., Roffi, L., Pastore, G., Chemello, L., and Dianzani, F. (1996) Antibodies to interferon (IFN) in hepatitis C patients relapsing while continuing recombinant IFN-alpha-2 therapy. *Clin. Exp. Immunol.* **104(3),** 384–387.
184. Olaso, V., Siles, M., Cordoba, J., Gisbert, C., Berenguer, M., Benages, R., and Berenguer, J. (1995) (Prevalence and clinical significance of autoantibodies in chronic hepatitis C treated with interferon alfa). (Spanish). *Rev. Esp. Enferm. Dig.* **87(8),** 577–581.
185. Marcellin, P., and Benhamou, J. P. (1994) Treatment of chronic viral hepatitis (review). *Baillieres Clin. Gastroenterol.* **8(2),** 233–253.
186. Poynard, T., Bedossa, P., Chevallier, M., Mathurin, P., Lemonnier, C., Trepo, C., Couzigou, P., Payen, J. L., Sajus, M., Costa, J. M., et al. (1995) A comparison of three interferon alpha-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. *N. Engl. J. Med.* **332(22),** 1457–1462.
187. Mitsui, T., Iwano, K., Masuko, K., Yamazaki, C., Okamoto, H., Tsuda, F., Tanaka, T., and Mishiro, S. (1992) Hepatitis C virus infection in medical personnel after needlestick accident. (See comments) *Hepatology* **16(5),** 1109–1114.
188. Lampertico, P., Rumi, M., Romeo, R., Craxi, A., Soffredini, R., Biassoni, D., and Colombo, M. (1994) A multicenter randomized controlled trial of recombinant interferon-alpha 2b in patients with acute transfusion-associated hepatitis C. *Hepatology* **19(1),** 19–22.
189. Takano, S., Satomura, Y., Omata, M. (1994) Effect of interferon beta on non-A, non-B acute hepatitis: a prospective, randomized, controlled-dose study. Japan Acute Hepatitis Cooperative Study Group. *Gastroenterology* **107,** 805–811.
190. Viladomiu, L., Genesca, J., Esteban, J. I., Allende, H., Gonzalez, A., Lopez-Talavera, J. C., Esteban, R., and Guardia, J. (1992) Interferon-alpha in acute posttransfusion hepatitis C: a randomized, controlled trial. *Hepatology* **15(5),** 767–769.
191. Hwang, S. J., Lee, S. D., Chan, C. Y., Lu, R. H., and Lo, K. J. (1994) A randomized controlled trial of recombinant interferon alpha-2b in the treatment of Chinese patients with acute post-transfusion hepatitis C. *J. Hepatol.* **21(5),** 831–836.
192. Naito, M., Hayashi, N., Hagiwara, H., Hiramatsu, N., Kasahara, A., Fusamoto, H., and Kamada, T. (1994) Serum hepatitis C virus RNA quantity and histological features of hepatitis C virus carriers with persistently normal ALT levels. *Hepatology* **19(4),** 871–875.

193. Coltorti, M., Romano, M., Persico, M., Morisco, F., Tuccillo, C., and Caporaso, N. (1995) Hepatitis C virus RNA in serum and liver histology in asymptomatic anti-HCV positive subjects. *Infection* **23(1),** 33–36.
194. Sangiovanni, A., Spinzi, G. C., Ceriani, R., Prada, A., Bissoli, F., Morales, R., Casiraghi, M. A., Sciariada, L., Colombo, E., Fossati, M., and Minoli, G. (1995) Randomized controlled trial of HCV healthy carriers treatment with interferon (IFN). *Hepatology* **22**(4 Part 2), 290A.
195. Altraif, I. H., Abdulla, A. S., al Sebayel, M. I., Said, R. A., al Suhaibani, M. O., and Jones, A. A. (1995) Hepatitis C associated glomerulonephritis. *Amer. J. Nephrol.* **15(5),** 407–410.
196. Stehman-Breen, C., Alpers, C. E., Couser, W. G., Willson, R., and Johnson, R. J. (1995) Hepatitis C virus associated membranous glomerulonephritis. *Clin. Nephrol.* **44(3),** 141–147.
197. Johnson, R. J., Gretch, D. R., Couser, W. G., Alpers, C. E., Wilson, J., Chung, M., Hart, J., and Wilson, R. (1994) Hepatitis C virus-associated glomerulonephritis. Effect of alpha-interferon therapy. *Kidney Intern.* **46(6),** 1700–1704.
198. Rostaing, L., Izopet, J., Baron, E., Duffaut, M., Puel, J., Durand, D., and Suc, J. M. (1995) Preliminary results of treatment of chronic hepatitis C with recombinant interferon alpha in renal transplant patients. *Nephrol. Dialysis Transplant.* **10(Suppl. 6),** 93–96.
199. Kakimoto, K., Takahara, S., Kokado, Y., Kameoka, H., Ishibashi, M., Okuyama, A., Kyo, M., and Nagano, S. (1994) (A case of allograft rejection induced by the interferon-alpha therapy to hepatitis type C after renal transplantation). (Japanese). *Acta Urol. Japan* **40(6),** 529–532.
200. Takahara, S., Kakimoto, K., Kokado, Y., Kameoka, H., Ishibashi, M., Kawada, S., Kyo, M., Nagano, S., Mikami, O., and Okuyama, A. (1995) Interferon alpha therapy for chronic active hepatitis type C after renal transplantation and allograft rejection. *Intern. Urol. Nephrol.* **27(4),** 479–485.
201. Feray, C., Samuel, D., Gigou, M., Paradis, V., David, M. F., Lemonnier, C., Reynes, M., and Bismuth, H. (1995) An open trial of interferon alfa recombinant for hepatitis C after liver transplantation: antiviral effects and risk of rejection. *Hepatology* **22**(4 Pt 1), 1084–1089.
202. Wright, T. L., Combs, C., Kim, M., Ferrell, L., Bacchetti, P., Ascher, N., Roberts, J., Wilber, J., Sheridan, P., and Urdea, M. (1994) Interferon-alpha therapy for hepatitis C virus infection after liver transplantation. *Hepatology* **20**(4 Pt. 1), 773–779.
203. Gane, E. J., Tibbs, C. J., Ramage, J. K., Portmann, B. C., and Williams, R. (1995) Ribavirin therapy for hepatitis C infection following liver transplantation. *Transpl. Intern.* **8(1),** 61–64.
204. Cattral, M. S., Krajden, M., Wanless, I. R., Rezig, M., Cameron, R., Greig, P. D., Chung, S. W., and Levy, G. A. (1996) A pilot study of ribavirin therapy for recurrent hepatitis C virus infection after liver transplantation. *Transplantation* **61(10),** 1483–1488.
205. Shiell, A., Briggs, A., and Farrell, G. C. (1994) The cost effectiveness of alpha interferon in the treatment of chronic active hepatitis C. (See comments) *Med. J. Australia* **160(5),** 268–272.
206. Nishiguchi, S., Kuroki, T., Nakatani, S., Morimoto, H., Takeda, T., Nakajima, S., Shiomi, S., Seki, S., Kobayashi, K., and Otani, S. (1995) Randomised trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. (See comments) *Lancet* **346(8982),** 1051–1055.

207. Perez, R., Pravia, R., Artimez, M. L., Giganto, F., Rodriguez, M., Lombrana, J. L., and Rodrigo, L. (1995) Clinical efficacy of intramuscular human interferon-beta vs interferon-alpha 2b for the treatment of chronic hepatitis C. *J. Viral Hep.* **2(2),** 103–106.
208. Kobayashi, Y., Watanabe, S., Konishi, M., Yokoi, M., Kakehashi, R., Kaito, M., Kondo, M., Hayashi, Y., Jomori, T., and Suzuki, S. (1993) Quantitation and typing of serum hepatitis C virus RNA in patients with chronic hepatitis C treated with interferon-beta. *Hepatology* **18(6),** 1319–1325.
209. Di Bisceglie, A. M., Shindo, M., Fong, T. L., Fried, M. W., Swain, M. G., Bergasa, N. V., Axiotis, C. A., Waggoner, J. G., Park, Y., and Hoofnagle, J. H. (1992) A pilot study of ribavirin therapy for chronic hepatitis C. *Hepatology* **16(3),** 649–654.
210. Di Bisceglie, A. M., Conjeevaram, H. S., Fried, M. W., Sallie, R., Park, Y., Yurdaydin, C., Swain, M., Kleiner, D. E., Mahaney, K., and Hoofnagle, J. H. (1995) Ribavirin as therapy for chronic hepatitis C: A randomized, double-blind, placebo-controlled trial. *Ann. Int. Med.* **123(12),** 897–903.
211. Brillanti, S., Garson, J., Foli, M., Whitby, K., Deaville, R., Masci, C., Miglioli, M., and Barbara, L. (1994) A pilot study of combination therapy with ribavirin plus interferon alfa for interferon alfa-resistant chronic hepatitis C. *Gastroenterology* **107(3),** 812–807.
212. Schvarcz, R., Yun, Z. B., Sonnerborg, A., and Weiland, O. (1995) Combined treatment with interferon alpha-2b and ribavirin for chronic hepatitis C in patients with a previous non-response or non-sustained response to interferon alone. *J. Med. Virol.* **46(1),** 43–47.
213. Wakita, T., and Wands, J. R. (1994) Specific inhibition of hepatitis C virus expression by antisense oligodeoxynucleotides: in vitro model for selection of target sequence. *J. Biol. Chem.* **269(19),** 14205–14210.
214. Hanecak, R., Brown Driver, V., Fox, M. C., Azad, R. F., Furusako, S., Nozaki, C., Ford, C., Sasmor, H., and Anderson, K. P. (1996) Antisense oligonucleotide inhibition of hepatitis C virus gene expression in transformed hepatocytes. *J. Virol.* **70(8),** 5203–5212.
215. Moradpour, D., Englert, C., Wakita, T., and Wands, J. R. (1996) Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. *Virology* **222(1),** 51–63.
216. Alt, M., Renz, R., Hofschneider, P. H., Paumgartner, G., and Caselmann, W. H. (1995) Specific inhibition of hepatitis C viral gene expression by antisense phosphorothioate oligodeoxynucleotides. *Hepatology* **22(3),** 707–717.
217. Mizutani, T., Kato, N., Hirota, M., Sugiyama, K., Murakami, A., and Shimotohno, K. (1995) Inhibition of hepatitis C virus replication by antisense oligonucleotide in culture cells. *Biochem. Biophys. Res. Commun.* **212(3),** 906–911.
218. Branch, A. D. (1996) A hitchhiker's guide to antisense and nonantisense biochemical pathways. *Hepatology* **24(6),** 1517–1529.
219. Smith, J. P. (1996) Treatment of chronic hepatitis C with amantadine-hydrochloride. *Gastroenterology* **110**(4 Suppl.), 1330 A.
220. Hong, Z., Ferrari, E., Wright Minogue, J., Chase, R., Risano, C., Seelig, G., Lee, C. G., and Kwong, D. (1996) Enzymatic characterization of hepatitis C virus NS3/4A complexes expressed in mammalian cells by using the herpes simplex virus amplicon system. *J. Virol.* **70(7),** 4261–4268.
221. Hahm, B., Han, D. S., Back, S. H., Song, O. K., Cho, M. J., Kim, C. J., Shimotohno, K., and Jang, S. K. (1995) NS3-4A of hepatitis C virus is a chymotrypsin-like protease. *J. Virol.* **69(4),** 2534–2539.

222. Tomei, L., Failla, C., Vitale, R. L., Bianchi, E., and De Francesco, R. (1996) A central hydrophobic domain of the hepatitis C virus NS4A protein is necessary and sufficient for the activation of the NS3 protease. *J. Gen. Virol.* **77(5),** 1065–1070.
223. Sakamoto, N., Wu, C. H., and Wu, G. Y. (1995) Inhibition of hepatitis C virus-directed translation by hammerhead ribozymes in vitro. *Hepatology* **22**(4 Part 2), 330A.
224. Carreno, V., and Quiroga, J. A. (1996) New and forthcoming therapies for viral hepatitis. *J. Hepatol.* **24**(Suppl. 2), 74–76.
225. Monjardino, J. (1996) Replication of hepatitis delta virus. *J. Virol. Hep.* **3,** 163–166.
226. Polish, L. B., Gallagher, M., Fields, H. A., and Hadler, S. C. (1993) Delta hepatitis: molecular biology and clinical and epidemiological features (review). *Clin. Microbiol. Rev.* **6(3),** 211–229.
227. Moestrup, T., Hansson, B. G., Widell, A., and Nordenfelt, E. (1983) Clinical aspects of delta infection. *Br. Med. J. Clin. Res. Ed.* **286(6359),** 87–90.
228. Caredda, F., Antinori, S., Re, T., Pastecchia, C., and Moroni, M. (1987) Course and prognosis of acute HDV hepatitis. *Prog. Clin. Biol. Res.* **234,** 267–276.
229. Rizzetto, M., Verme, G., Recchia, S., Bonino, F., Farci, P., Arico, S., Calzia , R., Picciotto, A., Colombo, M., and Popper, H. (1983) Chronic hepatitis in carriers of hepatitis B surface antigen, with intrahepatic expression of the delta antigen. An active and progressive disease unresponsive to immunosuppressive treatment. *Ann. Int. Med.* **98(4),** 437–441.
230. Fattovich, G., Boscaro, S., Noventa, F., Pornaro, E., Stenico, D., Alberti, A., Ruol, A., and Realdi, G. (1987) Influence of hepatitis delta virus infection on progression to cirrhosis in chronic hepatitis type B. *J. Infect. Dis.* **155(5),** 931–935.
231. Sagnelli, E., Felaco, F. M., Filippini, P., Pasquale, G., Peinetti, P., Buonagurio, E., Aprea, L., Pulella, C., Piccinino, F., Giusti, G. (1989) Influence of HDV infection on clinical, biochemical and histological presentation of HBsAg positive chronic hepatitis. *Liver* **9(4),** 229–234.
232. Farci, P., Mandas, A., Coiana, A., Lai, M. E., Desmet, V., Van Eyken, P., Gibo, Y., Caruso, L., Scaccabarozzi, S., Criscuolo, D., et al. (1994) Treatment of chronic hepatitis D with interferon alfa-2a. (See comments) *N. Engl. J. Med.* **330(2),** 88–94.
233. Hadziyannis, S. J. (1991) Use of alpha-interferon in the treatment of chronic delta hepatitis. *J. Hepatol.* **13(Suppl. 1),** S21–S26.
234. Gaudin, J. L., Faure, P., Godinot, H., Gerard, F., and Trepo, C. (1995) The French experience of treatment of chronic type D hepatitis with a 12–month course of interferon alpha–2b. Results of a randomized controlled trial. *Liver* **15(1),** 45–52.
235. Battegay, M., Simpson, L. H., Hoofnagle, J. H., Sallie, R., and Di Bisceglie, A. M. (1994) Elimination of hepatitis delta virus infection after loss of hepatitis B surface antigen in patients with chronic delta hepatitis. *J. Med. Virol.* **44(4),** 389–392.
236. Madejon, A., Cotonat, T., Bartolome, J., Castillo, I., and Carreno, V. (1994) Treatment of chronic hepatitis D virus infection with low and high doses of interferon-alpha 2a: utility of polymerase chain reaction in monitoring antiviral response. *Hepatology* **19(6),** 1331–1336.
237. Arrigoni, A., Ponzetto, A., Actis, G., and Bonino, F. (1983) Levamisole and chronic delta hepatitis (letter). *Ann. Int. Med.* **98(6),** 1024.
238. Craxi, A., Di Marco, V., and Volpes, R. (1990) Treatment with interferon alpha-2b of chronic HDV hepatitis in children. *J. Hepatol.* **11,** S175.

239. Berk, L., de Man, R. A., Housset, C., Berthelot, P., and Schalm, S. W. (1991) Alpha lymphoblastoid interferon and acyclovir for chronic hepatitis delta. *Prog. Clin. Biol. Res.* **364,** 411–420.
240. Rosina, E., Conoscitore, P., Giuliani, A., De Filippis, G., Smedile, A., Bauducci, M., Mangia, A., Borghesio, E., Martinotti, R., Bonino, F., Recchia, S., Piobbici, M., Bonino, F., Verme, G., Andriulli, A., and Rizzetto, M. (1994) Treatment of chronic hepatitis D with the thymosin derivative the Gamma-2, Results of a pilot study. *Hepatology* **20** (4 Part 2), 309.
241. Samuel, D., Zignego, A. L., Reynes, M., Feray, C., Arulnaden, J. L., David, M. F., Gigou, M., Bismuth, A., Mathieu, D., Gentilini, P., et al. (1995) Long-term clinical and virological outcome after liver transplantation for cirrhosis caused by chronic delta hepatitis. *Hepatology* **21(2),** 333–339.
242. Simons, J. N., Pilot-Matias, T. J., Leary, T. P., Dawson, G. J., Desai, S. M., Schlauder, G. G., Muerhoff, A. S., Erker, J. C., Buijk, S. L., Chalmers, M. L., Van Sant, C. L., and Mushahwar, I. K. (1995) Identification of two flavivirus-like genomes in the GB hepatitis agent. *Proc. Natl. Acad. Sci. USA* **92(8),** 3401–3405.
243. Karayannis, P., Petrovic, L. M., Fry, M., Moore, D., Enticott, M., McGarrdey, M. J., Scheuer, P. J., and Thomas, H. C. (1989) Studies of GB hepatitis agent in tamarins. *Hepatology* **9,** 186–192.
244. Schlauder, G. G., Dawson, G. J., Simons, J. N., Pilot-Matias, T. J., Gutierrez, R. A., Heynen, C. A., Knigge, M. F., Kurpiewski, G. S., Buijk, S. L., Leary, T. P., Muerhoff, A. S., Desai, S. M., and Mushahwar, I. K. (1995) Molecular and serologic analysis in the transmission of the GB hepatitis agents. *J. Med. Virol.* **46(1),** 81–90.
245. Simons, J. N., Leary, T. P., Dawson, G. J., Pilot-Matias, T. J., Muerhoff, A. S., Schlauder, G. G., Desai, S. M., and Mushahwar, I. K. (1995) Isolation of novel virus-like sequences associated with human hepatitis. *Nature Med.* **1(6),** 564–569.
246. Linnen, J., Wages, J., Jr., Zhang Keck, Z. Y., Fry, K. E., Krawczynski, K. Z., Alter, H., Koonin, E., Gallagher, M., Alter, M., Hadziyannis, S., Karayiannis, P., Fung, K., Nakatsuji, Y., Shih, J. W. K., Young, L., Piatak, M., Jr., Hoover, C., Fernandez, J., Chen, S., Zou, J. C., Morris, T., Hyams, K. C., Ismays, S., Lifson, J. D., Hess, G., Foung, K. H., Thomas, H., Bradley, D., Margolis, H., and Kim, J. P. (1996) Molecular cloning and disease association of hepatitis G virus: A transfusion-transmissible agent. *Science* **271(5248),** 505–508.
247. Karayiannis, P., Hadziyannis, S. J., Kim, J., Pickering, J. M., Piatak, M., Hess, G., Yun, A., McGarvey, M. J., Wages, J., and Thomas, H. C. (1997) Hepatitis G virus infection: clinical characteristics and response to interferon. *J. Viral Hep.* **4,** 37–44.

10

Hepatitis A Vaccines

John A. Lewis

1. Introduction

At this writing, inactivated vaccines that offer a safe, convenient, and durably efficacious alternative to the use of immune globulin (IG) are available as pre-exposure prophylaxis against hepatitis A virus (HAV) for both pediatric and adult populations. These vaccines are the medically useful products of four decades of cooperative scientific interactions between academic and industrial laboratories throughout the world. They have been well received in developed nations for groups at increased risk for hepatitis A, especially civilian travelers to regions where disease is endemic. The broader use of these vaccines to control community outbreaks is currently being explored, and both medical and pharmacoeconomic guidelines have been developed to rationalize the use of these vaccines in programs of widespread childhood immunizations. These guidelines will continue to be refined by the recent licensure of inactivated vaccines that require only a single primary dose for the induction of protective immunity *(3,8,34,141)*.

Despite an effort spanning several decades, live attenuated HAV vaccines have yet to be brought to licensure, although one candidate vaccine under development in China has been extensively studied in pediatric populations and may soon be relied on for the eradication of endemic disease in that country *(155)*. The licensure of live attenuated vaccines will require the resolution of difficult questions, among them the stability of the attenuated phenotype, transmission to unvaccinated cohorts, the durability of immunity, and the profound issue of safety that looms over any attempt to infect the liver with such a delicate touch that host immunity is engaged before the liver is extensively involved with the replication of the vaccine virus.

This chapter begins with an historical perspective of infectious hepatitis, its conventional prophylaxis with IG, and a description of the genetic and

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

antigenic structure of the etiologic agent HAV. It continues in the form of a chronological narrative to review the development and clinical evaluation of live attenuated HAV vaccine candidates and to review the development, manufacture, character, and clinical performance of inactivated HAV vaccines. The chapter concludes with a brief review of continuing efforts to develop subunit vaccines by recombinant DNA methods, which may extend vaccine prophylaxis against hepatitis A to the largest populations in endemic regions. The literature of hepatitis A disease and its control with vaccines is understandably vast, and an effort has been made in this chapter to supplement references to primary reports with references to reviews of breadth and incisiveness.

1.1. Epidemic Jaundice: A Modern Contagion Noted Often in Antiquity

Epidemic jaundice, a contagion of antiquity, was reported as early as the fifth century B.C. in the writings of the Babylonian Talmud and was known contemporaneously to the Greeks and Romans, warranting notice in the writings of Hippocrates in the fourth century B.C. *(101,259,260)*. Since these earliest times, the disease has been reported to occur in often spectacular epidemic form, perhaps none more widely debilitating than the 1988 epidemic in Shanghai, China during which nearly 300,000 cases of disease requiring 8000 hospitalizations and leading to nearly 50 deaths occurred due to the consumption of contaminated shellfish *(257)*.

The disease has campaigned frequently through history with the armies of many nations, invited along by disruptions of sanitation and the collisions of populations with varying susceptibility. The control of this "jaunesse de camps" has long been a concern of military medicine and, indeed, served to motivate, at Walter Reed Army Institute for Research, (WRAIR) Bethesda, MD, the development of the first prototype inactivated HAV vaccine to be evaluated in human clinical trials *(21,102,204,222,224)*.

The worldwide epidemiology of hepatitis A disease presents an ever-changing face, and reviews of the subject require frequent revision, as transitional developing nations gain control of endemic disease through improvements in sanitation and measures of public health *(95,216)*. This control, though it spares pediatric populations a mild and often asymptomatic disease, extends the term of susceptibility to adulthood at which time the morbidity and mortality of hepatitis A are increasingly severe, especially in older patients and those otherwise medically compromised *(3,95)*.

1.2. Prophylaxis with Immune Globulin (IG)

For nearly 50 years, immune globulin (IG) prepared from human plasma donations by the method of cold ethanol fractionation *(42)* reigned

as the sole form of prophylaxis against hepatitis A. First reported by Stokes and Neefe *(231)* as efficacious in the control of an outbreak of infectious hepatitis among summer campers, IG has since afforded passive protection in both pre- and post-exposure contexts ranging in efficacy from 50–91% *(3,229,256)*. This range reflects the interplay of numerous variables, among them the anti-HAV antibody titer of IG preparations, the dose administered, the term of observation, and the duration and burden of exposure to hepatitis A. Despite its efficacy, the appeal of IG prophylaxis is moderated by its character as a blood-derived product with the risks of transmission of human pathogens, the unpleasantness of its injection site pain, and its decline in efficacy with a half-life of 21–28 d *(135)*.

The efficacy of IG is realized through an antibody-mediated neutralization of the plasma viremia that develops following oral ingestion of materials contaminated with HAV. This viremia, if unchecked, proceeds to an infection of liver hepatocytes against which both the cell-mediated and humoral arms of the host immune system are ultimately aroused *(230)*.

Despite several decades of effort, using subhuman primates to model the pathogenesis of human disease, the primary site of HAV replication that gives rise to plasma viremia has yet to be convincingly demonstrated. Although HAV antigen has been detected by immunofluorescence in the duodenum of marmosets infected by intubation *(120)*, these results have yet to be reproduced and are criticized for the delivery of infectious inocula to tissues distal to the site of primary exposure under natural field conditions *(126,157,159)*.

More recent studies have detected infectious HAV and HAV antigen in saliva, following the application of virus to the posterior pharynx of a chimpanzee, suggesting a local oropharyngeal replication *(41)*, but similar experiments in the South American owl monkey *A. trivirgatus* failed to reproduce these findings, though HAV antigen demonstrable by immunofluorescence was detected in epithelial cells of intestinal crypts and in cells of the lamina propria of small intestine, from 3 d to 5 wk postinoculation *(14)*.

These studies in subhuman primates, though fraught with the difficulties of veterinary husbandry with highly infectious inocula, continue to be pursued in an effort to identify tissues of extrahepatic origin to which HAV replication might be adapted in order to generate HAV strains attenuated by the acquisition of a novel extrahepatic cell tropism. Variants of this phenotype would be expected to provide obvious advantages of safety in continuing efforts to develop useful live attenuated HAV vaccines *(41)*.

1.3. HAV: The Etiologic Agent of Hepatitis A

The etiology of epidemic jaundice remained mysterious until the early twentieth century when an infectious microbial agent was first reasoned to

be responsible. The subsequent futility of efforts to expose a bacteriological agent and the contemporaneous identification of a viral etiology for the icterus of yellow fever led pathologists to suspect a viral etiology for infectious hepatitis. Though suspected, the viral agent of infectious hepatitis would remain elusive, unseen, and uncharacterized for six more decades *(101,165,259,260)*.

1.3.1. Genetic Structure of HAV

The etiologic agent of infectious hepatitis, HAV, is now known to be a 27-nm nonenveloped, icosahedral virus with a single-stranded positive-sense RNA genome of nearly 7500 nucleotides. HAV is assigned taxonomically to the family Picornaviridae in which it resides as the sole member of the genus Heparnaviridae *(167)*.

The first complete nucleotide sequence of HAV and the genetic structure it implied was reported in 1985 for the LA strain by Najarian et al. at Chiron Corporation *(173)*, and since that time three additional sequences, of the HM 175, MBB, and GBM strains, have been described *(39,90,181)*. All four strains are highly conserved, sharing an overall homology of 92–95%, although several subregions of the genome, notably one of 168 nucleotides at the VP1/2A junction, have been identified as hypervariable among isolates *(114,115,208,209)*. The sequences of this region from more than 150 field isolates of human and subhuman HAV strains collected worldwide have been reported as a collaborative study by Robertson et al. at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, Jansen et al. at the University of North Carolina, and Moritsugu et al. at the National Institute of Health in Tokyo, Japan leading to the definition of seven HAV genotypes into four of which human strains have been assigned *(209)*.

The preponderance of human HAV isolates are grouped in Genotype I and share a VP1/2A subregion homology of 92–95%. All but seven of the remaining isolates are assigned to Genotype III, among which the subregion homology is 95% or greater. The subregion homology of the VP1/2A subregion between Genotypes I and III is approx 87%. Of the remaining seven strains, four are human isolates assigned to Genotypes II and VII, with a homology to Genotypes I and III of approx 80% *(209)*. These data demonstrate a close homology of most HAV strains, which, reinforced with studies of the antigenicity of Genotype I and III strains described below and cross-neutralization studies of various strains in vitro, support the assignment of the vast majority of HAV strains to a single HAV serotype *(134)*.

1.3.2. Antigenic Structure of HAV

The HAV genome codes for 10–11 genes in a continuous open reading frame of 7400 nucleotides. The majority of these genes are nonstructural and are involved in genome replication and the proteolytic processing of the

polyprotein translation product. Three genes at the 5' end of the genome, designated VP1, VP2, and VP3, encode structural proteins ranging in molecular weight from 25–30 kDa. These proteins, in 60 copies each, assemble to form the mature HAV capsid and in doing so create an immunodominant antigenic site that induces a vigorous neutralizing antibody response in mice as well as in humans *(183,184,227)*.

Murine monoclonal antibodies (MAb), which neutralize the growth of HAV in vitro, have been generated by McGregor et al. at Fairfield Hospital, Melbourne, Australia *(162)*, Hughes et al. at the Merck Research Laboratories (MRL) *(109)*, Tedder et al. at Middlesex Hospital, London, UK *(227)*, and Crevat et al. at Clonatec, Paris, France *(45)*. These MAbs have been assembled by Ping et al. at the University of North Carolina as a panel to resolve the antigenic structure of the HAV capsid into constituent epitopes *(227)*. Several of the epitopes that contribute to the immunodominant antigenic site have been defined with competitive binding assays in which each MAb of the panel was evaluated for its ability to compete with one select radiolabeled MAb for the binding to a limited quantity of HAV immobilized on a solid phase surface *(184)*. These experiments identified two monoclonal antibodies, K3-4C8 *(162)* and B5-B3 *(226)*, which, by failing to compete with each other for HAV binding, define at least two epitopes of the antigenic site. The immunodominance of these epitopes in the seroresponse to HAV in humans is reflected by the ability of the K3-4C8 and B5-B3 MAbs in combination to compete effectively with the binding of human convalescent antibody to HAV *(226)*.

Several of the amino acids that contribute to the K3-4C8 and B5-B3 epitopes have been identified by the nucleotide sequencing of the capsid region of HAV escape mutants isolated by their growth in cell culture in the presence of neutralizing HAV MAbs *(139,172,183,184,226)*. These amino acids include Asp 70 and Gln 74 of capsid protein VP3, and the nonlinear amino acids Ser 102, Val 171, and Ala 176 of VP1, whose geometries have been proposed, in the absence of the crystal structure of HAV, by computer modeling, using parameters of the crystal structures of other Picornaviridae *(150)*. This immunodominant antigenic site of HAV is very highly conserved among strains of Genotype I and Genotype III. This conclusion is supported by the demonstrations with MAbs that the antigenic structure of the HM175 and PA 21 isolates of Genotype I and III are essentially indistinguishable *(32)* and by the demonstration that polyclonal antisera raised in subhuman primates to infections with HM175 efficiently cross-neutralize the replication of the simian PA21 HAV strain in cell culture studies *(134)*. These data provide reasonable assurance that HAV vaccines developed with a strain of either Genotype I or Genotype III will be broadly protective against the preponderance of HAV strains worldwide.

2. The Modern Era of HAV Vaccine Development

2.1. Distinguishing Infectious Hepatitis from Serum Hepatitis

Infectious hepatitis was designated hepatitis A by McCallum in 1947 *(151)* to distinguish the disease from a hepatitis McCallum designated hepatitis B, which emerged as a clinical entity in the late nineteenth century with novel therapies that required parenteral applications. These therapies included the use of glycerinated lymph of human origin as an immunogen against small pox, the use of salversan therapy for the control of syphilis, and the use of yellow fever vaccine, whose preparation and potency relied on products from human serum *(259,260)*.

Several decades later, the pathogenesis of hepatitis A and hepatitis B were distinguished formally by the classical transmission studies in pediatric populations at the Willowbrook State School, Staten Island, NY reported by Krugman et al. *(127,128,259)*. These studies were conducted as carefully considered and ethically refined extensions of human transmission studies conducted during World War II to understand a rampant and debilitating disease. They made critical use of two serum pools, designated MS-1 and MS-2, which were obtained from Mir, one of 11 newly admitted students in whom consecutive bouts of hepatitis were induced by the administration of serum pools collected during prior outbreaks of hepatitis at Willowbrook *(128)*. Aliquots of the MS-1 and MS-2 pools were found to induce distinctly different disease when administered to pediatric recipients by intramuscular injection. The disease induced by the MS-1 typing pool developed with an incubation period of 30–38 d and followed a relatively mild, occasionally icteric clinical course marked by a 3–19-d period of liver transaminase elevations and abnormal thymol turbidity. By contrast, the hepatitis that developed in recipients of the MS-2 pool developed more slowly, with an incubation period of 50–100 d, culminating in a more severe and often icteric disease, characterized by elevated serum transaminase for periods of 50–100 d coincident with normal thymol turbidity *(128)*.

2.2. Identification of HAV by Immune Electron Microscopy

The Willowbrook studies and others that followed were profoundly important, not only in providing a fuller definition of the pathogenesis of hepatitis disease, but also in providing well-characterized and well-pedigreed infectious specimens with which continuing studies of the etiology and pathogenesis of hepatitis A could be confidently pursued *(24,128,161,164)*.

Indeed, it was with just such specimens that Feinstone et al. at the National Institute of Allergy and Infectious Disease (NIAID) reported the iden-

tification of the etiologic agent of hepatitis A in 1973 *(68)*. To search for the agent, aliquots of fecal specimens obtained from recipients of the MS-1 strain during acute phase disease were incubated with convalescent anti-HAV antibody, concentrated by centrifugation, and inspected by electron microscopy. This technique of immune electron microscopy (IEM) revealed in specimens from two patients small virus-like particles with a diameter of 27 nm, which were often aggregated and decorated heavily with antibody. Similar aggregates of 27-nm particles were observed with MS-1 fecal specimens incubated with serum from patients convalescent with hepatitis A, acquired naturally in Massachusetts or American Samoa, but could not be observed when these experiments were controlled with various rodent sera hyperimmune to hepatitis B *(68)*.

The IEM method was refined as an assay with HAV and anti-HAV antibody standards, which allowed the quantitation of antigen or antibody in clinical specimens by the aggregation state of HAV standards postincubation. Although limited in application by the requirements of electron microscopy, these IEM assays yielded insights into both the kinetics of host immune response to hepatitis A disease and the appearance of HAV in feces and proved useful in the characterization of hepatitis A disease transmitted in the subhuman primate models of disease under development at the time *(68)*.

2.3. *Transmission of Hepatitis A to Subhuman Primates*

2.3.1. Transmission to the Saguinus Genus of Marmosets

The first such subhuman primate model was developed with the *Saguinus* genus of marmosets by Deinhardt et al. at Presbyterian-St. Luke's Hospital, Chicago, IL *(51)*, who described a mild hepatitis in these South American monkeys infected intravenously with serum specimens obtained from a surgeon, G. B., during the acute phase of his hepatitis A disease. The transmitted disease was characterized by liver enzyme elevations of several weeks duration, coincident with histopathological changes in the liver, and was transmitted as the Barker strain through five marmoset passages, during which it increased markedly in virulence *(51)*.

The transmission of the Barker strain to marmosets was confirmed by Parks and Melnick at the Baylor College of Medicine, Houston, TX *(179)*, who reasoned, however, that insofar as the strain appeared to differ from the agent responsible for human disease in both its sensitivity to physical stress and the pathology it induced, it was perhaps more correctly considered a latent endogenous marmoset virus whose recrudescence during the G. B. transmission studies was induced by the stresses of captivity and experimentation. In support of this view, Parks and Melnick described the relative ease with which

liver enzyme elevations could be induced in marmosets with any of a variety of control human clinical specimens unrelated to infectious hepatitis *(180)*.

The Deinhardt laboratory responded to this criticism with further studies of disease transmission to marmosets of the *S. fuscicollis* or *S. nigricollis* species using coded specimens of preinfection and acute phase serum from volunteer recipients of the MS-1 strain of HAV *(105,106)*. These studies reported the reproducible transmission of disease with only acute phase specimens, 15 of 18 of which induced within 34–50 d a hepatocellular injury manifested by serum enzyme elevations and focal lobular necrosis with consequent lobular and portal inflammation *(106)*.

The reproducibility of the marmoset model was subsequently confirmed by Mascoli et al. at the Merck Research Laboratories (MRL) and International Center for Medical Research and Training in San José, Costa Rica *(156,190)* who transmitted hepatitis A to marmosets of the *S. mystax* species with well-pedigreed serum specimens obtained from natural outbreaks of hepatitis A in Costa Rica. These studies detailed the pathogenesis of an acute hepatitis that was induced within 28–77 d of an intravenous injection of acute phase serum specimens and progressed as a mild and generally nonfatal episode of morbidity characterized by serum transaminase elevations, coincident with focal or diffuse hepatocyte degeneration in parallel with sinusoidal distensions and mononuclear infiltrates. This histopathology, characteristic of human disease, could not be induced with serum specimens neutralized before inoculation with serum from patients convalescent from hepatitis A *(156,190)*.

2.3.2. *Transmission to Chimpanzees*

A short time later, Dienstag et al. at the NIAID *(56)* and Maynard et al. at the Phoenix Laboratories of the CDC, in Phoenix, AZ *(160,161)* reported the transmission of hepatitis A to chimpanzees, using fecal specimens of the MS-1 strain, which induced a disease similar to that transmitted by human specimens to marmosets *(57,187)*. The disease was generally mild and provoked, after a 2 wk period of incubation, serum aminotransferase elevations of several weeks duration, a liver pathology of periportal inflammation with monocytic infiltrates and hepatocyte degeneration, and, importantly, an abundance of 27-nm HAV particles in acute-phase fecal specimens that could be readily identified by IEM.

2.4. *Purification and Characterization of HAV from Experimentally Infected Subhuman Primates*

The fecal specimens of infected chimpanzees and the livers of infected marmosets, that contained titers of 10^{11} virus particles per gram of tissue, were appreciated as convenient and well-controlled reagents with which to

attempt a purification of HAV. Within a short time, relatively highly purified material amenable to biochemical and biophysical characterization was prepared by prototype purification processes, described by Bradley et al. at the CDC *(26,108,215)*, Dienstag et al. and Moritsugu et al. at the NIAID *(58,170)*, Locarnini et al. at Fairfield Hospital, Fairfield, Australia *(146)*, and Provost et al. at MRL *(192)*. These processes were developed with various combinations of velocity and isopycnic centrifugations in sucrose and CsCl, ion-exchange and size-exclusion chromatography, PEG precipitation, and detergent and organic extractions, techniques which, with subsequent refinements, would be adopted as elements of purification processes developed for the large-scale manufacture of human vaccines.

These efforts at purification provided information of taxonomic value that supported the assignment of hepatitis A to the Picornaviridae family *(25,27,28,43,44,192)*, and they identified physical and chemical methods for the inactivation of HAV, one of which, formaldehyde, would emerge as the agent of choice for the inactivation of HAV infectivity for the industrial manufacture of vaccines *(192)*.

2.5. *Development of Diagnostic Assays for Anti-HAV Antibody and HAV Antigen*

The purified products of these first essays in HAV purification were put to immediate practical use as reagents for the development of serodiagnostic assays for anti-HAV antibody. The first such assay, a Complement Fixation (CF) assay reported in 1975 by Provost et al. at MRL *(191)*, quantitated anti-HAV antibody in clinical specimens by its ability, once bound to HAV immobilized on a solid phase, to fix guinea pig complement and to promote the lysis of sheep red blood cells. Not long thereafter, Miller et al. at MRL *(168)* and Moritsugu et al. at the NIAID *(170)* described immune adherence hemagglutinin assays (IAHA), which were configured to reveal anti-HAV antibody in clinical specimens by its ability, once bound to HAV, to promote the aggregation of human O erythrocytes.

These assays were robust and amenable to large-scale serological testing and were used to describe the kinetics of antibody response to hepatitis A infection and the seroprevalence of HAV antibody in various populations *(168,170)*. Despite their virtues of sensitivity and specificity, CF and IAHA assays were dependent on biological reagents, which required careful control to minimize interassay variability, and they were superseded in a short while by radioimmunoassays (RIA) of the sort developed by Hollinger et al. at the CDC *(104)* and Purcell et al. at the NIAID *(203)*. These RIAs were configured as sandwich-type assays, dependent on a source of convalescent anti-HAV antibody which, applied noncovalently to a plastic surface,

allowed the capture of HAV from clinical or laboratory specimens that could be quantitated by its binding to a radioiodinated convalescent anti-HAV probe. With a simple modification of design, these RIAs could detect anti-HAV antibody in clinical specimens by its ability to compete with the binding of a radioiodinated anti-HAV antibody probe to a limited quantity of HAV immobilized on a solid phase.

These assays sustained continued investigations of the epidemiology and pathogenesis of hepatitis A and in one modified form or another, depending on local preferences, have supported the clinical development and manufacture of HAV vaccines around the world.

2.6. Propagation of HAV in vitro

2.6.1. The First Success at the Merck Research Laboratories (MRL)

The purified HAV products from subhuman primate specimens were appealing reagents, as well, with which to renew attempts to propagate HAV in cell culture in vitro, without which a large-scale manufacture of vaccine for human use was unthinkable. These renewed attempts were undertaken in the face of a misleading and discouraging literature that reported a cytopathogenic effect (CPE) to proceed rapidly from the inoculation of various cell cultures, including well-characterized cell lines with infectious human specimens *(24,145,164)*. On occasion, this CPE could be serially passaged with cell culture supernatants and on occasion was reported to be neutralized by incubation with sera from patients convalescent from hepatitis A, though these results proved essentially irreproducible *(24,145,164)*.

It was therefore an altogether unexpected but very welcome development when Provost et al. at MRL *(194)* reported in 1979 the propagation of HAV from livers of infected marmosets, without evidence of CPE, in either explant cultures of marmoset liver or monolayer cultures of the Fetal Rhesus Kidney 6 cell strain (FRhK6) *(243)*. The replication of virus in these cultures was detected several weeks after infection using the technique of immunofluorescence which identified HAV as a minute cytoplasmic fluorescent granulation. This immunofluorescence could be serially passaged with infected cell extracts and, importantly, could be neutralized for passage by incubation with serum from patients convalescent from hepatitis A but not by incubation with various control sera *(194)*.

This achievement at MRL was made possible, in the assessment of it authors, by technical refinements to the methods of immunofluorescence *(158)*, the commercial availability of well-controlled reagents for in vitro cell culture, and with "the ability to maintain healthy confluent cell cultures for moderate to long periods of time." *(198)*. The success at Merck was confirmed within the same year by Frosner et al. at the von Pettenkorfer

Institute, Munich, Germany, who propagated HAV from human fecal specimens in the Alexander human hepatoma cell line in which multiple copies of the hepatitis B genome are known to be integrated and transcriptionally active *(77)*. While neither of these first two successes would lie, for obvious reasons of regulatory concern, directly on the pathway of vaccine manufacture for human use, they demonstrated the feasibility of HAV propagation in vitro and encouraged a repetition of the work, using cell lines and HAV inocula whose pedigrees would be acceptable to national control authorities for human vaccine development.

Just such acceptable isolations were first reported by Flehmig at the University of Tubingen, Germany *(71)* in 1980, who propagated HAV directly from a human fecal specimen into the well-characterized FRhK4 cell strain *(244)* and passaged it further through a strain of human diploid fibroblasts *(244)*. This success was reproduced in the following year by Daemer et al. at the NIAID, who passaged HAV in vitro through a continuous cell line of African Green Monkey kidney (AGMK) *(47)*, by Provost et al. *(195)* who passaged HAV from clinical specimens in the FRhK6 cell strain; and by Gauss-Muller et al., who described the replication, after 208 d, of HAV from fecal specimens inoculated directly into cultures of human diploid fibroblasts *(83)*. Understandably, given the unpredictability of HAV isolations directly into human diploid fibroblast culture, most HAV strains for the development of human vaccines have been propagated first in cells of subhuman or human embryonic kidney origin and thereafter adapted with only minor difficulty to replication in human diploid fibroblast cell strains.

2.6.2. The Eccentricities of HAV Replication

The once intractable propagation of HAV in vitro has now been reported by patient investigators in numerous laboratories, using cell lines of both primate and nonprimate origin *(59,123,232)*. Without exception, these successes required several weeks or months following the inoculation of cell cultures with infectious specimens before assays for HAV were reproducibly positive. With each subsequent passage of infected cell lysates to newly established cultures, the interval required for the detection of HAV was abbreviated, until after a number of such passages, cell-culture adapted strains were developed, whose replication could be detected within several days of inoculation.

Despite their adaptation to in vitro replication, these strains continue to require several weeks for the modest accumulation of only 100 $TCID_{50}$ per cell, and although HAV can be detected in extracellular fluids, the preponderance of replicated virus is contained within the infected cell cytoplasm *(20,29,36,72,73,198,219,250)*. The replication of these strains proceeds

without evidence of CPE and without effect on host cell DNA, RNA, or protein synthesis *(147)*, an altogether inoffensive presence, explainable perhaps in part by the lack of an effective virally encoded genetic mechanism to commandeer host cell metabolism and by the rapidity with which newly replicated HAV genomes are encapsidated and isolated from a further taxation of host cell processes *(10,49,137)*.

The innocuity of the in vitro replication of these cell-culture adapted strains is further reflected in the ease with which cell lines of either fibroblast or epithelial origin can be infected and carried for long periods without a decline of fitness *(207,221,235)*. On occasion, persistently infected cell lines have given rise, presumably by genetic recombination, to HAV variants that replicate rapidly and induce an extensive CPE *(9,46,136,177)*. For these and other reasons of quality control, persistently infected cell cultures have not been adopted for manufacturing purposes, although they have proven useful for the laboratory scale production of HAV to support basic virological studies and for the recent development of plaque assays for neutralizing anti-HAV antibody in clinical specimens *(17,18)*.

Of the myriad technical impediments to the manufacture of inactivated HAV vaccines for human use, the eccentricities of HAV replication have proven to be among the most formidable. They have called on the ingenuity of biological process engineers to expand the scale of HAV production from laboratory exercises, sufficient to support small-scale preclinical studies in subhuman primate models, to an industrial manufacture adequate to support licensure and worldwide distribution.

3. Candidate Live Attenuated Vaccines

3.1. Development of Candidate Live Attenuated HAV Vaccines

The development of cell-culture adapted HAV strains was rationalized in terms of classical genetics as a continuous selection of naturally occurring variants in the initial virus inoculum and variants arising during in vitro replication, which were more ably suited to replication in the unnatural environment of cells of nonhepatocyte origin maintained in vitro at suboptimal (32–35°C) temperature. It was reasonable to assume that a selection of HAV variants that preferred the environment of an in vitro host was at the same time a selection against naturally virulent wild-type virus, and these cell-culture adapted strains were therefore recognized for their possible utility as live attenuated vaccine strains.

The evaluation of these strains as candidate vaccines has been pursued with the conviction that they could be manufactured at minimal cost and

Table 1
Live Attenuated HAV Vaccines Evaluated in Human Clinical Trials (1997)

Laboratory	Year	Strain	Route of Administration[a]	Effective dose $TCID_{50}$[b]	Clinical development
Merck Research Lab.	1986	CR 326F	Subcutaneous	$10^{6.3}$	Phase I
Zheijang Acad. Med. Sci.	1989	H2	Subcutaneous	$10^{6.3}$	Phase III
Smith Kline Beecham Biologicals	1991	HM 175	Intramuscular	$10^{6.3}$	Phase I

[a]Routes of administration refer to those routes through which seroconversion has been observed.
[b]The effective dose refers to that dose which in young adult populations induces antibody detectable by immunoassay in >90% of vaccines.

would induce safely, with a single dose, an immunity superior in character and durability to the immunity induced by the multidose regimens developed for inactivated vaccines. These attributes, if realized, would suit these vaccines ideally for prophylactic use by select populations in developed nations and for broader use in underdeveloped nations to support programs for the eradication of endemic disease *(155,225)*. The important question of whether an extensive regimen of in vitro cell passage would alter the natural hepatotropism of HAV and yield virus that would replicate innocuously in tissues proximal to the site of injection was unpredictable and could be answered only by preclinical and clinical investigations.

3.2. Evaluation of Candidate Live Attenuated Vaccines in Human Clinical Trials

At this writing, three cell-culture adapted strains have been studied in human clinical trials as live attenuated HAV vaccine candidates. These strains are presented in Table 1 according to the first report of their clinical performance. The general features of the behavior of these candidate vaccines are summarized below after which a more detailed account of each is provided.

All three strains were derived by extensive regimens of in vitro passage through cell substrates of various origins and were demonstrated to be appropriately attenuated in preclinical studies in subhuman primates in which they induced anti-HAV antibody (seroconversion) without histopathological or biochemical evidence of liver disease. These candidate vaccines have not proven immunogenic when administered by the oral route, even at

doses of 10^6 $TCID_{50}$, although doses of higher titer, if they can be manufactured in practice, may prove immunogenic by this route.

These candidate vaccine strains have proven to be mildly immunogenic when administered to adult vaccinees by intramuscular (IM) or subcutaneous (SC) routes at doses between 10^5 and 10^7 $TCID_{50}$. The most potent of these doses has tended to induce seroconversion in nearly all vaccinees within 3–5 wk of administration, although an occasional recipient has remained seronegative for several months after vaccination before convincing evidence of a seroresponse was obtained *(200)*. The geometric mean titers (GMTs) of anti-HAV antibody tend to persist without significant decline for at least 1 yr after vaccination. The durability of the seroresponse beyond 1 yr, which is often argued as a fundamental appeal of live attenuated vaccines, has not been reported for any of the three candidate vaccines.

Evidence for the replication of these strains in hepatic tissues of human recipients has been provided by the occasional recovery of infectious HAV, at generally low titers, from fecal specimens obtained during the period of seroconversion. This low-order replication is likely to minimize both the spread of HAV from vaccinees to nonvaccinated cohorts and the emergence of revertant wild-type virus. It does remain a formal possibility that seroconversion can be induced with these strains in the absence of replication merely by the antigenic mass of the 10^7 $TCID_{50}$ dose, which is known from studies with inactivated vaccines to be adequate to induce seroconversion *(222)*. It would be of fundamental interest for developers of these vaccines to apply to a cohort of vaccinee sera, assays of the sort developed by Robertson et al. *(210)* that detect antibody to nonstructural HAV proteins and can therefore discriminate between virus replication and antigen mass as the provocation for seroconversion.

The CR326F and HM 175 strains have been judged to be too attenuated to be useful as live attenuated vaccines for prophylaxis in adults, and they have not been evaluated in this population beyond Phase I studies. It remains to be demonstrated whether these strains might prove appropriately safe and immunogenic in pediatric populations, in which hepatitis A is decidedly a more benign disease. The CR326F and HM 175 strains have proven useful, however, as master seed virus for the manufacture of inactivated HAV vaccines, and in this context their superattenuated character endows these products with an unusually high degree of safety.

The H2 strain has been evaluated for safety and immunogenicity in both adult and pediatric recipients, and its demonstrated efficacy in extensive studies in pediatric populations suggests that licensure of the H2 strain in China to support national programs of disease eradication may be imminent.

3.2.1. The CR326F Strain Developed at MRL

The CR326F strain was the first cell culture adapted strain to be evaluated as an attenuated vaccine candidate. Its development at MRL established useful precedents for the preclinical and clinical development of other cell-culture adapted strains in both academic and industrial laboratories. The strain was described by Provost et al. in 1983 as an isolate from a fecal specimen of a Costa Rican patient, whose serum had been used previously for the definitive marmoset transmission studies *(196)*. Of several cell-culture adapted variants of the CR326F isolate developed with various regimens of cell culture passage at either 32°C or 35°C, one, designated the F variant, was extensively studied for attenuation in subhuman primates following its derivation by 15 serial passages through FRhK6 cells at 35°C and an additional eight passages at 35°C in the MRC-5 cell strain *(196,197)*.

The F variant was formulated for preclinical studies as a clarified MRC-5 cell lysate and evaluated for attenuation in chimpanzees and marmosets as an IV inoculum at doses of 10^7 $TCID_{50}$ and 10^6 $TCID_{50}$, respectively. This regimen induced seroconversion in chimpanzees within 40 d of administration and marmosets within 27 d, although elevations of serum transaminases were noted in 3 of 12 marmosets coincident with seroconversion. The F variant was therefore carried an additional eight passages through MRC-5 cell culture at 35°C to extinguish any trace of residual virulence, during which regimen it was cloned three times by endpoint dilution to yield the CR326F variant designated F' *(200)*.

The F' variant proved to be appropriately attenuated both for marmosets and chimpanzees, in which a mild histopathology of mononuclear infiltrations, though insufficient in scope to elevate serum transaminases, provided evidence of the replication of the virus in primate hepatocytes. The GMTs of the F' recipients were several-fold lower than the GMTs of the F variant recipients though sufficiently robust to protect against hepatitis A disease when seropositive animals were challenged by the IV route with 10^5 marmoset infectious doses of virulent HAV. No evidence of disease was observed in animals infected with fecal specimens from marmosets that had been previously infected with doses as high as 10^7 $TDIC_{50}$, providing evidence of the stability of the attenuated phenotype of the F' strain through serial animal passage *(202)*.

These preclinical studies encouraged the evaluation of the F' variant of the CR326F strain in human trials, first reported in 1986 by Provost et al. *(200)*. This trial enrolled 11 volunteers, who were administered a 1 mL subcutaneous dose of $10^{6.3}$ $TCID_{50}$ prepared as a clarified lysate of HAV-infected MRC-5 cell monolayers. No physical or biochemical evidence of hepatitis A

disease was observed in any vaccinee, six of whom seroconverted within a mean time of 45 d postvaccination to GMTs some hundred-fold lower than the GMTs of patients recently convalescent from naturally acquired hepatitis A disease *(200)*.

The second trial of the F' variant, a dose-escalating study reported by Midthun et al. in 1991 *(166)*, confirmed the suboptimal immunogenicity of the $10^{6.3}$ $TCID_{50}$ dose and demonstrated the superiority of a $10^{7.3}$ $TCID_{50}$ dose, which induced HAV antibody detectable by the most sensitive assays in all recipients within 6 mo of vaccination. This antibody was capable of the neutralization of HAV replication in the HAVARNA cell culture assay developed by Krah et al. at MRL *(124)* and persisted without decline to 12 mo postvaccination *(166)*. HAV was detected in the feces of one of the recipients of the $10^{7.3}$ $TCID_{50}$ dose even though attempts to isolate infectious virus from the specimen into MRC-5 cell culture were not successful *(166)*.

Although the CR326F strain has not been brought to licensure as an attenuated vaccine, its derivation has served as a critical precedent. It not only established the relationship between in vitro cell-culture adaptation and attenuation of HAV but demonstrated that attenuated strains could be propagated to titers in vitro, which would permit them to serve as Master Seed virus for the eventual production of inactivated HAV vaccines *(13)*.

3.2.2. *The H2 Strain Developed at Zheijang Medical Academy*

The H2 strain developed at the Zheijang Medical Academy was isolated from a fecal specimen of a pediatric patient and passaged extensively through monkey kidney cells at 35°C and 32°C *(152)*. It was subsequently adapted to the human diploid lung fibroblast strain KMB at 32°C and judged to be attenuated in preclinical evaluations in monkeys in which it induced seroconversion following parenteral, but not oral, delivery.

The H2 strain was first evaluated in human clinical trials in 1987 by Mao et al. *(153,154)* who described in a Phase I study of the safety and immunogenicity in 12 seronegative young healthy adults to whom it was administered as a 1 mL subcutaneous dose of $10^{6.5}$ $TCID_{50}$ *(152)*. This dose was well tolerated. No elevations of body temperature, SGPT (ALT), ICD, or LDH transaminases or physical symptoms of hepatitis A disease were noted in any vaccinee. All vaccinees seroconverted within 3 wk of vaccination and remained seropositive through 1 yr of observation with HAV antibody, which neutralized H2 in vitro culture. Infectious HAV was isolated in cell culture from occasional fecal specimens taken within 8–21 d of vaccination, although at levels below those required for detection of antigen by ELISA *(152)*.

The safety of the H2 strain in Phase I studies encouraged the evaluation of H2 vaccine in pediatric populations for whom a universal immunization has been contemplated as a means to control endemic disease in China. By 1992, more than 11,341 pediatric recipients were reported to have been immunized with the $10^{6.5}$ $TCID_{50}$ dose, which was reported to be well tolerated *(153,154,205)*. No significant local or systemic effects were reported among 3021 children evaluated through a 4-wk period after immunization. The dose was suitably immunogenic and promoted seroconversion within 4 wk of administration in 92.9% of recipients, nearly all of whom remained seropositive through 12 mo of observation *(154,155)*. Reductions in the potency of the dose to $10^{5.5}$ $TCID_{50}$ or $10^{5.0}$ $TCID_{50}$ diminished rates of seroconversions at 4 wk to 51–53% and 76–84% respectively, even though both rates were slightly higher when seroconversion was assayed at 12 wk postvaccination *(206)*. Infectious HAV was detected in occasional fecal specimens collected between 8 and 30 d postvaccination coincident with seroconversion, and no evidence of the transmission of the vaccine strain to unvaccinated contacts was demonstrated in a small-scale surveillance study *(155)*.

The protective efficacy of the H2 strain has been demonstrated in two pediatric populations, in a small trial in Hangzhou in which the vaccine was demonstrated efficacious with a statistical significance of $p < 0.025$ *(154)* and a substantially larger trial involving 2000 vaccinees in Shaoxing County, Zhejiang Province, in which the evidence of protective efficacy was reported as highly significant *(155)*. The performance of the H2 strain as a safe, immunogenic, and efficacious vaccine in pediatric populations suggests that its licensure and use in China as a vaccine to control endemic disease is imminent. The successful manufacture of this vaccine at the scale required for universal pediatric vaccination will require solutions to the exasperating difficulties of the large-scale propagation of HAV in vitro that will interest process engineers engaged with similar difficult exercises.

3.2.3. The HM 175 Strain Developed at NIAID and SmithKline Beecham Biologicals

The HM 175 strain was developed at the NIAID from a fecal specimen obtained during a natural outbreak of hepatitis A in Melbourne, Australia in 1976 *(94)*. The strain was isolated in AGMK cell culture and carried for 32 passages at 35°C by which regimen it was attenuated for replication in chimpanzees and marmosets *(122)*. The strain was finally adapted, through nine serial passages, to replication in MRC-5 cells *(11,80)*.

The HM 175 strain was evaluated for safety and tolerability in a Phase I study reported in 1992 by Sjogren et al. at WRAIR, which enrolled 14 adult volunteers, eight of whom received 1 mL by the oral route at potencies rang-

ing from 10^4 to 10^7 $TCID_{50}$ *(221,224)*. No local or systemic complaints were noted either after immunization or long-term follow-up. The vaccine proved inert by this oral route at all doses tested. Six of the vaccinees were administered 1 mL by im injection at doses of 10^5 $TCID_{50}$, 10^6 $TCID_{50}$, or 10^7 $TCID_{50}$. Seroconversion was observed within 3 wk of vaccination in all recipients of the two highest doses, which persisted without decline for at least 12 wk.

The genetic basis for the attenuation of the HM 175 vaccine strain has been studied extensively at the NIAID by Cohen et al., who reported the nucleotide sequence of the wild-type HM 175 strain and a cell-culture adapted derivative obtained by 32 passages through AGMK cells *(40)*. These strains were shown to differ by 24 nucleotide substitutions distributed throughout the genome, 16 of them in the coding regions specifying 12 amino acid substitutions. These substitutions have been shown, by a sequencing of the AGMK adapted strain at various intermediate levels of passage, to accumulate progressively through the process of adaptation *(233)* and to be retained through the passage of the AGMK adapted strain in MRC-5 cell culture, during which it acquired an additional 13 nucleotide changes through nine cell passages *(80,81)*.

The relative importance of the AGMK nucleotide changes to the phenotype of attenuation have been assessed by Emerson et al. at NIAID, who used techniques of recombinant DNA technology to construct a series of chimeric HAV viruses combining elements of wild-type and cell-culture adapted HAV genomes, which were evaluated for replication both in vivo and in vitro *(63–65)*. These thoughtfully conceived studies found chimeras that retained several critical nucleotide changes in the 2B/2C region and nucleotide changes in the 5' noncoding region to be most fully attenuated in vivo and most highly adapted in vitro. They provided evidence that the attenuation of HAV was dependent on a mosaic of nucleotide substitutions, a finding that would suggest that the phenotype of attenuation will prove appropriately stable through large-scale programs of disease eradication.

4. Inactivated HAV Vaccines

4.1. Development of Prototype

Experimentation along the pathway of inactivated HAV vaccine development was given impetus by Provost et al. at MRL in 1978 who described a prototype inactivated vaccine prepared with HAV that was partially purified from livers of infected marmosets and inactivated with 100 μg/mL formaldehyde at 35.5°C for 96 h *(193)*. This vaccine induced, without adju-

vant, a seroconversion of young adult marmosets of sufficient robustness, albeit after a 14-wk regimen of vaccinations at 2-wk intervals, to resist an IV challenge of 10^3 marmoset infectious doses of virulent HAV *(193)*.

By 1986, prototype formaldehyde-inactivated vaccines developed with cell-culture adapted HAV strains were reported simultaneously by Binn et al. at WRAIR *(21)* and by Provost et al. at MRL *(199)* and a year later by Flehmig et al. at the University of Tubingen, Germany *(74)*. These products were prepared with HAV from infected cell lysates that were either inactivated directly *(21)* or purified first and then inactivated with either formaldehyde *(199)* or β-propiolactone *(74)*. These vaccines were formulated as liquid suspensions without adjuvant and induced seroconversion with a single im or intraperitoneal (ip) dose in various rodent models of immunogenicity. They proved similarly immunogenic in the marmoset and owl monkey models of hepatitis A disease in which they induced, after several doses, an immunity sufficient in character and magnitude to protect seropositive animals from disease following intravenous challenges with virulent HAV strains *(21,131,199)*.

4.2. General Principles of Industrial Manufacture of Inactivated HAV Vaccines

The WRAIR and Tubingen vaccines were refined for experimentation in human trials and were evaluated, without adjuvant, in small Phase I trials in young healthy adults *(21,74)*. The vaccines proved to be only mildly immunogenic, requiring three or four dose regimens administered over a 6-mo interval to induce a seroresponse in most vaccinees *(76,222,223)*. These modest clinical results sustained, nonetheless, a cautious enthusiasm for the continued development of whole-virus inactivated vaccines despite their technical challenges. In time, this enthusiasm grew to a resolute commitment in light of the apparent overattenuation of live-virus vaccines candidates and the failure to develop subunit HAV vaccines by recombinant DNA technology that could induce, even in rodent models, a neutralizing antibody response of a magnitude that might be expected to be protective.

At this writing, five inactivated HAV vaccines have been registered for human use either in the United States, Europe, or Asia. These vaccines are presented in Table 2 in the chronological order of their first licensure. Although these products can be distinguished by either their purity, formulation, use of adjuvants or regimens of administration, their manufacture and control share several common features of procedure, which are described here first before the special character of each of these products is presented further along.

Table 2
Inactivated HAV Vaccines Licensed Worldwide (1997)

Vaccine	Manufacturer	Primary dose[a]		Booster dose	
		Adult	Pediatric	Adult	Pediatric
Havrix®	SmithKline Beecham Biologicals	1440 EU	720 EU	1440 U @ 6–12 mo	720 U @ 6–12 mo
Vaqta®	Merck and Co.	50 U	25 U	50 U @ 6 mo	25 U @ 6–18 mo
Epaxal®	Swiss Serum and Vaccine Inst.	500 U	500 U	500 U @ 12 mo	500 U @ 12 mo
Avaxim®	Pasteur Merieux Serums and Vaccines	160 U		160 U @ 6 mo	
Aimmugen®	Kaketsuken	0.5 μg[b]		0.5 μg @ 6 mo	

[a]Definitions of pediatric and adult population are variable.
[b]Two primary doses at 0 and 4 wk.

4.2.1. HAV Strains for Manufacture

All five vaccines have been developed with cell-culture adapted HAV strains isolated from human fecal specimens directly into cultures of primate epithelial cell origin and subsequently adapted, with a single exception to growth in the human diploid fibroblast cell strain MRC-5. This cell strain, derived from human embryonic lung tissue in 1974 by Jacobs at the Medical Research Council Laboratories, Mill Hill, UK *(113)* is karyotypically normal, and it has been approved by regulatory agencies throughout the world as a cell substrate for the manufacture of biologicals for human use.

4.2.2. HAV Vaccine Antigens

All five vaccines are prepared with HAV purified to varying degrees from infected cell lysates. Only Vaqta® (MRL) has been reported to be so thoroughly purified that only nanogram quantities of residual cell protein are present in the final vaccine formulation *(239)*.

The potencies of the vaccines are generally reported as Units, assigned by their manufacturers using RIAs or ELISAs calibrated with proprietary HAV standards, known to vary in HAV mass. These nominal units are misleading as a comparative basis of vaccine potency, which can be evaluated reliably only in head-to-head clinical trials of immunogenicity. Although it seems reasonable to suggest that all vaccines licensed for use according to a common regimen of administration are essentially equipotent and appropriate for interchangeable use, recently developed guidelines urge that vaccination regimens initiated with one product be completed without product substitution *(8)*.

4.2.3. Inactivation of HAV with Formaldehyde

All five vaccines are inactivated, following purification, by prolonged exposure to formaldehyde. This chemistry has not been reported, with a single exception, to compromise the antigenicity of HAV critical to the induction of protective immunity *(13,21,245)*. In this respect, the formaldehyde inactivation of HAV differs importantly from the inactivation of poliovirus, which suffers compromise to one of several epitopes important for the induction of humoral immunity *(69)*. Although alternatives to formaldehyde are known, notably UV irradiation and β-propiolactone which require hours rather than days for a thorough inactivation of HAV *(74,244)*, industrial manufacturers have adopted formaldehyde as the agent of choice. In so doing, manufacturers have availed themselves of four decades of experience with manufacturing practices that have ensured the safety of formaldehyde inactivated poliovirus vaccines.

The various HAV preparations are inactivated in buffered solutions at neutral pH at temperatures ranging from 35 to 37°C with concentrations of formaldehyde and terms of exposure ranging from 100 μg/mL for 15 days (d) to 400 μg/mL for 5 d. These variations reflect the dependence of the rate of HAV inactivation on the concentration of formaldehyde, and the stringency of these processes are therefore considered to be essentially indistinguishable *(13)*. These inactivation processes greatly exceed the requirements for a thorough inactivation of the infectivity of a bulk lot of vaccine and are adequate, assuming no deviation in the linear rate of inactivation, to ensure the absence of HAV infectivity in more than 10^{15} doses of vaccine *(13,21)*.

The thoroughness and consistency of these large-scale HAV inactivation processes are controlled by rigorous in vitro assays for HAV infectivity, which are conducted for especially long periods to allow the detection of any residual infectivity. The first inactivated HAV vaccines to be licensed were controlled to the standards of inactivation testing established for inactivated poliovirus vaccine manufacture, namely, a testing of 1500 doses or not more than 5% of each production lot *(38)*. More recently, with a fuller appreciation of the extent to which these inactivation processes have been mastered at manufacturing scale and a fuller recognition of the extent to which the master seed strains of some vaccines are known to be attenuated for humans, modifications in these testing regimens consistent with product safety have been proposed and are presently under advisement by national control authorities. The thoroughness of these inactivation processes, combined with the use of attenuated HAV strains as master seed virus for manufacture, endow these HAV vaccines with a profile of considerable safety.

4.2.4. Formulations of HAV Antigen

With a single exception, these inactivated vaccines are presented as aqueous formulations of 0.5 mL for children and adolescents and 1 mL for adults, to be administered as an IM injection. These vaccines differ in their use of various amino acids and simple sugars as stabilizers and phenoxyethanol or thimerosal as preservatives. Only Vaqta® is formulated without stabilizer and without preservative. Havrix®, Vaqta, and Avaxim® are adjuvanted by adsorption to aluminum. Epaxal® is adjuvanted with immunostimulatory virosome complexes, and Aimmugen ® is formulated without adjuvant as a lyophilized preparation.

4.3. Clinical Development of Inactivated Vaccines

4.3.1. Objectives of Clinical Development

Inactivated HAV vaccines have been developed to induce, safely and conveniently, an immunity equal in robustness and superior in durability to the immunity conferred by administration of IG. The first vaccines developed for clinical study were evaluated for safety and immunogenicity as three-dose regimens, administered as two primary doses at an interval of 2–4 wk, with a third booster dose administered at 24 wk. This regimen was adopted to respect traditions of administration developed for the licensure of other inactivated or nonreplicating vaccine antigens. The regimen was evaluated almost exclusively despite evidence that HAV vaccines could be formulated to a potency that would induce, with a single primary dose, a nearly universal seroconversion of young healthy adults to GMTs, which were several-fold greater than the GMTs of recipients of a standard doses of IG prophylaxis *(217,218)*. These GMTs were durable and persisted for at least 6 mo, at which time they could be boosted with a second dose to levels that exceeded by 50-fold or greater the GMTs of IG recipients *(217,218)*.

With the persuasive demonstration of the clinical efficacy of a single primary dose of Vaqta in a clinical trial in Monroe, NY *(247,248)*, the clinical development of inactivated HAV vaccines was freed from the paradigm of three-dose regimens and was focused confidently on the development of two-dose regimens formulated to potencies sufficient to induce with a single primary dose the seroconversion of >95% of both pediatric and adult populations, irrespective of age or physical condition.

The two-dose regimens that have been brought to licensure differ to some degree in the interval recommended between the administration of the primary and boosting doses. These differences reflect more the extent to which the durability of the seroresponse to the primary dose has been thoroughly assessed and less to any well-documented study of the effect of interval of administration on the peak GMTs following booster dose administration.

4.3.2. Serological Assays to Support Clinical Development

The clinical development of these vaccines has been supported by immunoassays configured as either RIAs or ELISAs that detect anti-HAV antibody in clinical specimens by its ability to compete with the binding of a radioiodinated or enzyme-linked anti-HAV antibody probe to a limited quantity of HAV immobilized on a solid-phase. These assays have been developed to be sensitive to the titers of anti-HAV antibody in recipients of IG shortly after administration and developed as quantitative assays by reference to a standard curve constructed with serial dilutions of a reference standard of IG assigned a nominal concentration of 100 IU/mL which allows the seroresponse to be expressed in milliInternational Units per milliliter (mIU/mL) *(85)*.

These assays have been developed either with reagents supplied with the commercially available HAVAB assay (Abbott Lab, Chicago, IL) *(50)* or with proprietary reagents prepared by vaccine manufacturers, and they have been configured as assays of competitive or noncompetitive design that differ in the conditions of incubation of serum specimen with convalescent anti-HAV antibody probe *(50,169)*. Not surprisingly, immunoassays that differ in reagents and configuration differ in relative sensitivity as well. Although they agree in general on the GMT of anti-HAV antibody in recipients of IG, these assays can differ by as much as fivefold in their assignment of GMTs to anti-HAV antibody in vaccinee serum from a given clinical trial *(19,53)*. These differences are most pronounced with sera obtained within the first several weeks and months following primary dose vaccination, and therefore they affect not only the GMTs of seroresponse but the apparent rapidity of that seroconversion by which criteria manufacturers seek to define the important attributes of their products.

This profusion of assays with varying sensitivities makes it difficult for the casual reader of the HAV vaccine literature to compare immunopotencies of various vaccines without strict attention to technical detail, and it can prompt the reasonable protest of a manufacturer whose product, assessed for immunopotency by one assay, is indirectly compared with a second vaccine whose immunopotency has been assessed by a different assay *(54)*. This dilemma of assay variability has persisted even with the development of commercially available ELISA immunoassays, notably the HAVAB IMX (Abbott Lab) and the Enzymun (Boehringer Mannheim, Mannheim, Germany), which though they agree on the GMTs of recipients of IG, disagree by several-fold when used to assay vaccinee sera, although essentially no difference in assay sensitivity is observed when sera taken after the booster dose are assayed *(53)*. In the final analysis, confusions engendered by the application of serological assays of variable sensitivity will continue until a standard serological assay is universally recognized and adopted to a

characterization of the immunopotency of vaccines developed by various manufacturers *(19,22,53,54)*.

4.3.3. Serologic Assays for Neutralizing HAV Antibody

The analysis of vaccine induced anti-HAV antibody by immunoassay has been supplemented by small-scale studies of neutralizing antibody using either the radioimmunofocus assay (RIFA) described by Lemon et al. *(133)* or the HAVARNA assay *(124)*. Neither of these assays is amenable to large-scale serological analyses, and they have been judiciously applied to serum from select trials to demonstrate the induction of biologically active antibody capable of the neutralization of HAV replication in vitro. These assays are configured with different designs and interpreted with different criteria for seropositivity and only recently have been directly compared *(143)*.

These neutralization assays have not proven to be significantly more sensitive than RIAs for the detection of anti-HAV antibody in vaccinee serum *(140,143)*. They can, however, qualitatively discriminate the anti-HAV antibody induced by a single protective dose of Vaqta from HAV antibody administered as IG prophylaxis, and this discrimination has provided insights into the character of the human seroresponse required for clinically efficacious protection *(143)*.

A radioimmunoprecipitation assay (RIPA) developed recently by Lemon et al. is by far the most sensitive serological assay for anti-HAV antibody, though its dependence on endogenously radiolabeled HAV reagents restricts the scope of its application *(140)*. Like the RIFA and HAVARNA assays, the RIPA can discriminate anti-HAV antibody induced by vaccination from antibody administered by passive immunoprophylaxis and with the HAVARNA and RIFA assays has allowed the definition of a standard serological response to inactivated vaccines, which can be expected to be protective *(143)*.

4.3.4. Demonstrations of Clinical Efficacy

Although subhuman primate models of hepatitis A disease were available for well-controlled laboratory demonstrations of the in vivo efficacy of inactivated vaccines and were used for that purpose *(13,21,55)*, clinical researchers felt the need to compel the licensure of vaccines for human use with demonstrations of efficacy in clinical trials under natural field conditions. While epidemiological circumstances of increased incidence of hepatitis A were well known and well characterized, the rates of disease in these settings remained low enough to pose difficult administrative issues. These difficulties were not insurmountable and indeed were mastered to provide a demonstration of the protective efficacy of a regimen of two primary doses of vaccine in a trial involving over 40,000 participants *(112)*.

It was, however, the unique epidemiology of a small community in upstate New York in which HAV disease appeared with a predictable and pervasive presence that yielded a demonstration of the efficacy of a single dose of Vaqta and so committed vaccine manufacturers to the development of two-dose regimens of vaccine that would be more easily embraced for their obvious virtues of convenience *(247)*.

4.4. Havrix (SmithKline Beecham Biologicals)

Havrix® (SmithKline Beecham Biologicals) is distinguished as the first inactivated HAV vaccine to be licensed for human use *(11,12,37,243)*. Havrix was licensed in Switzerland in 1991 as a three- 720 or 360 ELISA-unit (EU) dose regimen for adult and pediatric recipients and has been subsequently licensed and used widely at this regimen in more than 60 countries *(37)*. More recently, a two-dose regimen using either 1440 or 720 EU doses of Havrix for adult and pediatric recipients, respectively, has been developed and brought to licensure in the United States and Europe *(99,118)*.

4.4.1. Manufacture and Characterization of Havrix

Havrix is manufactured with the HM 175 strain of HAV, which was isolated and carried through 32 passages in AGMK cell culture and thereafter adapted through nine serial passages to growth in MRC-5 cells *(94)*. This strain has been reported to be attenuated for replication in subhuman primates *(122)* and superattenuated for replication in humans *(12,225)*. The virus is propagated in MRC-5 cell monolayers, released from infected cell harvests by freeze-thaw lysis and purified through a process of ultrafiltration and column chromatography. The purified HAV is ultrafiltered and inactivated at 250 μg/mL formaldehyde at 37°C for 15 d, during which it is ultrafiltered a second time to ensure the dispersity of HAV *(11,182)*.

The adult dose of Havrix is adsorbed to 0.5 mg of aluminum hydroxide, and formulated in PBS with 0.3% w/v amino acids and 0.05 mg/mL polysorbate as stabilizers and 0.5% w/v phenoxyethanol as preservative. The potency of Havrix is quantitated in EU where 1 EU is reported to correspond to an HAV mass of 0.42 nanograms (ngs) *(11)*. The inactivated HAV of an adult human dose is contained in a residuum of not more than 5 μg of MRC-5 cell protein and formaldehyde (<0.1 mg/mL) *(99,125,142,176)*.

Havrix is recommended for storage at 2–8°C at which temperature it is licensed with a shelf life of 2 yr. No decline in either the kinetics or GMT of seroconversion was reported in a study of Havrix administered at the date of expiration to young healthy adults as a three-dose regimen of 720 EU. *(205)*. The clinical immunopotency of Havrix has recently been reported to be stable for at least 7 d at 37°C *(253)*.

4.4.2. Clinical Evaluation of Havrix

The clinical evaluation of Havrix was begun in 1988 *(251)* and subsequently expanded to evaluate the safety and immunogenicity of various dosages and regimens of Havrix in 104 clinical studies, which enrolled more than 31,000 pediatric and adult recipients over a range of ages and ethnicity *(16,37,48,92,243)*. These trials defined for European licensure a three-dose regimen of 720 EU and 360 EU for adult and pediatric recipients, respectively, to be administered as two primary doses at an interval of 1 mo followed at an interval of 6–12 mo by a third, boosting dose *(99)*.

Havrix is well tolerated, with transient pain, soreness, or redness the most common injection site complaint, and headache, malaise, nausea, and anorexia the most common systemic complaints *(16,37)*. In a controlled study, only the frequency of injection site pain was statistically significantly greater in recipients of Havrix than recipients of an alum-adjuvanted hepatitis B vaccine (Engerix-B®) *(112)*.

Sera from vaccine trials with Havrix were assayed for anti-HAV antibody with a proprietary noncompetitive ELISA that defined a GMT of 20 mIU/mL, assayed in recipients of IG, as the criteria of seropositivity *(37)*. A subset of vaccinee sera was shown to be positive for neutralizing antibody by RIFIT assay and positive for antibody that could compete with the K3-4C8 and B5-B3 MAbs for binding to the immunodominant antigenic site of the HAV capsid *(52)*.

The 720 EU and 320 EU regimens induce seroconversion in >90% of vaccinees within 4 wk of the first of two primary doses and in >98% of vaccines, within 4 wk of the second, at which time the GMTs of vaccinees exceed by approximately tenfold the GMTs of recipients of a standard dose of IG *(16,37)*. These GMTs persist with only slight decline for 20 wk at which time they can be boosted within 4–8 wk of a third dose to GMTs that exceed by 100–300-fold the GMTs of IG recipients *(5)*.

4.4.3. Evaluation of the Protective Efficacy of Havrix

The protective efficacy of a primary regimen of two 360 EU doses of Havrix administered at an interval of 1 mo was demonstrated in 1992 in a trial in Kamphaeng Phet Province, Thailand, where hepatitis A disease is endemic at a rate of 119 cases per 100,000 people *(112)*. The conduct of this trial was overseen by a committee appointed by the Ministry of Public Health of Thailand and was greatly facilitated by administrative and organizational procedures developed during a previous study in the same province of a Japanese encephalitis virus vaccine. The trial enrolled a total of 40,119 children between the ages of 1 and 16 who were sorted by computer-generated block randomization to receive either two 320 EU primary doses of Havrix

or a similar regimen of a hepatitis B vaccine (Engerix-B). The vaccine was administered by IM deltoid injection and was well tolerated *(112)*. The health of the vaccinees was followed by a surveillance of local primary school attendance to identify children who, absent >2 d from school, were evaluated for elevations of serum alanine aminotransferase levels above 45 U/L and serological evidence of anti-HAV IgM antibody, findings that coincident with absenteeism defined a clinical case of hepatitis A. During the course of the trial, 6876 episodes of illness were identified, of which 40 fit the case criteria of hepatitis A disease. Of these, 32 were seen in recipients of Engerix-B and only 2 in recipients of Havrix. The incidence of disease in the control and vaccine groups differed significantly ($p < .0001$) and demonstrated convincingly a 94% protective efficacy of the two-primary-dose regimen administered within a 4 wk interval (C.I. 79–99%) *(112)*.

4.4.4. Development of a Two-Dose Regimen for Havrix

A two-dose regimen of Havrix has been recently developed and licensed for administration as a single primary dose of 720 or 1440 EU for pediatric and adult recipients, respectively, with a second booster dose of the same potency to be administered within an interval of 6–12 mo. This regimen has been reported to be safe and well tolerated in several small trials, and its convenience can be expected to improve the compliance and cost-effectiveness of vaccine prophylaxis with Havrix *(30,37,234,236,238,249)*

The single primary dose of 1440 EU promotes an apparently age-dependent seroconversion of 77–95% of recipients within 2 wk of administration and 95-100% of recipients within 4 wk at GMTs several-fold greater than those of IG recipients. The vaccine GMTs persist without decline for at least 12 mo *(30,37,171,236,238)*. The GMT of the seroresponse to the two 1440 EU dose regimen has been reported in a small trial to be superior at all time points to the seroresponse to a three 720 EU dose regimen, though the difference is small and its advantage for clinical protective efficacy will be difficult to assess *(148)*.

4.4.5. Persistence of the Serological Response to Havrix

The long-term persistence of Havrix-induced antibody has been most thoroughly studied in recipients of the three-dose regimen for whom a projection of anti-HAV antibody persistence for at least 10 yr beyond the third boosting dose was initially reported *(5,252)*. This projection has since been extended to a term of at least 20 yr as continued surveillance has revealed the kinetics of anti-HAV antibody decay to be composed of at least two distinguishable components, one of 6-mo duration, during which the peak GMTs observed 4–8 wk after the booster dose decayed by 60.9% and a second of at least 2 yr duration, during which the decay was moderated to a rate of 13.5–14.8% *(237)*.

Data for the persistence of antibody in recipients of the two-dose regimen of Havrix have not yet been reported, and they will be obviously germane to any recommendations of the use of this vaccine in pediatric recipients in programs of universal vaccination.

4.4.6. Administration of Havrix with IG

The effect of a concurrent administration of IG on the immunogenicity of Havrix has been evaluated in vaccinees 18–50 yr of age, who were administered a 5-mL gluteal injection of a commercially available IG with the first dose of a three-dose regimen of 720 EU of Havrix *(91,132)*. Although the co-administration of IG diminished by 1.8 to 2.9-fold the immunopotency of vaccine at all time points assayed, this active/passive mode of prophylaxis offers the advantages of both an immediate and durable protection to those who travel on short notice to regions of endemic disease and remain for extended periods, exposed to significant burdens of infectious virus. These advantages should outweigh any compromise to the long-term immunity induced by vaccination, which can be extended at a later date with a second booster if necessary. Studies of the immunogenicity of a 1440 EU dose of Havrix administered with IG have not been reported, even though this vaccine formulation is licensed for administration with IG *(99)*.

4.4.7. Administration of Havrix with a Hepatitis B Vaccine (Engerix-B)

A formulation of a 720 EU dose of Havrix with a 20-μg dose of the hepatitis B vaccine Engerix-B has been recently developed and evaluated in several small trials as a three-dose regimen in young healthy adults *(6,7,33,119)*. These trials have demonstrated this A/B combination to be safe and tolerable and to induce a seroresponse to both antigen components, indistinguishable in kinetics and GMTs to the immunity induced with each antigen alone. This combination may prove to be of prophylactic value to those whose professional or personal lifestyles expose them to risks of both infections.

4.4.8. Administration of Havrix with Vaccines for Travel

The immunogenicity of a single 1440 EU primary dose of Havrix has been evaluated in 110 young healthy adults who received either vaccine alone or vaccine and a yellow fever (YF 17D) vaccine prepared by Pasteur-Merieux to a potency of 1000 mouse LD_{50} *(86)*. No statistically significant effect of YF vaccination was observed on either the kinetics or GMTs of seroconversion to Havrix. The reciprocal effect of vaccination with HAV on the seroresponse to YF vaccination was not reported *(86)*.

4.4.9. Special Uses for Havrix

Havrix is recommended for administration by IM injection, preferably in the deltoid region *(99)*. Its immunopotency has been reported to be compromised by the intradermal route *(31)*. It does, however, retain sufficient potency by SC administration that a two-dose primary regimen of 1440 EU or 720 EU, administered within a 4 wk period, promotes a nearly universal seroconversion of adult and pediatric recipients with bleeding disorders to GMTs only several-fold below those reported for healthy recipients of a single dose of the same potency *(35,213,261)*. This regimen appears to be well tolerated and may sustain the use of this vaccine as prophylaxis against the occasionally rare contamination of FVIII preparations with infectious HAV *(213)*.

A 320 EU dose of Havrix has been formulated for delivery by jet injector and administered as a three-dose regimen to young healthy adults *(103)*. This method of delivery was reported to be superior by the criteria of kinetics and GMT of the seroresponse to that induced with the same dose delivered by needle injection at all time points following the administration of the first two doses ($p < 0.001$). No statistically significant differences in seroresponse were observed, however, between the two routes of delivery at times after the third dose *(103)*.

The immunogenicity of Havrix has been studied as a three-dose (720 EU) regimen in a small cohort of HIV positive men, 72% and 75% of whom were seropositive in response to the second and third dose of vaccine, respectively *(100)*. No evidence of an acceleration of HIV disease as a consequence of vaccination was observed in the study participants.

4.4.10. Use of Havrix to Control Community Outbreaks

The evaluation of Havrix as a means to control community outbreaks of hepatitis A has just recently begun, encouraged in part by preclinical studies in subhuman primates that demonstrated a moderating effect of postexposure vaccination on the severity of disease in both chimpanzees and marmosets infected orally with wild-type HAV *(55,211)*. These first attempts at outbreak control have required a blend of effective cooperation between various regional departments of health, favorable community sentiment, and the enthusiastic support of health care delivery personnel, and they have been recently rewarded with evidence that an intervention with vaccine can moderate the course of community-wide outbreaks of disease *(229)*.

The first of these demonstrations of effective intervention was reported by McMahon et al. at the Alaska Area Native Health Services, who described the use of a single 1440 EU or 720 EU dose of Havrix to control an outbreak of hepatitis A in rural communities in northwestern Alaska *(163)*. The trial

was conducted as a nonrandomized uncontrolled exercise enrolling 4930 participants. Following vaccination, 237 persons seropositive for anti-HAV IgM antibody were identified through a 60-wk surveillance period, 191 of whom were unvaccinated controls and 46 were vaccinees. This demonstration of the advantage of vaccination in Alaska, though not strictly controlled, is consistent with similar results of the apparent efficacy of a single dose of Havrix in the control of two large community-wide outbreaks in Slovakia *(189)*.

4.5. *Vaqta*

Vaqta is distinguished by its manufacture with a very highly purified preparation of virus and further distinguished as the only inactivated HAV vaccine for which the efficacy of a single primary-dose regimen has been demonstrated in human clinical trials *(13,246,247)*. The details of the purification process that carries the vaccine virus to a level of purity appropriate to products of the biotechnology industry have been widely published *(1,13,96,97,201,242)*.

Vaqta was licensed in Germany in 1995 as a two 50 U or 25 U dose regimen for adult or pediatric recipients (2–17 yr) and was licensed with the same regimen in the United States in 1996. These licensures culminated a 20 yr commitment of the Merck Research Laboratories to the development of a vaccine prophylaxis for hepatitis A disease *(201)*. The commitment was focused in 1987 on the development of an inactivated vaccine, and Vaqta was conceived at that time as a vaccine that would be manufactured to standards expected of the recombinant protein products of the biotechnology industry.

This standard of purity for Vaqta was intended to ensure the consistency of manufacture, the thoroughness of the process for inactivation, and the preparation of a product with appropriate safety and tolerability when administered widely to both pediatric and adult populations *(1,13,96,97)*. The purity of Vaqta is a result in equal measure of the well-documented hardiness of HAV, which makes it tolerant to extensive processing *(220)*; the ingenuity of biological process engineers, who developed highly productive cell culture systems for HAV propagation; and process biochemists, who developed extensive purification processing schemes that importantly yielded virus in sufficient quantity to sustain the manufacture of vaccine for worldwide distribution *(1)*.

4.5.1. Manufacture and Characterization of Vaqta

Vaqta is manufactured with a derivative of the F' variant of the CR326F strain of HAV designated P28, which was derived by the passage of the highly attenuated F' variant an additional 10 passages through MRC-5 cells at 32°C *(13,144,199)*. Despite its extensive adaptation to MRC-5 cell cul-

ture, this P28 variant requires a term of at least 20 d for the maximal accumulation of virus, during which term it replicates in the absence of CPE *(13)*. For the production of vaccine, the P28 variant is propagated in MRC-5 cell monolayers maintained at high cell density in Cell Cubes (Costar) to maximize the production of HAV *(1)*. The viability of these monolayers is ensured by a perfusion of the CellCubes with fresh nutrient medium, which is monitored on-line to allow adjustments to parameters critical to MRC-5 cell viability. On the twentieth day of culture, the MRC-5 cell monolayers are lysed by perfusion with hypotonic buffers containing detergents, releasing HAV into a lysate of MRC-5 cell protein *(1,96,97)*.

The purification process for Vaqta has been extensively described as a reasoned sequence of chromatographic procedures intermixed with organic extractions and PEG precipitations *(1,13,96,97)*. The process has been optimized using a high-performance size-exclusion chromatography (HPSEC) assay to characterize and improve the performance of each step for HAV recovery and the exclusion of MRC-5 cell protein. The process is initiated with a nuclease digestion of the MRC-5 cell lysate, which improves the consistency of the ion-exchange chromatography step by which HAV is first concentrated from the crude cell lysate. The column-bound HAV is eluted with high salt, concentrated further by PEG precipitation, and then resuspended and extracted with chloroform. The organic extract is further purified by ion-exchange chromatography and finally polished with HPSEC *(96,97)*.

The HAV product of this process is very highly purified. The 50 U dose of Vaqta contains less than 100 ng of nonviral protein and less than 4 pg of DNA *(175,176,239)*, and as expected with a purity of this order, only the capsid proteins of HAV can be detected by sensitive silver staining methods when purified virus preparations are analyzed by SDS/PAGE *(13)*. The exceptional purity of the virus preparation has permitted studies of the effects of ionic strength, pH, and temperature on the conformational stability of the virus, using the methods of intrinsic fluorescence emission spectroscopy, dynamic light scattering, HPLC, and fluorescent dye binding assays *(242)*. These studies have contributed to an understanding of the loss of infectivity as a consequence of capsid conformational changes and have served as valuable process development and monitoring tools to define the extremes of various operating conditions during the development of the HAV purification process.

The purified virus preparation is composed of both full and empty HAV capsids in the proportion of 1:3 that sediment in gradients of sucrose at 150S and 80S, respectively *(13)*. Both 150S and 80S capsids of HAV are known to react with the K3-4C8 and B5-B3 MAbs, which define epitopes of the immunodominant antigenic site of HAV *(214,246)*, and both capsid forms

are highly immunogenic in mice and *S. labiatus* marmosets, in which they induce a neutralizing antibody response sufficient to protect against the intravenous challenge of 10^4 infectious doses of wild-type HAV *(13,214,246)*. The 80S capsids are known by various analytical methods to lack an RNA genome and to be composed of the capsid proteins VP1, VP3, and notably VP0, which, as a precursor of the mature capsid protein VP2, identifies the 80S capsid as a precursor to and not a degradation product of the 150S capsid *(13)*.

The purified HAV preparations in phosphate-buffered saline at neutral pH are ultrafiltered and inactivated with 400 μg/mL formaldehyde for 5 d at 35°C, during which it is ultrafiltered again to ensure the dispersity of the HAV preparation. Under these conditions, the infectivity of the HAV is inactivated at a rate of 10^{12} $TCID_{50}$/24 hours with no evidence of a departure from linear inactivation kinetics through the extinction of 10^9 $TCID_{50}$, which can be assayed practically in the laboratory. At the end of the inactivation term, aliquots of the bulk product are assayed for complete inactivation through two consecutive 35 day periods of MRC-5 cell culture. The inactivated HAV antigen is adsorbed by coprecipitation to aluminum hydroxide and is further processed through a series of settle-decant operations to yield a vaccine product with less than detectable levels (<0.8 μg) of residual formaldehyde *(239)*.

The potency of Vaqta is reported in Units, assigned with an ELISA assay using a highly purified HAV standard, where 1 U is essentially equivalent to 1 ng of HAV *(13)*. The vaccine is formulated as either a 50 U dose in 1 mL with 0.45 mg of aluminum hydroxide for use in adults or a 25 U dose in 0.5 mL containing 0.22 mg aluminum hydroxide for pediatric and adolescent use. Neither formulation contains stabilizer or preservative. Vaqta is stable as an aqueous formulation and is licensed with a shelf-life of 2 yr when stored at the recommended temperature of 2–8°C. It is stable to elevated temperature as well, which makes it possible to distribute the vaccine according to license for up to 72 h at 37°C *(239)*.

4.5.2. Clinical Evaluation of Vaqta

Phase I clinical studies of the safety and immunogenicity of Vaqta were initiated in April 1989 *(61,62)* and were subsequently expanded in support of licensure to administer more than 10,000 doses of vaccine at various potencies and regimens to both adult and pediatric recipients *(175)*.

Vaqta is well tolerated. Local injection site reactions and minor adverse experiences of headache, nausea, and malaise are mild and transient and in placebo-controlled trials are no more frequent in recipients of Vaqta than in recipients of an alum placebo *(176,174,247)*. No serious vaccine-related adverse experiences in either pediatric or adult populations were observed in the clinical trial experience with Vaqta *(175,176)*.

The clinical development of Vaqta was supported with serological data derived with an immunoassay designated the m-HAVAB, developed at MRL to be a more-sensitive version of the commercially available HAVAB assay *(169)*. The m-HAVAB is configured as a competitive assay and is known by direct comparison to report GMTs of vaccine antibody some fivefold lower than other RIAs of noncompetitive design *(53,240)*. Sera from several select trials, including the efficacy trial in Monroe, NY, were extensively analyzed with RIFA and HAVARNA assays for neutralizing antibody to confirm the biological activity of antibody induced by Vaqta and to establish a standard for a seroresponse, known to be protective after a single primary dose *(143)*.

The Phase I clinical development of Vaqta was focused on the evaluation of the safety, tolerability, and immunogenicity of formulations of various potencies administered as a three dose regimen of two primary doses in a 2–4-wk interval, with a third booster dose at a minimal interval of 6 mo. These trials, reviewed extensively by Nalin et al., identified a 25 U dose to be sufficiently potent to induce with a single primary dose, a seroresponse in most young adult recipients within 4 wk of administration *(175,176,239)*.

The 25 U dose was subsequently studied by Shouval et al. at the Hadassah Medical School, Jerusalem, Israel *(216,217)* as a two-dose regimen of a single primary dose and a booster dose at 6 mo to allow the robustness of the seroresponse to the primary dose to be evaluated without the confounding effect of a second primary dose administered within a 4-wk period. The results of this study, presented in Fig. 1, found a single 25 U dose to induce seroconversion in 14 of 15 young adult recipients within 4 wk of administration to GMTs essentially indistinguishable from the GMTs of recipients of standard IG prophylaxis. These GMTs were observed to be durable and to persist without decline for at least 20 wk, at which time they were boosted 40–50-fold within 4–8 wk of the second 25 U dose to GMTs essentially indistinguishable from the GMTs of recipients of a three 25-U-dose regimens.

Similar rates of seroconversion that were durable through a 6-mo period of observation were reported in pediatric recipients of a 25 U dose of Vaqta reported by Block et al. *(23)* and they encouraged planning for the evaluation of the clinical efficacy of a single primary dose of vaccine. The fruition of this planning awaited the identification of a unique epidemiological circumstance in a small community in Monroe, NY, with an incidence of disease that would allow for a rigorous statistical demonstration of the protective efficacy of the antibody response to a single dose of vaccine in a 6-mo interval through which the antibody response was durable at GMTs essentially indistinguishable from recipients of IG *(247,248)*.

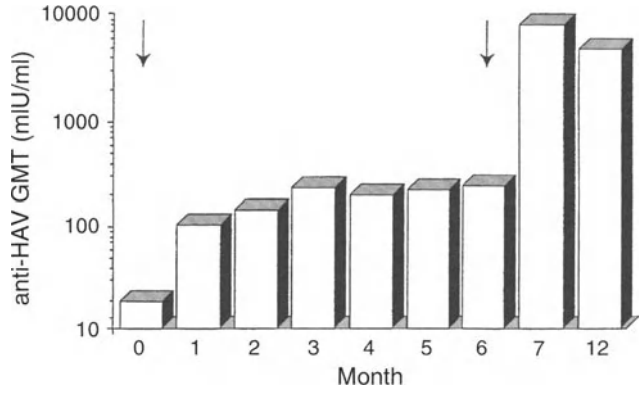


Fig. 1. Kinetics of appearance of anti-HAV antibody following a single primary dose of 25 U Vaqta followed by a booster of 25 U at 6 mo (n = 15). Arrows indicate the time of vaccination. (Reproduced with kind permission J. Hepatol. 335, 453–457.)

4.5.3. Clinical Evaluation of the Protective Efficacy of Vaqta in Monroe, NY

The protective efficacy of a single primary dose of Vaqta in the prevention of hepatitis A in pediatric recipients in Monroe, NY, was demonstrated in a now classical study reported in 1992 by Werzberger et al. *(246,247)*. The trial was conducted in the Hasidic Jewish community of Kiryas Joel in which hepatitis A had established from 1985 onward, a recurrent epidemic presence in the months of late summer and early fall, during which more than 300 clinical cases of disease in young children were recorded. This rather unique and predictable pattern of seasonal disease was reasoned to reflect the introduction of disease to the community by children from a sister Hasidic community in Brooklyn, NY, who vacationed during the summer at Kiryas Joel. These circumstances invited the possibility that a vaccination of children in the early summer months of 1991 could interdict epidemic disease that might be reasonably expected to recur in the fall of that year.

A double-blind placebo-controlled trial was therefore conceived to study the efficacy of a two-dose regimen of Vaqta administered as a single primary dose, with a booster to follow at 6 mo. This design was intended to allow for the demonstration of the efficacy of a single dose of Vaqta if the burden of disease introduced to Kiryas Joel in 1991 was similar in magnitude to that introduced in 1990, during which 100 cases of icteric disease were noted in children less than 13 yr of age.

The conduct of the trial was overseen by an independent monitoring committee, whose responsibilities included a general ethical oversight of the trial, a confirmation of the safety and tolerability of the vaccine, and the evaluation of episodes of illness reported by both active and passive surveillance that met the trial case definition of hepatitis A. For this trial, a case of hepatitis A was defined by a triad of a diagnostic level of IgM antibody to HAV, a serum alanine aminotransferase level at least twice the upper limit of normal during an episode of illness with no other obvious cause and one or more clinical signs or symptoms consistent with a diagnosis of hepatitis A, including fatigue, malaise, abdominal pain, emesis, elevated temperature, and discolorations of either stool or urine. Clinical cases of disease that occurred before the fiftiethth day following the administration of placebo or vaccine would be excluded from analysis in order to eliminate from consideration children who were infected before the start of the trial with HAV, which was known from continuing surveillance in the community.

Children who were demonstrated to be seronegative by fingerstick assay *(2)* were randomized to receive either vaccine or placebo according to a schedule for random number assignment that was based on the sequential order of injection. The assignment code was not revealed to study personnel or trial participants until the termination of the trial. Of the trial population of 1029 children, 519 were administered a single im 25 U dose of Vaqta and 518 children administered an alum placebo. Both Vaqta and alum placebo were well tolerated. No statistically significant differences in the frequency of local injection site reactions or the minor adverse experiences of headache, nausea, and malaise were observed in recipients of Vaqta or placebo. No adverse experiences were reported following the administration of either vaccine or placebo. *(247,248)*. The 25 U dose of Vaqta was highly immunogenic, inducing a seroresponse within 4 wk in all but one of 305 recipients to a GMT of 42 mIU/mL.

Vaccinations were begun in June 1991; by November 1991, active and passive surveillance had identified 25 cases of hepatitis A that met the clinical case definition and were known to occur more than 50 d after enrollment in the trial. All 25 cases were observed in recipients of placebo. No disease occurred in recipients of Vaqta, an efficacy of a single dose of vaccine of 100% with a significance of $p < 0.001$ with a 95% CI of 87.3%. The temporal distribution of all cases of hepatitis A evaluated by the monitoring committee in the course of the trial are presented in Fig. 2. This figure reveals evidence of the early onset of the protective efficacy of Vaqta, a protection noted as early as 21 d after vaccination, after which no cases of disease were reported in recipients of Vaqta to day 50, during which time nine cases of disease were recorded in placebo recipients.

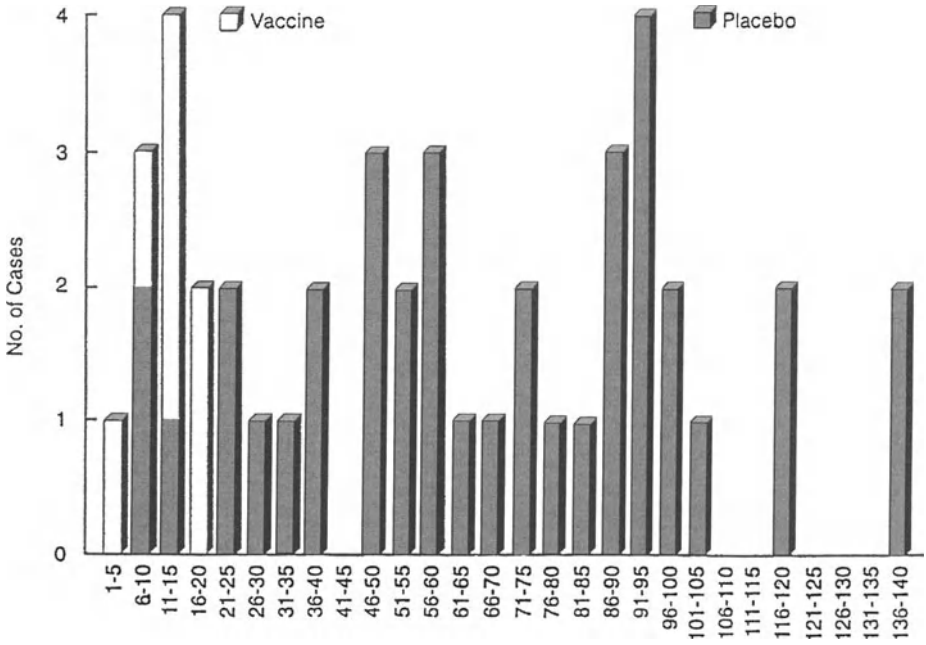


Fig. 2. Distribution of cases of clinical hepatitis A in the Vaqta and placebo groups, according to the length of time since injection. (Reproduced with kind permission N. Engl. J. Med. 335, 453–457.)

4.5.4. Persistence of the Anti-HAV Serological Response to Vaqta After the Primary and Booster Doses

With the demonstration of the efficacy of a single dose of vaccine in the Monroe trial, placebo recipients were immunized with a 25 U dose of Vaqta, and vaccinees were administered a booster dose of vaccine according to design. A cohort of vaccinees, whose permission was granted, were randomized to three groups to receive the booster dose of Vaqta at either 6, 12, or 18 mo, at which time they were serologically evaluated for the persistence of antibody to the single primary dose. This special amendment to the original trial design revealed the seroresponse to a single dose to be especially durable, to such an extent that 91% of the cohort observed for 12 or 18 mo remained seropositive at GMTs several-fold greater than the GMTs of IG recipients *(175)*. The GMTs of all three groups were elevated some 50–100-fold in response to the booster dose, even though the sample sizes were insufficient to allow a thorough statistical evaluation of the effect of interval before boosting on the GMT response to the boost *(175)*.

This special cohort of pediatric recipients provided additional serological data of interest, namely, the rapidity and robustness of the anamnestic response induced by a single dose of vaccine in all ten vaccinees whose HAV antibody titers had waned to undetectable levels before the administration of the booster dose at 6, 12, or 18 mo. These vaccinees responded to the booster dose to GMTs essentially indistinguishable from the GMTs of vaccinees who had remained seropositive throughout the course of observation *(175)*.

The long-term persistence of antibody induced by booster doses of Vaqta has been predicted, using a mathematical model based on individual subject estimates rather than group means *(254)*. This statistical modeling reveals the humoral immune response to a regimen of Vaqta to be especially durable to such an extent that over 40% of vaccinees can be expected to remain seropositive 30 yr after completing a course of immunization. It will remain of interest to determine whether vaccinees who have reverted to seronegativity many years after vaccination will be protected from clinical disease by an anamnestic response of the vigor demonstrated in pediatric recipients in Monroe. It is just such an anamnestic response to infection that Villarejos et al. have posited provides protection from disease in those recovered from natural infection who revert to seronegativity after long periods and respond to case contact disease with no evidence of an IgM response preceding the development of IgG antibody *(241)*.

4.5.5. Administration of Vaqta and IG

The effect of a coadministration of a standard dose of IG (0.06 mL/kg) with a 50 U adult dose of Vaqta has been reported in a study of 300 healthy adults 18–39 yr of age *(239)*. This study demonstrated a slight though statistically insignificant reduction in seropositivity in recipients of vaccine and IG (92%) compared with recipients of Vaqta alone (97%) at week 24 after immunization with the primary dose *(239)*. These groups were statistically distinguishable, however, by GMT at both week 24, at which time the GMT of vaccine and IG recipients was 83 mIU/mL, in contrast to the GMT of 137 mIU/mL of recipients of vaccine alone, and at week 28, 4 wk after a second dose of 25 U at week 28, (4872 mIU/mL and 6498 mIU/mL respectively) *(239)*. Although statistically distinguishable, the GMTs of both groups exceeded by some four- and fivefold, the GMTs required for protection both during the 24 wk following the primary dose of Vaqta and exceeded by some 100–200-fold the requirements for protection by humoral antibody alone following the boost.

4.6. Epaxal

The Swiss Serum and Vaccines Institute, Berne, Switzerland has developed and licensed Epaxal in Switzerland as a two 500 U dose regimen for adult and pediatric recipients to be administered over an interval of at least 1 yr *(67)*. This vaccine is distinguished by its use of immunopotentiating reconstituted influenza virosomes to adjuvant the immunogenicity of the inactivated HAV antigen *(87,88)*.

4.6.1. Manufacture and Characterization of Epaxal

Epaxal is manufactured with the RG-SB strain of HAV propagated in MRC-5 cell culture. The virus has been reported to be purified, for Phase I clinical trials, from cell lysates by a sequence of ultrafiltration, organic extraction with n-heptane, and velocity and isopycnic centrifugations through gradients of sucrose and CsCl *(88)*. The extent to which this process has been retained for the large-scale manufacture of vaccine has not been reported. The virus preparation is inactivated with formaldehyde for 10 d at 37°C *(88)*.

The purified and inactivated HAV is formulated by adsorption to the surface of unilamellar lipid structures composed of phosphatidylethanolamine and phosphatidylcholine, in which the hemagglutinin (HA) protein of influenza A virus is incorporated *(88)*. This formulation is thought to facilitate the delivery of the inactivated virus to antigen presenting cells, and so accelerate an immunoresponse *(87)*. Each 0.5 mL dose of Epaxal contains 500 RU of inactivated HAV in association with at least 5 μg of influenza hemagglutinin protein and not more than 350 μg of phospholipid. The vaccine is preserved with 15 μg thimerosal *(67)*.

4.6.2. Clinical Evaluation of Epaxal

The clinical evaluation of Epaxal, first reported in 1992, revealed a liposome formulation of inactivated HAV to be superior in both the kinetics and GMT of seroconversion to alum-adjuvanted formulations of the same antigen administered to young healthy adults *(88,117)*. All recipients of the liposome vaccine seroconverted within 14 d of vaccination and remained seropositive for at least 52 wk with GMTs approx 20-fold greater than the GMTs of IG recipients. Subsequent trials of either 500 EU or 1000 EU doses confirmed the potency of the liposome formulations for young healthy adults, in which seroconversion within 14 d of administration in >95% of recipients was routinely observed *(88,117,149,185,186)*.

The 500 EU dose is reported to be extremely well tolerated and in a direct and randomized trial superior to the tolerance to Havrix® *(107)*. Whereas a 500 EU dose of Epaxal and a 1440 EU dose of Havrix were equally immunopotent by the criteria of rates and GMTs of seroconversion,

only 17% of the recipients of Epaxal reported a local adverse effect after the first dose in contrast to 66% of the recipients of Havrix, a highly statistically significant ($p < 0.001$) difference. No differences in tolerability were observed with the second booster dose *(107)*.

The notable tolerability of Epaxal, if confirmed in other well-controlled trials in both pediatric and adult populations, may serve to constitute its distinctive appeal as other equally potent HAV vaccines are licensed that induce a near 100% seroconversion of all recipients within 14 d of vaccination. The persistence of antibody induced by the second booster dose of this vaccine has not yet been reported, and it will be of obvious interest to learn whether the presentation of inactivated HAV antigen with liposome adjuvantation will affect the durability of the humoral response.

4.7. Avaxim (Pasteur-Merieux Serums and Vaccines)

Pasteur-Merieux Serums and Vaccines has developed and licensed Avaxim as a two 160 U dose regimen for adults over the age of 16 to be administered over a 6-mo interval *(15)*.

4.7.1. Manufacture and Characterization of Avaxim

Avaxim is manufactured with the GBM strain of HAV, which was isolated directly into human embryo kidney cell culture from a human fecal specimen obtained during an outbreak of hepatitis A in 1975 *(70,90)*. The strain was subsequently adapted to growth in MRC-5 cell monolayers for the manufacture of vaccine *(15,71,72,73)*. A cell-culture adapted variant of the GBM strain, derived by serial passage through human embryonic kidney cells and human embryonic fibroblasts, has been reported to be attenuated for replication in chimpanzees at doses of 10^7 $TCID_{50}$, and it is reasonable to assume that the master seed virus for Avaxim derived by a similar regimen is similarly attenuated *(75)*. The complete nucleotide sequence of the wild-type GBM strain has been reported *(90)*.

The vaccine manufacturing process has been briefly described as a sequence of tangential ultrafiltration and chromatography of a filtered cell lysate followed by formaldehyde inactivation *(82)*. The extent to which the HAV is purified by this process has not been reported. The HAV preparation is inactivated with 100 μg/mL formaldehyde at 37°C for 14 d with constant agitation, after which the inactivated virus product is adsorbed to aluminum hydroxide.

The vaccine is stabilized with Medium 199, contains 12.5 μg of residual formaldehyde and 0.5 mg aluminum hydroxide in an 0.5-mL adult dose *(15)*. Avaxim is preserved with 2-phenoxyethanol and is recommended for storage at 2–8°C, at which temperature it is licensed with a shelf life of 2 yr *(15)*.

4.7.2. Clinical Evaluation of Avaxim

The clinical evaluation of Avaxim began in 1992 with studies of the safety and immunogenicity of a two 160 U dose regimen in adults over the age of 16, administered at a minimal interval of 6 mo *(15)*. This regimen was well tolerated and induced seroconversion in >90% of recipients within 4 wk of a single primary dose of vaccine delivered by im deltoid injection *(82,89,240)*. The immunopotency of a two-dose regimen of Avaxim and a three-dose 720 EU regimen of Havrix have been directly compared in a multicentered study in 840 healthy adults and found to be essentially equipotent *(240)*.

4.8. Aimmugen

Kaketsuken has developed and licensed Aimmugen as a three-dose 0.5 μg regimen for adults over the age of 16 to be administered as a two-dose primary regimen over a 4 wk interval with a third booster dose of the same potency at 6 mo *(4)*. Aimmugen is distinguished as the only licensed inactivated HAV vaccine preserved in lyophilized form before resuspension in liquid form for administration *(4)*.

4.8.1. Manufacture and Characterization of Aimmugen

Aimmugen is manufactured with the KRM 003 strain of HAV, which was adapted to replication in the GL 37 strain of AGMK cells though 72 serial passages, during which it was cloned four times by limiting dilution passage. The virus for clinical trials was purified from AGMK cell lysates by an extensive process of clarifying centrifugations, PEG precipitation, organic extraction, digestions with Proteinase K and DNAase I, concentration with organic solvents, and finally size-exclusion chromatography that yields a highly purified preparation by the criteria of SDS/PAGE analysis. The extent to which this process has been retained for large-scale manufacture has not been reported. The purity of the licensed Aimmugen product has not been described *(111)*.

The HAV preparation is inactivated with 100 μg/mL formaldehyde at 37°C for 12 d and assayed for residual infectivity by incubation of aliquots of the bulk-inactivated product in GL37 cell monolayer cell culture. The inactivated product is suspended in phosphate buffered saline, stabilized by the addition of lactose, D-sorbitol, L-sodium glutamate, arginine hydrochloride, and polysorbate 80, and then lyophilized from a 1-mL vol. Aimmugen is recommended for storage at temperatures below 10°C, under which conditions the vaccine is licensed with a 3-yr shelf life *(4)*.

4.8.2. Clinical Evaluation of Aimmugen

The clinical evaluation of Aimmugen was initiated in 1988 with evaluations of the safety and immunogenicity of various formulations adminis-

tered as a three-dose 0.5 μg regimen of two primary doses over a 2–4 wk interval with a third booster dose at 24 wk *(111)*. Of these formulations, a lyophilized adjuvant-free preparation proved to be superior in immunogenicity to an aqueous formulation and only twofold less immunopotent than an alum formulation, and for these reasons the lyophilized formulation has been carried forward through clinical trials *(111)*.

The immunogenicity of the vaccine has been reported from several clinical studies conducted in cohorts of healthy adults with a mean age of 28–34 yr at doses of 0.25 μg, 0.5 μg, and 1.0 μg *(78,79,111)*. These doses are generally well tolerated and immunogenic, promoting seroconversion of all recipients within 4 wk of the second dose to GMTs of antibody detected by RIA and by neutralizing antibody within several-fold of recipients of IG.

The immunopotency of a three-dose 0.5-mg regimen has been evaluated in a small-scale trial of hemodialysis patients and shown to induce rates and GMTs of seroconversion indistinguishable from those observed in normal healthy recipients of Aimmugen *(129)*.

5. Non-Classical Approaches for the Developments of Vaccines for Hepatitis A

The licensure of five inactivated HAV vaccines since 1991 offers safe, convenient, efficacious, and widely available alternatives to the use of IG under most circumstances of prophylaxis. These vaccines have been well received in developed nations for groups at increased risk for hepatitis A, and their use will expand as the titers of anti-HAV antibody in IG preparations and hence the efficacy of passive immunoprophylaxis continues to decline. The development of inactivated HAV vaccines continues to be pursued in several national laboratories throughout the world, encouraged by health authorities, and the formulation of these products can be expected to be tailored to fill the particular medical needs dictated by unique national circumstances of endemic disease *(60,130)*.

The broader use of the HAV vaccines already licensed will be dictated by pressing issues of cost effectiveness. These pharmacoeconomic issues continue to motivate the preclinical development of subunit HAV vaccines, which in theory can be developed and manufactured at greatly reduced cost. These developmental efforts have focused on judiciously selected peptide components of the immunodominant antigenic site of the HAV capsid linked to various proteins and formulated with different adjuvants, various purified HAV capsid proteins, and, more recently, 80S empty HAV capsids which can be assembled by the expression of the capsid protein region of HAV in various recombinant virus systems.

Although the promise of an inexpensive subunit vaccine prophylaxis for hepatitis A has yet to be fulfilled, the prospects for such a triumph have not dimmed and will certainly be brightened with a more sophisticated understanding of the antigenic structure of the HAV capsid. Such a reward must await the resolution of the crystal structure of the HAV capsid, which has not yet been reported and with the development of large-scale methods for the production and purification of HAV capsids prepared with recombinant DNA methods.

5.1. Recombinant Approaches to HAV Vaccine Development

5.1.1. Subunit Peptide or Capsid Protein Vaccines

The subunit approach to the development of HAV vaccines was pioneered by Emini et al. at MRL, who identified by computer modeling a 13-residue peptide spanning amino acids 11–25 of the HAV VP 1 capsid protein, which shared surface accessibility homologies with peptides of the VP1 of poliovirus that contributed to the major antigenic site of poliovirus *(66)*. This peptide, free in solution, reacted by ELISA with convalescent anti-HAV antibody and was therefore linked covalently to various carrier proteins and reported to induce an antibody response to HAV in rabbits or guinea pigs. This antibody, though it competed in immunoassays with convalescent human anti-HAV antisera for binding to HAV and was capable of the neutralization of the growth of HAV in cell culture, was nonetheless weak when compared with the neutralizing responses induced in these rodent models with whole virus immunogens *(66)*.

The immunogenicity of the HAV VP1 peptide has been compared for immunogenicity when covalently coupled to keyhole limpet hemocyanin, incorporated into tetrameric branched lysine cores or presented encapsulated in multilamellar liposomes *(98)*. Of these formulations, the liposome formulation was most immunopotent though it remained several thousandfold less immunopotent on a mass basis than the native HAV capsid.

The VP1 peptide has been developed by Lemon et al. as a polio chimera by the insertion of the peptide into the B-C loop of the VP1 protein of the Sabin strain type 1-poliovirus and studied for immunopotency in rabbits, guinea pigs, and mice *(138)*. This chimera and a series of other chimeras developed with peptides that included amino acids from VP1 and VP3, which contribute to the immunodominant antigenic site of HAV induced significant titers of antipeptide detected by ELISA but induced in only a few animals neutralizing antibody at generally low titer *(138)*.

These results with various HAV capsid peptides have discourage continued investigations with peptide approaches to HAV immunoprophylaxis, though they will certainly be invigorated as information derived from the

crystal structure of HAV is combined with technical advances in the synthesis of peptidomimetics with appropriate geometries for immunogenicity. The immunogenicity of these peptide sequences in the larger context of the capsid proteins was first reported in 1985 by Hughes et al. at MRL, who immunized mice with either the VP1, VP2, or VP3 capsid proteins of HAV purified from denatured whole virus capsids by SDS/PAGE gels *(110)*. All three proteins induced anti-HAV responses which reacted robustly with HAV capsids, and although these antibodies were shown to compete with convalescent antisera in binding assays, they were only weakly neutralizing when studied with HAV propagation assays in vitro. The immunogenicity of the VP1 and VP3 capsid protein was subsequently confirmed in a number of laboratories using methods of recombinant DNA technology to express the VP1 and VP3 proteins *(178)* as Trp E *(84,116)* or β-galactosidase fusions *(188)*, and in general these studies have reported only modest neutralizing antibody titers, which have discouraged the vigorous pursuit of these subunit approaches. Though only weakly immunogenic, these fusion proteins have been demonstrated to efficiently prime for the induction of stable neutralizing antibody responses in rabbits immunized with a subimmunogenic dose of HAV and their use in conjunction with greatly reduced antigenic masses of HAV will continue to draw interest *(84,188)*.

5.1.2. HAV Vaccines Developed with Recombinant Viruses

Recombinant DNA methods that permit the introduction of genetic elements encoding the capsid region of HAV into various virus vectors have more recently been explored in an attempt to produce HAV capsids which present the epitopes of capsid proteins in natural conformations essential to induction of neutralizing antibody. The utility of vaccinia virus vectors for the synthesis of the structural proteins of the HAV capsid proteins VP1, VP2, and VP3 following the infection of Vero, MRC-5, or HeLa cells in vitro have been explored in several laboratories *(93,121,255,258)*. These studies have reported the synthesis and appropriate cleavage of the structural polyprotein *(93)* and the production of particulate antigens, which sediment as 70S empty capsids in gradients of sucrose and CsCl and that appear by electron microscopy to resemble the empty capsids produced as a consequence of in vivo and in vitro infections with HAV *(121,259)*. These particulate antigens have been shown to induce a neutralizing antibody response that affords protective immunity in marmosets against a challenge with wild-type HAV *(121)*.

While these experiments with recombinant vaccinia virus demonstrate that appropriate cleavage of the capsid polyprotein of HAV could be realized in eukaryotic systems without the intercession of other HAV proteins, the continued development of these systems for the production of vaccines

for human use must confront issues of productivity and the need for further purification which are problems familiar to manufacturers of currently licensed vaccines. These same issues will vex the development of HAV vaccines using empty virus capsids synthesized in insect cells following infection with recombinant baculovirus vectors *(212,228)*. These empty capsids have been demonstrated to induce neutralizing antibody in rodent models, and studies of their assembly from 14S precursors has provided fundamentally important information on the assembly of the HAV capsid and the conformational pathways that lead to the acquisition of epitopes which induce protective immunity *(212,228)*.

Acknowledgment

I want very much to thank my many colleagues in the Merck Research Laboratories and the Merck Vaccine Division, especially Dr. Philip Provost and Dr. Maurice Hilleman, for their encouragement and numerous thoughtful suggestions. Their contributions have greatly improved this discussion of the vaccines that control HAV, on which they have long focused their productive attention.

References

1. Aboud, R. A., Aunins, J. G., Buckland, B. C., DePhillips, P. A. ., Hagen, A. J., Hennessey, J. P. Jr., Junker, B., Lewis, J. A., Oliver, C. N., Orella, C. J., Sitrin, R. D. (1994) *Hepatitis A virus vaccine*. Patent Application WO 94/03589, Published Feb. 17, 1994.
2. Adler, R. and Shouval, D. (1993) A finger-stick assay for determination of immunity to hepatitis A: a preliminary report. *J. Hepatol.* **18**(Suppl. 2), S27-S31.
3. Adler, R. and Shouval, D. (1996) Hepatitis A prophylaxis: vaccine or immunoglobulin? *Clin. Immunother.* **4**, 261–272.
4. Aimmugen®, Kaketsukan, Kumamoto, Japan, package circular.
5. Ambrosch, F., Wiedermann. G., Andre, F. E., D'Hondt. E., Delem, A., and Safary A. (1991) Comparison of HAV antibodies induced by vaccination, passive immunization and natural infection, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), *Proc. 1990 Intern. Symp. Viral Hep. Liver Dis.* Williams and Wilkins, Philadelphia, PA, pp. 98–100.
6. Ambrosch, F., Andre, F. E., Delem, A., D'Hondt, E., Jonas, S., Kunz, Ch., Safary, A., and Wiedermann, G. (1992) Simultaneous vaccination against hepatitis A and B: results of a controlled study. *Vaccine* **10**(Suppl. 1), 142–145.
7. Ambrosch, F., Widermann, G., Andre, F. E., Delem, A., Gregor, H., Hofmann, H., D'Hondt, E., Kundi, M., Wynen, J., and Kunz, Ch (1994) Clinical and immunological investigation of a new combined hepatitis A and hepatitis B vaccine. *J. Med. Virol.* **44**, 452–456.
8. American Academy of Pediatrics, Committee on Infectious Disease. (1996) Prevention of hepatitis A infections: guidelines for use of hepatitis A vaccine and immune globulin. *Pediatrics* **98**, 1207–1215.

9. Anderson, D. A. (1987) Cytopathology, plaque assay, and heat inactivation of hepatitis A virus strain HM 175. *J. Med. Virol.* **22**, 35–44.
10. Anderson, D. A., Ross, B. C., and Locarnini, S. A. (1988) Restricted replication of hepatitis A virus in cell culture: encapsidation of viral RNA depletes the pool of RNA available for replication. *J. Virol.* **62**, 4201–4206.
11. Andre, F. E., Hepburn, A., and D'Hondt, E. (1990) Inactivated candidate vaccines for hepatitis A. *Prog. Med. Virol.* **37**, 72–95.
12. Andre, F. E. (1995) Approaches to a vaccine against hepatitis A; development and manufacture of an inactivated vaccine. *J. Inf. Dis.* **171**(Suppl. 1), 33–39.
13. Armstrong, M. E., Giesa, P. A., Davide, J. P., Redner, F., Waterbury, J. A., Rhoad, A. E., Keys, R. D., Provost, P. J., and Lewis, J. A. (1993) Development of the formalin-inactivated hepatitis A vaccine VAQTA from the live attenuated virus strain CR326F. *J. Hepatol.* **18**(Suppl. 1), S20–S26.
14. Asher, L. V., Binn, L. N., Mensing, T. L., Marchwicki, R. H., Vassell, R. A., and Young, G. D. (1995) Pathogenesis of hepatitis A in orally inoculated owl monkeys (A. trivirgatus). *J. Med. Virol.* **47**, 260–268.
15. AVAXIM™. Pasteur Merieux Serum and Vaccines, Marnes-La-Coquette, France, package circular.
16. Bader, T. F. (1996) Hepatitis A vaccine. *Amer. J. Gastroenterol.* **91**, 217–222.
17. Barrett, P. N., Meyer, H., Wachtel, I., Eibl, J., and Dorner, F. (1996) Determination of the inactivation kinetics of hepatitis A virus in human plasma products using a simple $TCID_{50}$ assay. *J. Med. Virol.* **49**, 1–6.
18. Beales, L. P., Wood, D. J., Minor, P. D., and Saldanha J. A. (1996) A novel cytopathic microtiter plate assay for hepatitis A virus and anti-hepatitis A neutralizing antibodies. *J. Vir. Meth.* **59**, 147–154.
19. Berger, R., Just, M., and Althaus, B. (1993) Time course of hepatitis A antibody production after active, passive and active/passive immunization: the results are highly dependent on the antibody test system used. *J. Virol. Meth.* **43**, 287–298.
20. Binn, L. N., Lemon, S. M., Marchwicki, R. H., Redfield, R. R., Gates, N. L., and Bancroft, W. H. (1984) Primary isolation and serial passage of hepatitis A virus strains in primate cell culture. *J. Clin. Microbiol.* **20**, 28–33.
21. Binn, L. N., Bancroft, W. H., Lemon, S. M., Marchwicki, R. H., LeDuc, J. W., Trahan, C. J., Staley, E. C., and Keenan, C. M. (1986) Preparation of a prototype inactivated hepatitis A virus vaccine from infected cell cultures. *J. Inf. Dis.* **153**, 749–756.
22. Binn, L. N., MacArthy, P. O., Marchwicki, R. H., Sjogren, M. H., Hoke, C. H., Burge, J. R., and D'Hondt, E. (1992) Laboratory tests and reference reagents employed in studies of inactivated hepatitis A vaccine. *Vaccine* **10**(Suppl. 1), S102–105.
23. Block, S. L., Hedrick, J. A., Tyler, R. D., Smith, R. A., Calandra, G. B., Patterson, C., Lewis, J. A, Sitrin, R. D., Miller, W. J., Schwartz, S., Wiens, B. L., and Nalin, D. R. (1993) Safety, tolerability, and immunogenicity of a formalin inactivated hepatitis A vaccine trial in rural Kentucky children. *Ped. Infect. Dis. J.* **12**, 976–980.
24. Boggs, J. D., Melnick, J. L., Conrad, M. E., and Felsher, B. (1970) Viral hepatitis: clinical and tissue culture studies. *JAMA* **214**, 1041–1046.
25. Bradley, D. W., Hornbeck, C. L., Gravelle, C. R., Cook, E. H., and Maynard, J. E. (1975) CsCl banding of hepatitis A associated virus like particles. *J. Inf. Dis.* **31**, 304–305.
26. Bradley, D. W., Hornbeck, C. L., Cook, E. H., and Maynard, J. E. (1975) Purification of hepatitis A virus from chimpanzee stools. *J. Virol.* **22**, 228–231.

27. Bradley, D. W., McCaustland, K. A., Schreeder, M. T., Cook, E. H., Gravelle, C. R., and Maynard, J. E. (1977) Multiple buoyant densities of hepatitis A virus in cesium chloride gradients. *J. Med. Virol.* **1**, 219–226.
28. Bradley, D. W., Fields, H. A., McCaustland, K. A., Cook, E. H., Cravelle, C. R., and Maynard, J. E. (1978) Biochemical and biophysical characterization of light and heavy density hepatitis A virus particles. *J. Med. Virol.* **2**, 175–187.
29. Bradley, D. W., Schable, C. A., McCaustland, K. A., Cook, E. A., Murphy, B. L., Fields, H. A., Ebert, J. W., Wheeler, C., and Maynard, J. E. (1984) Hepatitis A virus: growth characteristics of in vivo and in vitro propagated wild and attenuated virus strains. *J. Med. Virol.* **14**, 373–386.
30. Briem, H. and Safary, A. (1994) Immunogenicity and safety in adults of hepatitis A vaccine administered as a single dose with a booster six months later. *J. Med. Virol.* **44**, 443–445.
31. Brindle, R. J., Morris, C. A., Berger, R., and Kurtz, J. B. (1994) Inadequate response to intradermal hepatitis A vaccine. *Vaccine* **12**, 483–484.
32. Brown, E. A., Jansen, R. W., and Lemon, S. M. (1989) Characterization of a simian hepatitis A virus (HAV): antigenic and genetic comparison with human HAV. *J. Virol.* **63**, 4932–4937.
33. Bruguera, M., Bayas, J., Vilella, A., Tural, C., Gonzalez, A., Vidal, J., Dal-Re, R., and Salleras, L. (1996) Immunogenicity and reactogenicity of a combined hepatitis A and B vaccine in young adults. *Vaccine* **14**, 1407–1411.
34. Bulletin of the World Health Organization. (1995) *Public Health Control of Hep. A* **73**, 15–20.
35. Carlsson, U., Brundin, L., Eliasson, I., Hansson, B. G. (1996) Hepatitis A vaccination by intracutaneous low dose administration: a less expensive alternative. *Scand. J. Inf. Dis.* **28**, 435-438.
36. Chaudhary, R. K., Parker, C., and Mo, T. (1988) Tissue culture-grown hepatitis A virus vaccine, in *Viral Hepatitis and Liver Disease* (Zuckerman, A. J., ed.), Alan R. Liss, New York, pp. 97–99.
37. Clemens, R., Safary, A., Hepburn, A., Roche, C., Stanbury, W. J., and Andre, F. E. (1995) Clinical experience with an inactivated hepatitis A vaccine. *J. Inf. Dis.* **171**(Suppl. 1), S44-S49.
38. Code of Federal Regulations: Food and Drug Administration Human Health and Services, USA. **1993**, 21CFR 630. 1–630. 8.
39. Cohen, J. I., Ticehurst, J. R., Purcell, R. H., Buckler-White, A., and Baroudy, B. M. (1987) Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. *J. Virol.* **61**, 50–59.
40. Cohen, J. I., Rosenblum, B., Ticehurst, J. R., Daemer, R. J., Feinstone,S. M., and Purcell, R. H. (1987) Complete nucleotide sequence of an attenuated hepatitis A virus: comparison with wild-type virus. *Proc. Natl. Acad. Sci. USA* **84**, 2497–2501.
41. Cohen, J. I., Feinstone, S. and Purcell, R. H. (1989) Hepatitis A virus infection in a chimpanzee: duration of viremia and detection of virus in saliva and throat swabs. *J. Inf. Dis.* **5**, 887–890.
42. Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L. (1946) Preparation and properties of serum and plasma proteins. *J. Am. Chem. Soc.* **68**, 459–475.
43. Coulepsis, A. G., Locarnini, S. A., Westaway, E. G., Tannock, G. A., and Gust, I. D. (1982) Biophysical and biochemical characterization of hepatitis A virus. *Intervirology* **18**, 107–127.

44. Coulepsis, A. G., Locarnini, S. A., Ferris, A. A. Lehmann, N. I., Gust, I. D. (1978) The polypeptides of hepatitis A virus. *Intervirology* **10**, 24–31.
45. Crevat, D., Crance, C. J., Chevrinais, A. M., Passagot, J., Biziagos, E., Somme, G., and Deloince, R. (1990) Monoclonal antibodies against an immunodominant and neutralizing epitope on hepatitis A virus antigen. *Arch. Virol.* **13**, 95–98.
46. Cromeans, T., Sobsey, M., and Fields, H. A. (1987) Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. *J. Med. Virol.* **22**, 45–56.
47. Daemer, R. J., Feinstone, S. M., Gust, I. D., and Purcell, R. H. (1981) Propagation of human hepatitis A virus in African green monkey kidney cell culture: primary isolation and serial passage. *Infect. Immun.* **32**, 388–393.
48. Davidson, M., Krugman, S., and Sandman, L. A. (1992) Inactivated hepatitis A vaccine: safety and immunogenicity study in health professionals. *Vaccine* **10**(Suppl. 1), S119–S121.
49. De Chastonay, J. and Siegl, G. (1987) Replicative events in hepatitis A virus infected MRC-5 cells. *Virology* **157**, 268–275.
50. Decker, R. H., Overby, L. R., Ling, G. M., Frosner, G. G., Deinhardt, F., and Boggs, J. (1979) Serologic studies of transmission of hepatitis A in humans. *J. Inf. Dis.* **139**, 74–82.
51. Deinhardt, F., Holmes, A. W., Capps, R. B., and Popper, H. (1967) Studies on the transmission of human viral hepatitis to marmoset monkeys. I. Transmission of disease, serial passages and description of liver lesions. *J. Exp. Med.* **125**, 673–688.
52. Delem, A., Safary, A., De Namur, F., Hauser, P., and D'Hondt, E. (1993) Characterization of the immune response of volunteers vaccinated with a killed vaccine against hepatitis A. *Vaccine* **11**, 479–484.
53. Delem, A. D. (1992) Comparison of modified HAVAB and ELISA for determination of vaccine induced anti-HAV response. *Biologicals* **20**, 289–291.
54. D'Hondt, E. and Delem, A. (1994) Inactivated virosome hepatitis A vaccine. *Lancet* **343**, 1165–1166.
55. D'Hondt, E., Purcell, R. H., Emerson, S. U., Wong, D. C., Shapiro, M., and Govindarajan, S. (1995) Efficacy of an inactivated hepatitis A vaccine in pre and post exprosure conditions in marmosets. *J. Inf. Dis.* **171**(Suppl. 1), S40-S43.
56. Dienstag, J. L., Feinstone, S. M., Purcell, R. H., Hoofnagle, J. H., Barker, L. F., London, W. T., Popper, H., Peterson, J. M., and Kapikian, A. Z. (1975) Experimental infection of chimpanzees with hepatitis A virus. *J. Inf. Dis.* **132**, 532–545
57. Dienstag, J. L., Popper, H., and Purcell, R. H. (1976) The pathology of viral hepatitis types A and B in chimpanzees: a comparison. *Amer. J. Pathol.* **85**, 131–148.
58. Dienstag, J. L., Schulman, A. N., Gerety, R. J., Hoofnagle, J. H., Lorenz, D. E., Purcell, R. H., and Barker, L. F. (1976) Hepatitis A antigen isolated from liver and stool: Immunologic comparison of antisera prepared in guinea pigs. *J. Immunol.* **117**, 876–881.
59. Dotzauer, A., Feinstone, S. M., and Kaplan, G. (1994) Susceptibility of non-primate cell lines to hepatitis A virus infection. *J. Virol.* **68**, 6064–6068.
60. Elbert, L. B., Lisitzina, E. A., Kusov, Y. Y, and Balayan, M. S. (1992) Immunogenicity of inactivated purified tissue culture vaccine against hepatitis A assessed in laboratory rodents. *Vaccine* **10**, 828–832.
61. Ellerbeck, E., Lewis, J. A., Midthun, K., Gershman, K., Nalin, D. R., McCaughtry, M., Provost, P. J., and Calandra, G. B. (1991) Use of a live-attenuated hepatitis A virus to prepare a highly purified formalin-inactivated hepatitis A vaccine, in *Viral*

Hepatitis and Liver Disease (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), *Proc. 1990 Intern. Symp. Viral Hep. Liver Dis.* Williams and Wilkins, Philadelphia, PA, pp. 91–93.

62. Ellerbeck, E. F, Lewis, J. A., Nalin, D. R., Gershman, K., Miller, W. J., Armstrong, M. E., Davide, J. P., Rhoad, A. E., McGuire, B., Calandra, G., Provost P. J., and Midthun, K. (1991) Safety profile and immunogenicity of an inactivated vaccine derived from an attenuated strain of hepatitis A virus. *Vaccine* **10**, 668–672.
63. Emerson, S. U., McRill, C., Rosenbluth, B., Feinstone, S., and Purcell, R. H. (1991) Mutations responsible for adaptation of hepatitis A virus to efficient growth in cell culture. *J. Virol.* **65**, 4882–4886.
64. Emerson, S. U., Huang, Y. K., McRill, C., Lewis, M., and Purcell, R. H. (1992) Mutations in both the 2B and 2C genes of hepatitis A virus are involved in adaptation to growth in cell culture. *J. Virol.* **66**, 650–654.
65. Emerson, S. U., Huang, Y. K., and Purcell, R. H. (1993) 2B and 2C mutations are essential but mutations throughout the genome of HAV contribute to adaptation to cell culture. *Virology* **194**, 475–480.
66. Emini, E. A., Hughes, J. V., Perlow, D. S., and Boger, J. (1985) Induction of hepatitis A virus neutralizing antibody by a virus-specific synthetic peptide. *J. Virol.* **55**, 836–839.
67. Epaxal®, Swiss Serum and Vaccine Institute, Berne, Switzerland, package circular.
68. Feinstone, S. M., Kapikian, A. Z., and Purcell, R. H. (1973) Hepatitis A: detection by immune electron microscopy of viruslike antigen associated with acute illness. *Science* **182**, 1026–1028.
69. Ferguson, M., Wood, D. J., and Minor, P. D. (1993) Antigenic structure of poliovirus in inactivated vaccines. *J. Gen. Virol.* **74**, 685–690.
70. Flehmig, B., Franck, H., Frosner, G., and Gerth, H. J (1977) Hepatitis A virus particles in stools of patients from a natural hepatitis outbreak in Germany. *Med. Microbiol. Immunol.* **163**, 209–214.
71. Flehmig, B. (1980) Hepatitis A virus in cell culture: Propagation of different hepatitis A virus isolates in a fetal rhesus monkey kidney cell line (FRhK4) *Med. Microbiol. Immunol.* **168**, 239–248.
72. Flehmig, B. (1981) Hepatitis A virus in cell culture: growth characteristics of hepatitis A virus in Frhk–4/cells. *Med. Microbiol. Immunol.* **170**, 73–81.
73. Flehmig, B. (1981) Hepatitis A virus in cell culture: propagation of hepatitis A virus in human embryo kidney cells and human embryo fibroblast strains. *Med. Microbiol. Immunol.* **170**, 83–89.
74. Flehmig, B., Haage, A., and Pfisterer, M. (1987) Immunogenicity of a hepatitis A virus vaccine. *J. Med. Virol.* **22**, 7–16.
75. Flehmig, B., Mauler, R. F., Noll, G., Weinmann, E. and Gregersen, J. P. (1988) Progress in the development of an attenuated hepatitis A vaccine, in *Viral Hepatitis and Liver Disease* (Zuckerman, A. J., ed.), Alan R. Liss, New York, pp. 87–90
76. Flehmig, B., Heinricy, U., and Pfisterer, M. (1989) Immunogenicity of a killed hepatitis A vaccine in seronegative volunteers. *Lancet* **1**, 1039–1041.
77. Frosner, G. G., Deinhardt, F., Scheid, R., Gauss-Muller, V., Holmes, N., Messelberger, V., Siegl, G., and Alexander, J. J. (1979) Propagation of human hepatitis A virus in a hepatoma cell line. *Infection* **7**, 303–305.
78. Fujiyama, S., Iino, S., Odoh, K., Kuzuhara, S., Watanabe, H., Tanaka, M., Mizuno, K., and Sato, T. (1992) Time course of hepatitis A virus antibody titer after active and passive immunization. *Hepatology* **15**, 983–989.

79. Fujiyama, S., Odoh, K., Kuramoto, I., Mizuno, K., Tsurusaki, R., and Sato, T. (1994) Current seroepidemiological status of hepatitis A with a comparison of antibody titers after infection and vaccination. *J. Hepatol.* **21**, 641–645.
80. Funkhouser, A. W., Purcell, R. H., D'Hondt, E., and Emerson, S. U. (1994) Attenuated hepatitis A virus: genetic determinants of adaptation to growth in MRC–5 cells. *J. Virol.* **68**, 148–157.
81. Funkhouser, A. W., Raychaudhuri, G., Purcell, R. H., Govindarajan, S., Elkins, R., and Emerson, S. U. (1996) Progress toward the development of a genetically engineered attenuated hepatitis A virus vaccine. *J. Virol.* **70**, 7948–7957.
82. Garin, D., Vidor, E., Wallon, M., Fanget, B., Brasseur, P., Delolme, H., Caron, F., Mojon, M., Gravey, A., Humbert, G., Flehmig, B., and Peyron, F. (1995) Good immunogenicity of GBM strain inactivated hepatitis A vaccine in healthy male adults. *Vaccine* **13**, 220–224.
83. Gauss-Muller, V., Frosner, G. G., and Deinhardt, F. (1981) Propagation of hepatitis A virus in human embryo fibroblasts. *J. Med. Virol.* **7**, 233–239.
84. Gauss-Muller, V., Mingquan, Z., von der Helm, K., and Deinhardt, F. (1990) Recombinant proteins VP1 and VP3 of hepatitis A virus prime for neutralizing response. *J. Med. Virol.* **31**, 277–283.
85. Gerety, R. J., Smallwood, L. A., Finlayson, J. S., and Tabor, E. (1983), Standardization of the antibody to hepatitis A virus (anti-HAV) content of immunoglobulin. *Dev. Biol. Standards* **54**, 411–416.
86. Gil, A., Gonzalez, A., Dal-Re, R., and Calero, J. R. (1996) Interference assessment of yellow fever vaccine with the immune response to a single-dose inactivated hepatitis A vaccine (1440 EU) A controlled study in adults. *Vaccine* **4**, 1028- 1030.
87. Gluck, R. (1992) Immunopotentiating reconstituted influenza virosomes and other adjuvants for improved presentation of small antigens. *Vaccine* **10**, 915–919.
88. Gluck, R., Mischler, R., Brantschen, S., Just, M., Althaus, B., and Cryz, S. J., Jr. (1992) Immunopotentiating reconstituted influenza virosome (IRIV) vaccine delivery system for immunization against hepatitis A. *J. Clin. Invest.* **90**, 2491- 2495.
89. Goilav, C., Zuckerman, J., Lafrenz, M., Vidor, E., Lauwers, S., Ratheau, C., Benichou, G., and Zuckerman, A. (1995) Immunogenicity and safety of a new inactivated hepatitis A vaccine in a comparative study. *J. Med. Virol.* **46**, 287–292.
90. Graff, J., Normann, A., Feinstone, S. M., and Flehmig, B. (1994) Nucleotide sequence of wild-type hepatitis A virus GBM in comparison with two cell culture-adapted variants. *J. Virol.* **68**, 548–554.
91. Green, M. S., Cohen, D., Lerman, Y., Sjogren, M., Binn, L. N., Zur, S., Slepon, R., Robin, G., Hoke, C., Bancroft, W., Safary, A., Danon, Y., and Wiener, M. (1992) Depression of the immune response to an inactivated hepatitis A vaccine administered concomitantly with immune globulin. *J. Inf. Dis.* **168**, 740–743.
92. Green, M. S., Cohen, D., Lerman, Y., Sjogren, M., Binn, L., Zaur, S., Slepon, R., Robin, G., Hoke, C., Bancroft, W., Safary, A., Danon, Y., and Wiener, M. (1994) A trial of the reactogenicity and immunogenicity of an inactivated hepatitis A vaccine. *Isr J. Med. Sci.* **30**, 485–488.
93. Guo, K. J., Gao, F., Liu, C. B., Ruan, L., and Chu, C. M. (1992) Characteristics and immunogenicity of hepatitis A virus antigens expressed by a recombinant vaccinia virus, in *Vaccines*, Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 295–298.
94. Gust, I. D., Lehmann, N. I., Crowe, S., McCrorie, M., Locarnini, S. A., and Lucas, C. R. (1985) The origin of the HM 175 strain of hepatitis A virus. *J. Inf. Dis.* **151**, 365–367.

95. Hadler, S. C. (1991) Global impact of hepatitis A virus infection: changing patterns, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), *Proc. 1990 Intern. Symp. Viral Hep. Liver Dis.* Williams and Wilkins, Philadelphia, PA, pp. 14–20.
96. Hagen, A. J., Oliver, C. N., and Sitrin, R. D. (1996) Optimization of polyethyleneglycol precipitation of hepatitis A virus used to prepare VAQTA, a highly purified inactivated vaccine. *Biotechnol. Prog.* **12**, 406–412.
97. Hagen, A. J., Aboud, R. A., DePhillips, P. A., Oliver, C. N., Orella, C. J., and Sitrin, R. D. (1996) Use of a nuclease enzyme in the purification of VAQTA, a hepatitis A vaccine. *Biotechnol. Appl. Biochem.* **23**, 209–215.
98. Haro, I., Pinto, R. M., Gonzalez-Dankaart, J. F., Perez, J. A., Reig, F., and Bosch, A. (1995) Anti-hepatitis virus antibody response elicited in mice by different forms of a synthetic VP1 peptide. *Microbiol. Immunol.* **39**, 485–490.
99. Havrix, SmithKline Beecham, Philadelphia, PA, package circular.
100. Hess, G., Clemens, R., Bienzle, U., Schonfeld, C., Schunck, B., and Bock, H. L. (1995) Immunogenicity and safety of an inactivated hepatitis A vaccine in anti-HIV positive and negative homosexual men. *J. Med. Virol.* **46**, 40–42.
101. Hilleman, M. R. (1993) Hepatitis and hepatitis A vaccine: a glimpse of history. *J. Hepatol.* **18**(Suppl. 2), S5–S10.
102. Hoke, C. H., Binn, L. N., Egan, J. E., DeFraites, R. F., MacArthy, P. O., Innis, B. L., Eckels, K. H., Dubois, D., D'Hondt, E., Sjogren, M. H., Rice, R., Sadoff, J. C., and Bancroft, W. H. (1992) Hepatitis A in the US army: epidemiology and vaccine development. *Vaccine* **10**(Suppl. 1), S75–S79.
103. Hoke, C. H., Egan, J. E., Sjogren, M. H., Sanchez, J., DeFraites, R. F., MacArthy, P., Binn, L. N., Rice, R., Burke, A., Kimes, M. H., Erikson, L., Boscia, J., Moonsamy, G. I., D'Hondt, E., and Bancroft, W. H. (1995) Administration of hepatitis A vaccine to a military population by needle and jet injector and with hepatitis B vaccine. *J. Inf. Dis.* **171**(Suppl. 1), S53–S60.
104. Hollinger, F. B., Bradley, D. W., Maynard, J. E., Dreesman, G. R., and Melnick, J. L. (1975) Detection of hepatitis A viral antigen by radioimmunoassay. *J. Immunol.* **15**, 1464–1466.
105. Holmes, A. W., Wolfe, L., Rosenblate, H., and Deinhardt, F. (1969) Hepatitis in marmosets: induction of disease with coded specimens from a human volunteer study. *Science* **165**, 816–817.
106. Holmes, A. W., Wolfe, Deinhardt, F., and Conrad, M. E. (1971) Transmission of human hepatitis to marmosets: further coded studies. *J. Inf. Dis.* **124**, 520–521.
107. Holzer, B. R., Hatz,C., Schmidt-Sissolak, D., Gluck, R., Althaus, B., and Egger, M. (1996) Immunogenicity and adverse effects of inactivated virosome versus alum-adsorbed hepatitis A vaccine: a randomized controlled trial. *Vaccine* **14**, 982–986.
108. Hornbeck, C. L., Gravelle, C. R., Hollinger, F. B., and Bradley, D. W. (1975) Agarose gel filtration of concentrated fecal extracts containing virus-like particles associated with hepatitis A. *Intervirology* **6**, 309–314.
109. Hughes, J. V., Stanton, L. W., Tomassini, J. E., Long, W. J., and Scolnick, E. M. (1984) Neutralizing monoclonal antibodies to hepatitis A virus: partial localization of a neutralizing antigenic site. *J. Virol.* **52**, 465–473.
110. Hughes, J. V. and Stanton, L. W. (1985) Isolation and immunizations with hepatitis A viral structural proteins: induction of antiprotein, antiviral and neutralizing responses. *J. Virol.* **55**, 395–401.

111. Iino, S., Fujiyama, S., Horiuchi, K., Jyo, K., Kuwabara, Y., Sato, S., Saika, S., Morita, M., Odoh, K., Kuzuhara, S., Watanabe, H., Tanaka, M., and Mizuno, K. (1992) Clinical trial of a lyophilized inactivated hepatitis A candidate vaccine in healthy adult volunteers. *Vaccine* **10**, 323–328.
112. Innis, B. L., Snitbhan, R., Kunasol, P., Laorakpongse, T., Poopatanakool, W., Kosik, C. A., Suntayakorn, S., Suknuntapong, T., Safary, A., Tang, D. B., and Boslego, J. W. (1994) Protection against hepatitis A by an inactivated vaccine. *JAMA* **271**, 1328–1334.
113. Jacobs, J. P. (1976) The status of human diploid cell strain MRC–5 as an approved substrate for the production of viral vaccines. *J. Biol. Stand.* **4**, 97–99.
114. Jansen, R. W., Newbold, J. E., and Lemon, S. M. (1985) Combined immunoaffinity cDNA-RNA hybridization assay for detection of hepatitis A virus RNA in clinical specimens. *J. Clin. Microbiol.* **22**, 984–989.
115. Jansen, R. W., Siegl, G., and Lemon, S. M. (1990) Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. *Proc. Natl. Acad. Sci. USA* **87**, 2867–2871.
116. Johnston, J. M., Harmon, S. A., Binn, L. N., Richards, O. C., Ehrenfeld, E., and Summers, D. F. (1988) Antigenic and immunogenic properties of a hepatitis virus capsid protein expressed in E. coli. *J. Inf. Dis.* **157**, 1203–1211.
117. Just, M., Berger, R., Drechsler, H., Brantschen, S., and Gluck, R. (1992) A single vaccination with an inactivated hepatitis A liposome vaccine induces protective antibodies after only two weeks. *Vaccine* **10**, 737–739.
118. Kallinowski, B., Gmelin, K., Kommerell, B., Bock, H. L., Clemens, R., Scheiermann, N., Wohland, B., Hofmann, F., and Theilmann, L. (1992) Immunogenicity, reactogenicity and consistency of a new inactivated hepatitis A vaccine a randomized multicenter study with three consecutive vaccine lots. *Vaccine* **10**, 500–501.
119. Kallinowski, B., Bock, H. L., Clemens, R., and Theilmann, L. (1996) Immunogenicity and reactogenicity of a combined hepatitis A/B candidate vaccine: first results. *Liver* **16**, 271–273.
120. Karayiannis, P., Jowett, T., Enticott, M., Moore, D., Pignatelli, M., Brenes, F., Scheuer, P. J., and Thomas H. C. (1986) Hepatitis A replication in tamarins and host immune response in relation to pathogenesis of liver cell damage. *J. Med. Virol.* **18**, 261–276.
121. Karayiannis, P., O'Rourke, S., McGarvey, M., Luther, S., Waters, J., Goldin,R., and Thomas, H. C. (1991) A recombinant vaccinia virus expressing hepatitis A virus structural polypeptides: characterization and demonstration of protective immunogenicity. *J. Gen. Virol.* **72**, 2167–2172.
122. Karron, R. A., Daemer, R. J., Ticehurst, J., D'Hondt, E., Popper, H., Mihalik, K., Phillips, J., Feinstone, S. M., and Purcell, R. H. (1988) Studies of prototype live hepatitis A virus vaccines in primate models. *J. Inf. Dis.* **157**, 338–345.
123. Kojima, H., Shibayama, T., Sato, A., Suzuki, S., Ichida, F., and Hamad, C. (1981) Propagation of human hepatitis A virus in conventional cell lines. *J. Med. Virol.* **7**, 272–286.
124. Krah, D. L., Amin, R. D., Nalin, D. R., and Provost, P. J. (1991) A simple antigen reduction assay for the measurement of neutralizing antibodies to hepatitis A virus. *J. Inf. Dis.* **63**, 634–637.
125. Krause, D. S. (1996) Hepatitis A vaccines. *JAMA* **276**, 449.
126. Krawczynski, K. K., Bradley, D. W., Murphy, B. L., Ebert, J. W., Anderson, T. E., Doto, I. L., Nowoslawski, A., Duermeyer, W., and Maynard, J. E. (1981) Pathoge-

netic aspects of hepatitis A virus infection in enterically inoculated marmosets. *Am. J. Clin. Pathol.* **76**, 698–706.
127. Krugman, S., Ward, R., and Giles, J. P. (1962) The natural history of infectious hepatitis. *Amer. J. Med.* **32**, 717–728.
128. Krugman, S., Giles, J. P., and Hammond, J. (1967) Infectious hepatitis: evidence for two distinctive clinical, epidemiological, and immunological types of infection. *JAMA* **200**, 365–373.
129. Kuramoto, I., Fujiyama, S., Matsushita, K., and Sato, T. (1994) Immune response after hepatitis A vaccination in haemodialysis patients: comparison with hepatitis B vaccination. *J. Gastroenterol. Hepatol.* **9**, 228–231.
130. Kusov,Y., Elbert, L. B., Nelga, I. V., Grishina, G. K., Dunaevski, O. A., Kharin, N. V., Maslov, Y. N., Drozdov, S. G., and Balayan, M. S. (1991) Immunogenicity trial of inactivated hepatitis A virus vaccine in human volunteers. *Vaccine* **9**, 540–541.
131. LeDuc, J. W., Lemon, S. M., Keenan, C. M., Graham, R. R., Marchwicki, R. H., and Binn, L. H. (1983) Experimental infection of the New World owl monkey (Aotus trivirgatus) with hepatitis A virus. *Infect. Immun.* **40**, 766–772.
132. Leentvaar-Kuijpers, A., Coutinho, R. A., Brulein, V., and Safary, A. (1992) Simultaneous passive and active immunization against hepatitis A. *Vaccine* **10**(Suppl. 1), S138–S141.
133. Lemon, S. M., Binn, L. N., and Marchwicki, R. H. (1983) Radioimmunofocus assay for quantitation of hepatitis A virus in cell culture. *J. Clin. Microbiol.* **17**, 834–839.
134. Lemon, S. M. and Binn, L. N. (1983) Antigenic relatedness of two strains of hepatitis A virus determined by cross-neutralization. *Infect. Immun.* **42**, 418–420.
135. Lemon, S. M. and Binn, L. N. (1985) Serum neutralizing antibody response to hepatitis A virus. *J. Inf. Dis.* **148**, 1033–1049.
136. Lemon, S. M., Murphy, P. C., Shields, P. A., Ping, L. H., Feinstone, S. M., Cromeans, T., and Jansen, R. W. (1991) Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during persistent infection: evidence for genetic recombination. *J. Virol.* **65**, 2056–2065.
137. Lemon, S. M., Whetter, L., Chang, K. H., and Brown, E. A. (1992) Why do hepatitis viruses replicate so poorly in cell cultures? *FEBS Micro. Lett.* **100**, 455–461.
138. Lemon, S. M., Barclay, W., Ferguson, M., Murphy, P., Jing, L., Burke, K., Wood, D., Katrak, K., Sangar, D., Minor, P., and Almond, J. (1992) Immunogenicity and antigenicity of chimeric picornaviruses which express hepatitis A virus (HAV) peptide seqeuences: evidence for a neutralization domain near the amino terminus of VP1 of HAV. *Virology* **188**, 285–295.
139. Lemon, S. M. (1992) Hepatitis A virus: current concepts of the molecular virology, immunobiology and approaches to vaccine development. *Rev. Med. Virology* **2**, 73–87.
140. Lemon, S. M. (1993) Immunologic approaches to assessing the response to inactivated hepatitis A vaccine. *J. Hepatol.* **18**(Suppl. 2), S15-S19.
141. Lemon, S. M. and Shapiro, C. N. (1994) The value of immunization against hepatitis A. *Infect. Agents Dis.* **1**, 38–49.
142. Lemon S. M. and Thomas, D. L. (1997) Vaccines to prevent viral hepatitis. *N. Engl. J. Med.* **336**, 196–204.
143. Lemon, S. M., Murphy, P. C., Provost, P. J., Chalikonda, I., Davide, J. P., Schofield. T. L., Nalin, D. R., and Lewis, J. A. (1997) Immunoprecipitation and virus neutralization assays demonstrate qualitative differences between the protective antibody

responses to an inactivated hepatitis A vaccine and passive immunization with immune globulin. *J. Inf. Dis.* **176**, 9–19.
144. Lewis, J. A., Armstrong, M. E., Larson, V. M., Emini, E. A., Midthun, K., Ellerbeck, E., Nalin, D., Provost, P. J., and Calandra, G. B. (1991) Use of a live attenuated hepatitis A vaccine to prepare a highly purified formalin-inactivated hepatitis A vaccine, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), *Proc. 1990 Intern. Symp. Viral Hep. Liver Dis.* Williams and Wilkins, Philadelphia, PA, pp. 94–97.
145. Liebhaber, H., Krugman, S., Giles, J. P., and McGregor, D. (1964) Recovery of cytopathic agents from patient with infectious hepatitis: isolation and propagation in cultures of human diploid lung fibroblasts (WI–38) *Virology* **24**, 109–113.
146. Locarnini, S. A., Coulepsis, A. G., Ferris, A. A., Lehmann, N. I, and Gust, I. D. (1978) Purification of hepatitis A virus from human feces. *Intervirology* **10**, 300- 308.
147. Locarnini, S. A., Coulepsis, A. G., Westaway, E. G., and Gust, I. D. (1981) Restricted replication of human hepatitis A in cell culture: intracellular biochemical studies. *J. Virol.* **37**, 216–225.
148. Lonergan, G. (1995) Hepatitis A vaccine: which dose is best? *JAMA* **273**, 999.
149. Loutan, L., Bovier, P., Althaus, B., and Gluck, R. (1994) Inactivated virosome hepatitis A vaccine. *Lancet* **343**, 322–324.
150. Luo, M., Rossman, M., and Palmenberg, A. C. (1988) Prediction of three- dimensional models for foot and mouth disease virus and hepatitis A virus. *Virology* **166**, 503–514.
151. MacCallum, F. O. (1947) Homologous serum hepatitis. *Lancet* **2**, 691–692.
152. Mao, J., Dong, D. X., Zhang, H. Y., Chen, N. L., Zhang, X. Y., Huang, H. Y., Xie, R. Y., Zhou, T. J., Wan, Z. J., Wang, Y. Z., Hu, Z. H., Cao, Y. Y., Li, H. M., and Chu, C. M. (1989) Primary study of attenuated live hepatitis A vaccine (H2 Strain) in humans. *J. Inf. Dis.* **159**, 621–624.
153. Mao, J. S (1990) Development of live, attenuated hepatitis A vaccine (H2 Strain). *Vaccine* **8,** 523–524.
154. Mao, J. S., Dong, D. X., Zhang, S. Y., Zhang, H. Y., Chen, N. L., Huang, H. Y., Xie, R. Y., Chai, C. A., Zhou, T. J., Wu, D. M., and Zhang, H. C. (1991) Further studies of attenuated live hepatitis A vaccine (H2 strain) in humans, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), *Proc. 1990 Intern. Symp. Viral Hep. Liver Dis.* Williams and Wilkins, Philadelphia, PA, pp. 110–113.
155. Mao-J. S., Chen, N. L., Huang, H. Y., Chai, S. A., Dong, D. X., Cao, Y. Y., Zhang, H. Y., Wu, D. M., Zhang, S. Y. (1992) Development of live attenuated hepatitis A vaccine H2 strain. *Chin. Med. J.* **105**, 189–193.
156. Mascoli, C. C., Ittensohn, O. L., Villarejos, V. M., Arguedas, J. A., Provost, P. J., and Hilleman, M. R. (1973) Recovery of hepatitis agents in the marmoset from human cases occurring in Costa Rica. *Proc. Soc. Exp. Biol. Med.* **142**, 276–282.
157. Mathiesen, L. R., Drucker, J., Lorenz, D., Wagner, J. A., Gerety, R. J., and Purcell, R. H. (1977) Localization of hepatitis A antigen in marmoset organs during acute infection with hepatitis A virus. *Infect. Immun.* **138**, 369–377.
158. Mathiesen, L. R., Feinstone, S. M., Purcell, R. H., and Wagner, J. A. (1977) Detection of hepatitis A antigen by immunofluorescence. *Infect. Immun.* **18**, 524–530.
159. Mathiesen, L. R., Moller, A. M., Purcell, R. H., London, W. T., and Feinstone, S. M. (1980) Hepatitis A virus in the liver and intestine of marmosets after oral inoculation. *Infect. Immun.* **28**, 45–48.

160. Maynard, J. E., Bradley, D. W., Gravelle, C. R., Ebert, J. W., and Krushak, D. H. (1975) Preliminary studies of hepatitis A in chimpanzees. *J. Inf. Dis.* **131**, 194–197.
161. Maynard, J. E., Lorenz, D., Bradley, D. W., Feinstone, S. M., Krushak, D. H., Barker, L. F., and Purcell, R. H. (1977) Review of infectivity studies in non-human primates with virus-like particles associated with MS-1 hepatitis. *Amer. J. Med. Sci.* **270**, 81–85.
162. McGregor, A. W., Kornitschuk, M., Hurrell, G. R., Lehmann, N. I., Coulepsis, A. G., Locarnini, S. A., and Gust, I. D. (1983) Monoclonal antibodies against hepatitis A virus. *J. Clin. Microbiol.* **18**, 1237–1243.
163. McMahon, B. J., Beller, M., Williams, J., Schloss, M., Tanttilla, H., and Bulkow, L. (1996) A program to control an outbreak of hepatitis A in Alaska using an inactivated hepatitis A vaccine. *Arch. Pediatr. Adolesc. Med.* **150**, 733–739.
164. Melnick, J. L., and Boggs, J. D. (1972) Human volunteer and tissue culture studies of viral hepatitis. *Can. Med. Assoc. J.* **106**, 461–467.
165. Melnick, J. L. (1995) History and epidemiology of hepatitis A virus. *J. Inf. Dis.* **171**(Suppl. 1), S2–S8.
166. Midthun, K., Ellerbeck, E., Gershman, K., Calandra, G., Krah, D., McCaughtry, M., Nalin, D., and Provost, P. (1991) Safety and immunogenicity of a live attenuated hepatitis A virus vaccine in seronegative volunteers. *J. Inf. Dis.* **163**, 735–739.
167. Miller, M. J. (1995) Viral taxonomy. *Clin. Infect. Dis.* **16**, 612.
168. Miller, W. J., Provost, P. J., McAleer, W. J., Ittensohn, O. L., Villarejos, V. M., and Hilleman, M. R. (1975) Specific immune adherence assay for human hepatitis A antibody. Application to diagnostic and epidemiologic investigations. *Proc. Soc. Exp. Biol. Med.* **149**, 254–261.
169. Miller, W. J., Clark, W., Hurni, W., Kuter, B., Schofield, T., and Nalin, D. R. (1993) Sensitive assays for hepatitis A antibodies. *J. Med. Virol.* **41**, 201–204.
170. Moritsugu, Y., Dienstag, J. L., Valdesuso, J., Wong, D. C., Wagner, J., Routenberg, J. A., and Purcell, R. H. (1976) Purification of hepatitis A antigen rom feces and detection of antigen and antibody by immune adherence hemagglutination. *Infect. Immun.* **13**, 898–908.
171. Muller, R., Chriske, H., Deinhardt, F., Jilg, J., Theilmann, L., Hess, G., Hofmann, F., Hopf, U., Stickl, H., Maiwald, H., Bienzle, U., Schofield, C., Zimmermann, H., Dietrich, M., May, B., Bock, H. L., Clemens, R., and Bogaerts, H. (1992) Hepatitis A vaccination: schedule for accelerated immunization. *Vaccine* **10**(Suppl. 1), S124–S125.
172. Nainan, O. V., Brinton, M. A., and Margolis, H. S. (1992) Identification of amino acids located in the antibody binding sites of human hepatitis A virus. *Virology* **191**, 984–987.
173. Najarian, R., Caput, D., Gee, W., Potter, S. J., Renard, A., Merryweather, J., Van Nest, G., and Dina, D. (1985) Primary structure and gene organization of human hepatitis A virus. *Proc. Natl. Acad. Sci. USA* **82**, 2627–2631.
174. Nalin, D. R., Kuter, B., Brown, L., Patterson, C., Calandra, G. B., Werzberger, A., Shouval, D., Ellerbeck, E., Block, S. L., Bishop, R., Wiens, B., Schwartz, S. W., Lewis, J. A., Sitrin, R. D., Provost P. J., Miller, W. J., and Ryan, J. L. (1993) Worldwide experience with the CR326F-derived inactivated hepatitis A virus vaccine in pediatric and adult populations: an overview. *J. Hepatol.* **18**(Suppl. 2), S51-S55.
175. Nalin, D. R. (1995) VAQTA: Hepatitis A vaccine, purified, inactivated. *Drugs Future* **20**, 24–29.
176. Nalin, D. R. (1996) Hepatitis A vaccines. *JAMA* **276**, 450.
177. Nasser, A. M. and Metcalf, T. G. (1987) Production of cytopathology in FRhK6 cells by BSC-1 passaged hepatitis A virus. *Appl. Envirn. Microbiol.* **53**, 2967–2971.

178. Ostermayr, R., von der Helm, K., Gauss-Muller, V., Winnacker, E. L., and Deinhardt, F. (1987) Expression of hepatitis A virus cDNA in E. coli: antigenic VP1 recombinant protein. *J. Virol.* **61**, 3645–3647.
179. Parks, W. P. and Melnick, J. L. (1969) Attempted isolation of hepatitis viruses in marmosets. *J. Inf. Dis.* **120**, 539–547.
180. Parks, W. P., Melnick, J. L., Voss, W. R., Singer, D. B., Rosenberg, H. S., Alcott, J., and Casazza, A. M. (1969) Characterization of marmoset hepatitis virus. *J. Inf. Dis.* **120**, 548–559.
181. Paul, A. V., Tada, H., von der Helm, K., Wissel, T., Kiehn, R., Wimmer, E., and Deinhardt, F. (1987) The entire nucleotide sequence of the genome of human hepatitis A virus (MBB isolate) *Virus Res.* **8**, 153–171.
182. Peetermans, J. (1992) Production, quality control, and characterization of an inactivated hepatitis A vaccine. *Vaccine* **10**(Suppl. 1), S99–S101.
183. Ping, L. H., Jansen, R. W., Stapelton, J. T., Cohen, J. I., and Lemon, S. M. (1988) Identification of an immunodominant antigenic site involving the capsid protein VP3 of hepatitis A virus. *Proc. Natl. Acad. Sci. USA* **85**, 8281–8285.
184. Ping, L. H., and Lemon, S. M. (1992) Antigenic structure of human hepatitis A virus defined by analysis of escape mutants selected against murine monoclonal antibodies. *J. Virol.* **66**, 2208–2216.
185. Poovorawan, Y., Theamboonlers, A., Chumdermpadetsuk, S., Gluck, R., and Cryz, S. J., Jr. (1994) Control of a hepatitis A outbreak by active immunization of high-risk susceptible subjects. *J. Inf. Dis.* **169**, 228–229.
186. Poovorawan, Y., Theamboonlers, A., Chumdermpadetsuk, S., Gluck, R., and Cryz, S. J., Jr. (1995) Safety, immunogenicity, and kinetics of the immune response to a single dose of virosome-formulated hepatitis A vaccine. *Vaccine* **13**, 891–893.
187. Popper, H., Dienstag, J. L., Feinstone, S. M., Alter, H. J., and Purcell, R. H. (1980) The pathology of viral hepatitis in chimpanzees. *Virchows Arch A. Path. Anat. Histol.* **387**, 91–106.
188. Powdrill, T. F. and Johnston, J. M. (1991) Immunologic priming with recombinant hepatitis A virus capsid proteins produced in E. coli. *J. Virol.* **65**, 2686–2690.
189. Prikazsky, V., Olear, V., Cernoch, A., Safary, A., and Andre, F. E. (1994) Interrruption of an outbreak of hepatitis A in two villages by vaccination. *J. Med. Virol.* **44**, 457–459
190. Provost, P. J., Ittensohn, O. L., Villarejos, V. M., Arguedas, J. A., and Hilleman, M. R. (1973) Etiologic relationship of marmoset-propagated CR326 hepatitis A virus to hepatitis in man. *Proc. Soc. Exp. Biol. Med.* **142**, 1257–1267.
191. Provost, P. J., Ittensohn, O. L., Villarejos, V. M., and Hilleman, M. R. (1975) A specific complement fixation test for human hepatitis A employing CR326 virus antigen, diagnosis and epidemiology. *Proc. Soc. Exp. Biol. Med.* **148**, 962–969.
192. Provost P. J., Wolanski, B. S., Miller, W. J., Ittensohn, O. L., McAleer, W. J., and Hilleman, M. R. (1975) Physical, chemical, and morphologic dimensions of human hepatitis A virus strain CR326. *Proc. Soc. Exp. Biol. Med.* **148**, 532- 539.
193. Provost, P. J. and Hilleman, M. R. (1978) An inactivated hepatitis A vaccine prepared from infected marmoset liver. *Proc. Soc. Exp. Biol. Med.* **159**, 201- 203.
194. Provost, P. J. and Hilleman, M. R. (1979) Propagation of human hepatitis A virus in cell culture in vitro. *Proc. Soc. Exp. Biol. Med.* **160**, 213–221.
195. Provost, P. J., Giesa, P. A., McAleer, W. J., and Hilleman M. R. (1981) Isolation of hepatitis A virus in vitro in cell culture directly from human specimens. *Proc. Soc. Exp. Biol. Med.* **167**, 201–206.

196. Provost, P. J., Banker, F. S., Giesa, P. A., McAleer, W. J., Buynak, E. B., and Hilleman, M. R. (1982) Progress toward a live, attenuated human hepatitis A vaccine. *Proc. Soc. Exp. Biol. Med.* **170**, 8–14.
197. Provost, P. J., Conti, P. A., Giesa, P. A., Banker, F. S., Buynak, E. B., McAleer, W. J., and Hilleman, M. R. (1983) Studies in chimpanzees of live, attenuated hepatitis A vaccine candidates. *Proc. Soc. Exp. Biol. Med.* **172**, 357–363.
198. Provost P. J. (1984) In vitro propagation of hepatitis A virus, in *Hepatitis A* (Gerety, R. J., ed.), Academic, New York, pp. 245–261.
199. Provost, P. J., Hughes, J. V., Miller, W. J., Giesa, P. A., Banker, F. S., and Emini, E. A. (1986) An inactivated hepatitis A viral vaccine of cell culture origin. *J. Med. Virol.* **19**, 23–31.
200. Provost, P. J., Bishop, R. P., Gerety, R. J., Hilleman, M. R., McAleer, W. J., Scolnick, E. M., and Stevens C. E. (1986) New findings in live, attenuated hepatitis A vaccine development. *J. Med. Virol.* **20**, 165–175.
201. Provost, P. J., Emini, E. A., Lewis, J. A., and Gerety, R. J. (1988) Progress toward the development of a hepatitis A vaccine, in *Viral Hepatitis and Liver Disease* (Zuckerman, A. J., ed.), Alan R. Liss, New York, pp. 83–83.
202. Provost, P. J., Banker, F. S., Wadsworth, C. W., and Krah, D. L. (1991) Further evaluation of a live hepatitis A vaccine in marmosets. *J. Med. Virol.* **34**, 227- 231.
203. Purcell, R. H., Wong, D. C., Moritsugu Y. Dienstag, J., Routenberg J. A., and Boggs, J. D. (1976) A microtiter solid-phase radioimmunoassay for hepatitis A antigen and antibody. *J. Immunol.* **116**, 349–356.
204. Purcell, R. H. (1993) The discovery of the hepatitis viruses. *Gastroenterology* **104**, 955–963.
205. Rajan, E., Al Blouschi, S., O'Farrell, B., Shattock, A., Courtney, M. G., Safary, A., and Fielding, J. F. (1996) Two year hepatitis A vaccine is as good as new. *Vaccine* **14**, 1439–1441
206. Ran, L. A., Wang, D. Z., Duan, Q. Y., Yan, T. Y., Liu, Q. L., Luo, Y. G., Yang, D. Z., Yang, D. F., Xiao, Z., Xie, Q. J., Li, W. D., and Luo, S. B. (1993) Safety and immunogenicity of live attenuated hepatitis A virus vaccine H2 strain in humans. *Chin. Med. J.* **106**, 604–607.
207. Robertson, B. H., Khanna, B., Brown, V. K., and Margolis, H. S. (1988) Large- scale production of hepatitis A virus in cell culture: effect of type of infection on virus yield and cell integrity. *J. Gen. Virol.* **69**, 2129–2134.
208. Robertson, B. H., Khanna, B., Nainan, O. V., and Margolis, H. S. (1991) Epidemiological patterns of wild-type hepatitis A virus determined by genetic variation. *J. Inf. Dis.* **163**, 286–292.
209. Robertson, B. H., Jansen, R. W., Khanna, B., Totsuka, A., Nainan, O. V., Siegl, G., Widell, A., Margolis, H. S., Isomura, S., Ito, K., Ishizu, T., Moritsugu, Y., and Lemon, S. M. (1992) Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. *J. Gen. Virol.* **73**, 1365–1377.
210. Robertson, B. H., Jia, X. Y., Tian, H., Margolis, H. S., Summers, D. F., and Ehrenfeld, E. (1993) Antibody response to non-structural proteins of hepatitis A virus following infection. *J. Med. Virol.* **40**, 76–82.
211. Robertson, B. H., D'Hondt, Spelbring, J., Tian, H. W., Krawczynski, K., and Margolis, H. S. (1994) Effect of postexposure vaccination in a chimpanzee model of hepatitis A virus infection. *J. Med. Virol.* **43**, 249–251.
212. Rosen, E., Stapelton, J. T., and McLinden, J. (1993) Synthesis of immunogenic hepatitis A virus particles by recombinant baculovirus *Vaccine* **11**, 706–712.

213. Rothschild, C., Pol, S., Mattlinger, B., Reijasse, D., Torchet, M. F., and Gazengel, C. (1995) Vaccination against hepatitis A virus in French hemophilic children. *Vox. Sang.* **69**, 80–81.
214. Ruchti, F., Siegl, G., and Weitz, M. (1991) Identification and characterization of incomplete hepatitis A virus particles. *J. Gen. Virol.* **72**, 2159–2166.
215. Schulman, A. N., Dienstag, J. L., Jackson, D. R., Hoofnagle, J. H., Gerety, R. J., Purcell, R. H., and Barker, L. F. (1976) Hepatitis A antigen particles in liver, bile and stool of chimpanzees. *J. Inf. Dis.* **134**, 80–83.
216. Shapiro, C. N. and Margolis, H. S. (1993) Worldwide epidemiology of hepatitis A infection. *J. Hepatol.* **18**(Suppl. 2), S11-S14.
217. Shouval, D., Ashur, Y., Adler, R., Lewis, J., Armstrong, M., Davide, J., McGuire, B., Kuter, B., Brown, L., Miller, W., Elkana, Y., and Nalin, D. (1993) Single and booster dose responses to an inactivated hepaitis A virus vaccine: comparison with immune serum globulion prophylaxis. *Vaccine* **11**(Suppl. 1), S9-S14.
218. Shouval, D., Ashur, Y., Adler, R., Lewis, J. A., Miller, W., Kuter, B., Brown, L., and Nalin, D. R. (1993) Safety, tolerability, and immunogenicity of an inactivated hepatitis A vaccine: effects of single and booster injections and comparison to administration of immune globulin. *J. Hepatol.* **18**(Suppl. 2), S32–37.
219. Siegl, G., deChastonay, J., and Kronauer, G. (1984) Propagation and assay of hepatitis A virus in vitro. *J. Virol. Meth.* **9**, 53–67.
220. Siegl, G., Weitz, A., and Kronauer, G. (1984) Stability of hepatitis A virus. *Intervirology* **22**, 218–226.
221. Simmonds, R. S., Szucs, G., Metcalf, T. G., and Melnick, J. L. (1985) Persistently infected cultures as a source of hepatitis A virus. *Appl. Envirn. Micrbiol.* **49**, 749–755.
222. Sjogren, M. H., Binn, L. N., Eckels, K. H., LeDuc, J. W., Trahan, C. J., Burke, D. S., and Bancroft, W. H. (1987) Inactivated hepatitis A vaccine: production and preliminary human trial. *Gastroenterology* **92**, 1778.
223. Sjogren, M. H., Eckels, K. H.,Binn, L. N., Dubois, D. R., Hoke, ChH., Burke, D. S., and Bancroft, W. H. (1987) Safety and immunogenicity of an inactivated hepatitis A vaccine, in *Viral Hepatitis and Liver Disease* (Zuckerman, A. J., ed.), Alan R. Liss, New York, pp. 94–97.
224. Sjogren, M. H., Hoke, C. H., Binn, L. N., Eckels, K. H., Dubois, D. R., Lyde, L., Tsuchida, A., Oaks, S., Marchwicki, R., Lednar, W., Chloupek, R., Ticehurst, J., and Bancroft, W. H. (1991) Immunogenicity of an inactivated hepatitis A vaccine. *Ann. Int. Med.* **114**, 470–471.
225. Sjogren, M. H., Purcell, R. H., McKee, K., Binn, L., Macarthy, P., Ticehurst, J., Feinstone, S., Caudill, J., See, A., Hoke, C., Bancroft, W., and D'Hondt E. (1992) Clinical and laboratory observations following oral or intramuscular administration of a live attenuated hepatitis A vaccine candidate. *Vaccine* **10**(Suppl. 1), S135–137.
226. Stapelton, J. T., Jansen, R. W, and Lemon, S. M. (1985) Serum neutralizing antibody to hepatitis A virus in immune serum globulin and in the sera of human recipients of immune serum globulin. *Gastroenterology* **89**, 637–642.
227. Stapelton, J. T. and Lemon, S. M. (1987) Neutralization escape mutants define a dominant immunogenic neutralization site on hepatitis A virus. *J. Virol.* **61**, 491–498.
228. Stapelton, J. T., Raina, V., Winokur, P., Walters, K., Klinzman, D., Rosen, E., and McLinden J. H. (1993) Antigenic and immunogenic properties of recombinant hepatitis A virus 14S and 70S subviral particles. *J. Virol.* **67**, 1080–1085.

229. Stapelton, J. T. (1992) Passive immunization against hepatitis A. *Vaccine* **10**(Suppl. 1), S45–S47.
230. Stapelton, J. T. (1995) Host immune response to hepatitis A virus. *J. Inf. Dis.* **171**(Suppl. 1), S9–S14.
231. Stokes, J. and Neefe, J. R. (1945) The prevention and attenuation of infectious hepatitis by gamma globulin. *JAMA* **129**, 270–272.
232. Takeda, Y., Ashida, M., and Hamada, C. (1996) Growth of hepatitis A virus in murine cells. *Acta. Virologica* **40**, 201–208.
233. Tedeschi, V., Purcell, R. H., and Emerson, S. U. (1993) Partial characterization of hepatitis A viruses from three intermediate passage levels of a series resulting in adaptation to growth in cell culture and attenuationof virulence. *J. Med. Virol.* **39**, 16–22.
234. Tilzey, A. J., Palmer, S. J., Barrow, S., Perry, K. R., Tyrell, H., Safary, A., and Banatvala, J. E. (1992) Effect of hepatitis A vaccination schedule on immune response. *Vaccine* **10**(Suppl. 1), S121–S123.
235. Vallbracht, A., Hofmann, L., Wurster, K. G., and Flehmig, B. (1984) Persistent infection of human fibroblasts by hepatitis A virus. *J. Gen. Virol.* **65**, 609–615.
236. Van Damme, P., Mathei, C., Thoelen, S., Meheus, A., Safary, A., and Andre, F. E. (1994) Single dose inactivated hepatitis A vaccine: rationale and clinical assessment of the safety and immunogenicity. *J. Med. Virol.* **44**, 435–445.
237. Van Damme, P., Thoelen, S., Cramm, M., DeGroote, K., Safary, A., and Banatvala, J. E. (1992) Inactivated hepatitis A vaccine: reactogenicity, immunogenicity, and long-term antibody persistence. *J. Med. Virol.* **44**, 446–451.
238. Van Damme, P., Thoelen, S., Cramm, M., and Meheus, A. (1996) Safety and immunogenicity of a high potency inactivated hepatitis A vaccine. *J. Travel Med.* **3**, 83–90.
239. Vaqta®, Merck and Co., package circular.
240. Vidor, E., Xueref, C., Blondeau, C., Bajard, A., Francon, A., Goudeau, A., Peyron, F., Brasseur, P., and Zuckerman, A. (1996) Analysis of the antibody response in humans with a new inactivated hepatitis A vaccine. *Biologicals* **4**, 235–242.
241. Villarejos, V. M., Jaime Serra, C., Anderson-Visona, K., and Mosley, J. W. (1982) Hepatitis A virus infection in households. *Amer. J. Epidemiol.* 115, 577–586.
242. Volkin, D. B., Burke, C. J., Marfia, K. E., Oswald, C. B., Wolanski, B., and Middaugh, C. R. (1997) Size and conformational stability of the hepatitis A virus used to prepare VAQTA, a highly purified inactivated vaccine. *J. Pharm. Sci.* **86**, 666–673.
243. Wagstaff, A. J., Plosker, G. L., and Balfour J. (1996) Inactivated hepatitis A vaccine (HM 175 Strain) *Clin. Immunother.* **6**, 68–88.
244. Wallace, R. E., Vasington, P. J., Petriccianni, J. C., Hopps, H. E., Lorenz, D. E., and Kadanka, Z. (1973) Development and characterization of cell lines from subhuman primates. *In vitro* **8**, 333–341.
245. Wang, C.-H., Tschen, S-Y., and Flehmig, B. (1995) Antigenicity of hepatitis A virus after ultra-violet inactivation. *Vaccine* **13**, 835–840.
246. Weitz, M., Finkel-Jimenez, B., and Siegl, G. (1991) Empty hepatitis A virus particles in vaccines, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), *Proc. 1990 Intern. Symp. Viral Hep. Liver Dis.* Williams and Wilkins, Philadelphia, PA.
247. Werzberger, A., Mensch, B., Kuter, B., Brown, L., Lewis, J., Sitrin, R., Miller, W., Shouval, D., Wiens, B., Calandra, G., Ryan, J., Provost, P., and Nalin, D. (1992) A

controlled trial of a formalin-inactivated hepatitis A vaccine in healthy children. *N. Engl. J. Med.* **327**, 453–457.
248. Werzberger, A., Kuter, B., Shouval, D., Mensch, B., Brown, L., Wiens, B., Lewis, J., Miller, W., Sitrin, R., Provost, P., and Nalin D. (1993) Anatomy of a trial: a historical view of the Monroe inactivated hepatitis A protective efficacy trial. *J. Hepatol.* **18**(Suppl. 2), S46–50.
249. Westblom, T. U., Gudipai, S., DeRousse, C., Midkiff, B. R., and Belshe, R. B. (1994) Safety and immunogenicity of an inactivated hepatitis A vaccine: effect of dose and vaccination schedule. *J. Inf. Dis.*, **169**, 996–1001.
250. Wheeler, C. M., Fields, H. A., Schable, C. A., Meinke, W. J., and Maynard, J. E. (1986) Adsorption, purification and growth characteristics of hepatitis A virus strain HAS–15 propagated in fetal rhesus monkey kidney cells. *J. Clin. Microbiol.* **23**, 434–440.
251. Wiedermann, G., Ambrosch, F., Kollaritsch, H., Hofmann, H., Kunz, Ch., D'Hondt, E., Delem, A., Andre, F. E., Safary, A., and Stephenne, J. (1990) Safety and immunogenicity of an inactivated hepatitis A candidate vaccine in healthy adult volunteers. *Vaccine* **8**, 581–584.
252. Wiedermann, G., Ambrosch, F., Andre, F. E., D'Hondt, E., Delem, A., and Safary, A. (1992) Persistence of vaccine-induced antibody to hepatitis A virus. *Vaccine* **10**(Suppl. 1), S129–S131.
253. Wiedermann, G., Ambrosch, F., Andre, F. E., Delem, A., D'Hondt, E., and Safary, A. (1994) Thermostability of an inactivated hepatitis A vaccine stored at 37°C for one week. *J. Med. Virol.* **44**, 442–443.
254. Wiens, B. L., Bohidar, N. R., Pigeon, J. G., Egan, J., Hurni, W., Brown, L., Kuter, B. J., and Nalin, D. R. (1996) Duration of protection from clinical hepatitis A disease after vaccination with VAQTA. *J. Med. Virol.* **49**, 235–241.
255. Winokur, P. L., McLinden, J. H., and Stapelton, J. T. (1991) The hepatitis A virus polyprotein expressed by a recombinant vaccinia virus undergoes proteolytic processing and assembly into viruslike particles. *J. Virol.* **65**, 5029–5036.
256. Winokur, P. L. and Stapelton, J. T. (1992) Immunoglobulin prophylaxis for hepatitis A. *Clin. Inf. Disease* **163**, 580–586.
257. Yao, G. B. (1990) Clinical spectrum and natural history of viral hepatitis A in 1988 Shanghai epidemic, in *Viral Hepatitis and Liver Disease* (Hollinger, F. B., Lemon, S. M., and Margolis, H. S., eds.), *Proc. 1990 Intern. Symp. Viral Hep. Liver Dis.* Williams and Wilkins, Philadelphia, PA.
258. Zhu, N. L., Li, G. D., and Wang, Y. (1994) Physiochemical and immunological characterization of hepatitis A virus nucleocapsids expressed in a vaccinia virus/T7/EMCV system. *Arch. Virol.* **135**, 443–449.
259. Zuckerman, A. (1976) Twenty five centuries of viral hepatitis, in *Perspectives in Viral Hepatitis. Rush-Presbyterian St. Luke's Med. Bull.* **15**, 53–82.
260. Zuckerman A. (1983) The history of viral hepatitis from antiquity to the present, in *Viral Hepatitis: Laboratory and Clinical Science* (Deinhardt, F. and Deinhardt, J., eds.), M. Dekker, New York, pp. 2–32.
261. Zuckerman, J. N., Moore, S., Smith, J., Tyrrell, H., Baxter, A., and Lee, C. A. (1996) A study to assess the immunogenicity, reactogenicity and safety of hepatitis A vaccine administered subcutaneously to patients with congenital coagulation disorders. *Haemophilia* **2**, 235–239.

11

Hepatitis B Vaccines

Steven Specter

1. Introduction

Hepatitis B virus (HBV) chronically infects 200–300 million people worldwide. The resulting acute and chronic disease that results from contact with these individuals or their blood is a leading cause of liver disease, including hepatocellular carcinoma, and mortality. In fact, HBV is second only to smoking as a cause of human cancers *(1)*. No better reasons could be cited for the vital need for safe, effective, and economically feasible vaccine. Vaccines for HBV have been the subject of numerous research articles, book chapters, and monographs. For the benefit of the reader only a small amount of that information will be summarized here. Greater depth of information can be acquired from some of these monographs *(2–4)* and an excellent chapter in Fields Virology by Hollinger *(5)*.

The discovery of Australia antigen in the early 1960s was the beginning of the unraveling of the cause of serum hepatitis and the ultimate development of a vaccine. A good history of the story of the discovery of the Australia antigen and its relationship to hepatitis can be found in a keynote address delivered by its discoverer, Nobel Laureate Baruch Blumberg *(6)*. Subsequent to the determination that Australia antigen was a protein associated with HBV, the viral particle was visualized in 1970 *(7)*. These discoveries initiated a process that has led to diagnostic tools that have reduced the passage of hepatitis via blood transfusion, enhanced the detection of the etiology of most causes of hepatitis, and led to vaccine development for HBV, resulting in a vaccine that was first approved by the FDA in 1981.

HBV vaccine development has proceeded in a manner unique in comparison with all other viral vaccines that preceded its development. Classic viral vaccines had been developed by propagation of virus either in animals, eggs, or cell culture. This was followed either by natural attenuation of the

From: *Viral Hepatitis: Diagnosis, Therapy, and Prevention*
Edited by: S. Specter © Humana Press Inc., Totowa, NJ

virus or inactivation by a physical or chemical process. However, the production of excess hepatitis B virus surface antigen (HBsAg) in the plasma of patients provided a ready source of antigenic material that could be used as a vaccine.

Studies in the early 1970s by Krugman and coworkers indicated that heat treatment of plasma from HBV-infected individuals resulted in immunogenic material that could prevent hepatitis B disease *(8–10)*. Vaccine development next benefited from the successful passage of HBV to chimpanzees, which was first reported in 1973 *(11–13)*. This established a model in which vaccine efficacy and safety of administration could be evaluated. Further study ultimately led to the discovery that the concentration of HBsAg particles reaches 10^{10}–10^{12} per milliliter of blood. *(14)*. When the crude preparations of diluted HBsAg were tested, it became clear that this noninfectious preparation yielded immunogenic material that could be used for vaccine production. Beginning in 1975 experimental HBV vaccines consisting of inactivated HBsAg that was purified from positive human plasma were being tested in the United States, Europe, and the Far East *(15–21)*.

2. Antigenic Nature of HBsAg

HBsAg has three major antigenic determinants, two of which are allelic, "d,y" and "w,r," as well as "a," which is common to all strains that have so far been identified *(13)*. There are 10 reported subtypes of HBV, with the predominate types being adw, ayw, and adr, in terms of geographical distribution *(22–25)*. The subtype ayr is not found commonly but is observed in isolated pockets in Oceania. In the Far East, some less common and unusual subtypes have been identified including, awr, adwr, adyr, adyw, and adywr *(13)*. These subtypes represent antigenic make-up on individual particles indicating some form of genetic recombination or other mechanism that has resulted in phenotypic mixing.

It is the common nature of the "a" determinant to all types and subtypes that has allowed the vaccine to generate long-term immunity to all identified subtypes of HBV.

3. Vaccine Development

As indicated above, the earliest attempts at assessing the ability to develop a vaccine resulted in determination that heat inactivation of plasma from HBV-infected individuals yields immunogenic material. Thus, early vaccines were prepared from plasma donors that had high HBsAg but appeared to be in good health. The 22-nm HBsAg particle was separated

from infectious 42-nm HBV virions by ultracentrifugation, as part of a nine-step process involving pepsin digestion at pH 2, 8 *M* urea treatment, molecular sieve filtration, and treatment with formalin (1:4000 for 72 h at 37°C) as steps in inactivation of any infectious virus present *(26)*. The inactivation process destroys HBV and most other known animal viruses, including human retroviruses. This vaccine was administered as 20 μg HBsAg with 0.5 mg alum/dose in 1 mL and thimerosal as a preservative. This is currently licensed in the United States as Hepatavax-B (Merck, Sharp and Dohme, West Point, PA). However, the plasma-derived HBV vaccines are no longer in common use.

Worldwide, there are approximately a dozen such plasma-based vaccines. The earliest vaccine trials using the plasma-derived vaccine were performed in chimpanzees, as noted above. The vaccine was demonstrated to be safe and effective at inducing protective immunity *(27–29)*. The transition to human trials was slowed by caution and fear of transmission of infections *(30)*. The first licensed vaccine became available for use in the United States in 1982 *(31)*. Protective levels of antibody can be measured within weeks of the first two doses in most adults *(32,33)*.

However, in the later 1980s, the plasma-derived vaccines fell out of favor because of concerns regarding contamination of the blood supply with the human immunodeficiency virus (HIV), which causes AIDS. Despite convincing evidence of the safety of these vaccines *(34)*, they have been replaced by vaccines that are not derived from human serum or plasma. This pushed the development of recombinant vaccines that could be produced in eukaryotic vectors and were free of any contaminating human viruses.

More recently, the gene that codes for HBsAg has been placed in a plasmid carried by the yeast, *Saccharomyces cerevisiae*; the expressed protein is then purified, adsorbed to alum, and given in solution containing thimerosal. The protein is the predicted 24 kDa, slightly smaller than the natural HBsAg, since it lacks sugar side chains. Nevertheless, it provides protection against HBV infection *(35)*. Several yeast-encoded HBV vaccines have been licensed *(35)*.

The two most commonly used yeast-derived HBV vaccines are Engerix-B (SmithKline Beecham, Philadelphia, PA) and Recombivax HB (Merck, Sharp and Dohme), which are licensed in most countries. A general survey of HBV vaccines was reported in 1993 and indicated that at least five yeast-based vaccines were commercially available *(35)*. The yeast-based vaccines are presently the most commonly used HBV vaccines. Both Energix-B and Recombivax HB are nonglycosylated HBsAg particles that have been purified by physicochemical methods, alum absorbed, and preserved with thimerosal *(36)*.

4. Vaccines and Their Administration

The current recommendations for use of all U.S.-licensed HBV vaccines is a regimen of three immunizations. After the initial vaccination, the booster doses are given at 1 and 6 mo after the first dose. HBV vaccination is recommended for newborn infants within 12 h of birth if their mother is infected with HBV, and is in all cases recommended before a newborn leaves the hospital *(37–39)*. Alternatively, there is still a holdover of a four-dose regimen with doses being administered at birth, 1, 2, and 12 mo thereafter *(40)*. For children that are not vaccinated prior to leaving the hospital after birth, initiation of the series is recommended at the first visit to the doctor, usually at 1–2 mo of age. The series is then continued at 1 and 6 mo after the initial injection. For adolescents and adults who have not been vaccinated, the recommendation to vaccinate is extended to all those that are at high risk. The recommended dose is 10 μg for infants and 20 μg for adults *(41)*.

5. Recommendations for Vaccination

The use of HBV vaccine has become routine in the United States and other developed nations. Current vaccine recommendations are available in several publications, including the Immunization Practices Advisory Committee (ACIP), the American Academy of Pediatrics, the American College of Physicians, and the Technical Advisory Group of the World Health Organization. The most current guidelines of the ACIP are published in Morbidity and Morality Weekly Reports (MMWR) *(41,42)*.

5.1. Immunization Schedules and Dosages

Currently there are two recombinant HBV vaccines licensed for use in the United States. They are Recombivax HB and Energix-B, both of which are alum-adsorbed particles of HBsAg that have been genetically engineered into the common yeast *Saccharomyces cerevisiae* and produced synthetically. Routine vaccination involves a three-dose regimen for both infants and adults, with the first booster at 1–2 mo and the second booster at 6–18 mo after the initial injection, respectively. The Energix B has an alternative four-dose regimen at 0, 1, 2, and 12 mo for normal individuals and 0, 1, 2, and 6 mo for dialysis patients. However, there is not strong evidence that this regimen provides any additional benefit to the three-dose regimen. Dosages vary depending on the preparation used, the age of the recipient, state of health, and whether vaccine is administered pre- or postexposure (Table 1) *(8)*.

5.2. Preexposure Immunization

The use of HBV vaccine is now recommended universally for infants shortly after birth and prior to leaving the hospital for the initial administra-

Table 1
ACIP Recommended Dosages for HBV Immunization/Prophylaxis

Group	HBIG[a]	Energix B (μg)	Recombivax HB (μg)
Preexposure			
Infants and children ≤10 yr	NA[b]	10[c]	2.5[c]
Adolescents 11–19 yr	NA	20[c]	5[c]
Adults ≥20 yr	NA	20[c]	10[c]
Dialysis patients/ immunocompromised host	NA	40[c]	40[c]
Postexposure			
Perinatal	0.5 mL	10	5
Sexual	0.06 mL/kg	20	10
Vaccinated responder	NA	none or 1 dose—see age chart[d]	
Non-responder	0.06 mL/kg × 2 only or 0.06 mL/kg		
		20	10
Undetermined	nothing[d] or 0.06 mL/kg		
		20	10

[a]Hepatitis B immune globulin (HBIG) is administered intramuscularly.
[b]Not applicable.
[c]Vaccine is given at 0, 1, and 6 mo.
[d]Check anti-HBs antibody level first, if not sufficient (≥10 mIU), give one vaccine booster dose.

tion, with boosters at 1–2 mo later and at 6–18 mo of age. Alternatively, vaccine may begin at 2 and 4 mo, with the third administration at 6–18 mo.

In addition to newborns, HBV vaccination is recommended for all health-care professionals as well as for several other high-risk groups (Table 2). In a recent study of vaccination in health-care settings, it was clearly demonstrated that far too few employees (approx 2/3) are receiving the full three-dose regimen. Nevertheless, the number of cases of HBV infections acquired as a result of exposure during professional activities has dropped dramatically for health-care workers in the United States, from 17,000 in 1983 to 400 in 1995 *(44)*. Those at highest risk, hemodialysis unit workers, doctors, and nurses, had the highest vaccination rate (nearly 75%), whereas dieticians and other support personnel tended to have lower compliance rates.

For vaccination of adolescents, 11–19 yr old, the dose is twice that used for infants, for adults it is 2–4 times the infant dose, depending on the preparation used, and for high-risk groups, such as dialysis patients or immunocompromised hosts, the dose is 2–4 times the normal adult dose depending on preparation (*see* Table 1).

Table 2
High-Risk Individuals for Whom HBV Vaccination is Recommended[a]

Emergency service providers, e.g., paramedics, police officers, fire fighters
Ethnic groups with high HBV infection rates and those from endemic areas of HBV infection, e.g., Alaskan natives, Chinese, Filipinos, Haitians, Indochinese, Koreans, Native Americans
Health-care workers, e.g., physicians, dentists, nurses, and blood and laboratory technicians
Hemodialysis patients and medical personnel
Household contacts and sex partners of HBV carriers
International travelers
Military personnel
Morticians and embalmers
Patients and staff from certain institutions, e.g., for developmentally disabled, long-term correctional institutions
People with multiple sex partners, heterosexual and homosexual
Recipients of certain blood products

[a]Adapted from American Liver Foundation *(43)*.

For individuals who have responded appropriately, there are currently no recommendations for revaccination (booster) under normal circumstances. In special cases, revaccination can be considered. Individuals exposed to HBsAg+ blood by the percutaneous or permucosal route should be tested for anti-HBs antibody. The ACIP recommends that any such exposure in an individual showing ≤10 mIU should result in a booster vaccination. Similarly, the ACIP recommends that dialysis patients be tested annually for anti-HBs antibody and should be boosted whenever their titer is < 10 mIU *(1)*.

5.3. Postexposure Prophylaxis and Immunization

It should be noted that if infants are born to HBsAg+ mothers they are highly likely to become chronic HBV carriers unless treated. Thus, administration of hepatitis B immune globulin (HBIG) is recommended in all such cases, concomitant with HBV vaccine (Table 1). It should be noted that vaccine and HBIG should be administered in separate anatomical locations. The HBIG is given as a single im bolus, and a normal HBV vaccination schedule of three doses is then followed.

For nonvaccinated individuals who are exposed to HBV, either through sexual contact or sharing of items such as toothbrushes or razors, both HBIG and HBV vaccine should be administered as soon as possible after exposure has been recognized. The HBIG is given as a single im bolus, and a normal

HBV vaccination schedule of three doses is followed. Immunoprophylaxis and vaccination are not recommended for casual household exposure to an individual with acute HBV infection, unless the exposed individual is an infant under 12 mo of age. By contrast, for household contacts of those with chronic hepatitis, vaccination is recommended.

Individuals who are acutely exposed to blood should be assessed as to whether immunoprophylaxis is necessary. In all cases, if this is a nonvaccinated individual, a vaccination series should be initiated. If the blood to which the individual has been exposed is HBsAg+, then HBIG should be given as described (Table 1). If the individual has been vaccinated, testing should be done to be certain that there are anti-HBs antibody at ≥10 mIU/mL by RIA or are positive in an ELISA. If individuals have been vaccinated but are nonresponders, then HBIG and one additional dose of vaccine should be administered.

6. Vaccine-Induced Immunity

Previous studies indicate that 90% or more of healthy individuals receiving a three-dose regimen of HBV vaccine will develop protective immunity. This has been defined as antibody levels ≥10 mIU/mL anti-HBV antibody as measured by RIA or as a positive test as measured by ELISA *(45)*. Antibody responses do wane following vaccination but protective levels are maintained for many years *(46,47)*. Levels often fall below the 10 mIU/mL level, but it is believed that immunologic memory is maintained via T- and B-memory lymphocytes. This has been demonstrated in studies in which a booster was given 5–7 yr after the primary series and yielded a rapid anamnestic response *(48,49)*.

In those who do not respond appropriately to the initial series of vaccinations, an additional booster or a repeated series may be tried, but this approach has had varying success. The issue of revaccination of individuals who have completed the three-dose regimen but who do not show adequate titers of antibody (≥10 mIU) is still under discussion. The ACIP recommends revaccination for individuals who fail to respond to the initial three-dose regimen by using one or more boosters. Response rates for such individuals receiving one booster have been shown to vary from 18 to 54% for nonresponders to 33 to 83% for hyporesponders (positive but <10 mIU) *(50–52)*. A complete 3 booster revaccination has been shown to generate a 44–75% response rate *(53–55)*. In recent studies, Chedid and coworkers indicated that the defect may be poor response by type 1 helper T lymphocytes *(56)*. Thus, future vaccine approaches may need to be designed to overcome this defect.

7. Hepatitis D and HBV Vaccine

Hepatitis D virus or delta hepatitis virus (HDV) is a defective single-stranded RNA virus with negative polarity with similarities to viroids or virusoids, similar to those seen in plants *(57)*. A major defect in HDV is the lack of a surface protein, which the virus obtains from HBV. Thus, the viral particle contains HBsAg (*see* chapter 5). This fortuitous occurrence allows for immunization against HBV to also provide protection against disease caused by HDV. HDV causes significant morbidity and mortality, mainly as a result of superinfection of HBV-infected individuals *(58)*. HDV is a highly prominent cause of fulminat viral hepatitis *(59)* and a cause of chronic hepatitis that leads to cirrhotic livers more rapidly than does HBV alone *(60)*. Thus, HBV vaccination can prevent both HBV and HDV infections. By contrast, there are no reports of successful prophylaxis via immunization with HBV vaccine against superinfection by HDV in HBV carriers.

8. Future Prospects

The control of the spread of HBV infection is inhibited by a variety of factors. These include

1. the failure to effectively implement widespread immunization,
2. a need to improve the vaccine so that a higher percentage of effective responses occurs in individuals undergoing the 3 dose regimen,
3. decreasing cost so that highly endemic regions can afford universal vaccination of infants, and
4. identifying the length of time protection lasts so that an appropriate time period is identified before an additional booster is needed.

The most immediate goal for the future is the initiation of a program for elimination of HBV worldwide via universal immunization of infants. This practice is now recommended by the ACIP in the United States and the World Health Organization (WHO) worldwide. While it is clear that this will take quite some time to accomplish, routine vaccination of infants for HBV is now ongoing in the United States and some other developed nations. It must be noted that while the United States and some European nations where HBV has a higher prevalence practice routine vaccination, many nations, including European countries where HBV incidence is low, have not instituted this practice. By contrast, it has been difficult getting to individuals who exhibit high-risk behaviors for acquiring HBV, such as drug abusers and those who are highly promiscuous. Thus, the spread of disease in adults has not been significantly diminished. In moderate-to-high-incidence nations the recommendation for universal vaccination of infants has

been in place since 1987 *(45)*. Nevertheless this is difficult to accomplish because of inadequate delivery systems and the high cost of the vaccine. While many vaccines are administered at a cost of under $2.00, the three-dose regimen for HBV vaccine approximates $100. Thus, future goals in this regard include development of a more effective vaccine administration program, especially in high-incidence areas, such as Asia and Africa. In addition, vaccines must be developed that allow for a more cost-effective administration to large numbers of individuals.

Approaches to vaccination for the future include the development of synthetic peptide vaccines *(61–64)*, antiidiotype peptides *(65)*, and recombinant vaccines *(66,67)*. In one interesting approach, HBsAg was fused to malarial antigen, with the resulting protein being more antigenic than either the hepatitis or malarial protein *(67)*. Thus, fusion proteins are gaining prominence for future vaccine use. It is hoped that newer vaccines will address overcoming nonresponsiveness, either by using different antigens, such as fusion proteins, or using vaccine-adjuvant combinations that are more immunogenic *(68–70)*. Such approaches have looked at using the preS antigen and core antigen of HBV (HBcAg) *(71,72)*. These antigens may be important in individuals who are genetically nonresponsive to HBsAg *(73)*. However, there are additional studies that indicate that preS2 and HBcAg may lack the necessary immunogenicity to elicit effective immunity *(51,74)*. Furthermore, the immunization of the immunocompromised host is not as likely to benefit from using different antigens, as they will most likely require more potent immunogens. The approaches to increase immunogenicity are to use adjuvants such as microbial products, synthetic materials, or natural human products, such as the cytokines interleukin–2 (IL-2), IL-12, or interferon gamma *(68,75–78)*. These are all in the developmental stages and are likely to take several years before they are used clinically.

Recombinant vaccines are being developed using many different approaches. These include more-effective yeast-based production systems *(79,80)*, higher eukaryotic production systems *(81–83)*, use of different genetic sequences to yield more immunogenic materials, live recombinant vaccines in vectors, such as other viruses (e.g., vaccinia, adenovirus) *(84,85)* or bacteria (e.g., *Escherichia coli*, *Salmonella*) *(86,87)*, or use of naked nucleic acid vaccines *(88)*.

Another exciting development in the area of recombinant vaccines is the genetic engineering of plants to carry viral genes. In recent years, viral genes have been placed in certain fruits or vegetables in the hope that eating these plant products will produce immunization in a highly cost-effective manner *(89,90)*. While this is still in the early developmental stages, it holds great promise for use in underdeveloped nations.

9. Conclusions

Effective and safe vaccines for HBV are available and in use mainly in developed nations. Their potential to reduce HBV disease worldwide is being realized, although they have not yet achieved their full potential. This requires more assertive approaches to universal immunization of infants and immunization of high-risk individuals. Development of more cost-effective and more immunogenic vaccines will also increase our ability to provide more widespread protective immunity. Many experimental approaches are already in development and should yield success over the next few years. These methods and their worldwide application should result in a highly significant decrease in the transmission of HBV over the next few decades and the reduction in the number of HBV carriers. Although the chronic nature of HBV infection will limit our ability to eliminate this infection, by universally protecting the world population from birth, this goal can be realized. Ultimately, the public health efforts of worldwide vaccination should remove HBV as a significant pathogen; however, one might expect that this would require several decades to achieve.

Acknowledgment

The author thanks Dr. Gordon Dreesman for providing much of the research materials that were used in the preparation of this chapter.

References

1. Gold, E. (1996) Hepatitis history. *J. Am. Med. Assoc.* **275**, 31.
2. Ellis, R. W., ed. (1993) *Hepatitis B Vaccines in Clinical Practice*. Marcel Dekker, New York.
3. Maupas, P. and Guesry, P., eds. (1981) *Hepatitis B Vaccine*, Elsevier/North-Holland, Amsterdam.
4. Muraskin, W. (1995) *The War Against Hepatitis B: A History of the International Task Force on Hepatitis B Immunization*, University of Pennsylvania, Philadelphia.
5. Hollinger, F. B. (1996) Hepatitis B virus, in *Fields Virology*, 3rd ed. (Fields, B. N., Knipe, D. M., and Howley, P. M., eds.), Lippincott-Raven, Philadelphia, PA, pp. 2738–2807.
6. Blumburg, B. S. (1984) Keynote address: the Australia antigen story, in *Hepatitis B. The Virus, the Disease, and the Vaccine* (Millman, I., Eisenstein, T. K., and Blumberg, B. S., eds.), Plenum, New York, pp. 1–31.
7. Dane, D. S., Cameron, C. H., and Briggs, M. (1970) Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet* **1**, 695–698.
8. Calendra, G. B. and West, D. J. (1997) Recommendations for prevention of hepatitis B with vaccine, in *Hepatitis B Vaccines in Clinical Practice* (Ellis, R. W., ed.), Marcel Dekker, New York, pp. 1–16.

9. Krugman, S., Giles, J. P., and Hammond, J. (1971) Viral hepatitis, type B (MS-2 strain): studies on active immunization. *J. Am. Med. Assoc.* **217**, 41–45.
10. Krugman, S., Giles, J. P., and Hammond, J. (1970) Hepatitis virus: effects of heat on the infectivity and antigenicity of the MS-1 and MS-2 strain. *J. Infect. Dis.* **122**, 423–436.
11. Barker, L. F., Maynard, J. E., Purcell, R. H., Hoofnagle, J. H., Berquist, K. R., and London, W. T. (1975) Viral hepatitis, type B, in experimental animals. *Amer. J. Med. Sci.* **270**, 189–195.
12. Barker, L. F., Chisari, F., McGrath, P. P., Dalgard, D. W., Kirschstein, R. L., Almedia, J. D., Edgington, T. S., Sharp, D. G., and Peterson, M. R. (1973) Transmission of type B viral hepatitis to chimpanzees. *J. Infect. Dis.* **127**, 648–662.
13. Robinson, W. S. (1983) Hepatitis B virus, in *Viral Hepatitis: Laboratory and Clinical Science* (Deinhardt, F. and Deinhardt, J. eds.), New York, Marcel Dekker, pp. 57–116.
14. Kim, C. Y. and Tilles, J. G. (1970) Purification and biophysical characterization of hepatitis B antigen. *J. Clin. Invest.* **52**, 1176–1186.
15. Gerety, R. J., Tabor, E., Purcell, R. H., and Tyeryar, F. J. (1979) Summary of an international workshop on hepatitis B vaccines. *J. Infect. Dis.* **140**, 642–648.
16. Hilleman, M. R. (1993) Plasma-derived hepatitis B vaccine: a breakthrough in preventive medicine, in *Hepatitis B Vaccines in Clinical Practice* (Ellis, R. W., ed.), Marcel Dekker, New York, pp. 17–39.
17. Hilleman, M. R., Buynak, E. B., Roehm, R. R., Tytell, A. A., Bertland, A. U., and Lampson, G. P. (1975) Purified and inactivated human hepatitis B vaccine: progress report. *Amer. J. Med. Sci.* **270**, 401–404.
18. Maupas, P., Goudeau, A., Coursaget, P., Drucker, J., Barin, F., and Andre, M. (1978)Immunization against hepatitis B in man: a pilor study of two years' duration, in *Viral Hepatitis* (Vyas, G. N., Cohen, S. N., and Schmid, R., eds.), Franklin Institute, Philadelphia, PA, pp. 539–556.
19. Purcell, R. H., and Gerin, J. L. (1975) Hepatitis B subunit vaccine: a preliminary report of safety and efficacy tests in chimpanzees. *Amer. J. Med. Sci.* **270**, 395–399.
20. Reerick-Brongers, E. E., Reesink, H. W., Brummelhuis, H. G. J., van Elven, E. H., Schut, L. J. T., van den Ende, M. E., and Balner, H. (1981) Preparation and evaluation of heat-inactivated HBsAg as a vaccine against hepatitis B, in *Viral Hepatitis—1981 Intern. Symp.* (Szmuness, W., Alter, H. J., and Maynard, J. E., eds.), Franklin Institute, Philadelphia, PA, pp. 437–750.
21. Tao, G. M., Huang, T. Y., Feng, P. F., Wu, C. H., Liu, Y. C., Li, H. F., and Wang, C. C. (1978) A preliminary study on hepatitis B vaccine. *China Med. J.* **4**, 101–110.
22. Bancroft, W. H., Mundon, F. K., and Russell, P. K. (1972) Detection of additional determinants of hepatitis B antigen. *J. Immunol.* **109**, 842–848.
23. Bancroft, W. H., Holland, P. V., Mazzur, S., Courouce, A. M., and Madalinski, K. (1976) The geographical distribution of HBsAg subtypes. *Bibl. Haematol. Basel* **42,** 42–59.
24. Le Bouvier, G. L. (1971) The heterogeneity of Australia antigen. *J. Infect. Dis.* **123**, 671–675.
25. Mazzur, S., Burget, S., and Blumberg, B. S. (1974) Geographical distribution of Australia antigen determinants d, y, w. *Nature* **247,** 38–39.
26. Hollinger, B. F. (1991) Hepatitis B virus, in *Viral Hepatitis*, 2nd ed. (Hollinger, F. B., Robinson, W. S., Purcell, R. H., Gerin, J. L., and Ticehurst, J., eds.), Raven, New York, pp. 73–138.
27. McAuliffe, V. J., Purcell, R. H., and Gerin, J. L (1980) Type B hepatitis: a review of current prospects for a safe and effective vaccine. *Rev. Infect. Dis.* **2**, 470–492.

28. Murphy, B. L., Maynard, J. E., and Le Bouvier, G. L. (1974) Viral sub-types ands cross protection in hepatitis B virus infections of chimpanzees. *Intervirology* **3**, 378–381.
29. Purcell, R. H. and Gerin, J. L. (1975) Hepatitis B subunit vaccine: A preliminary report of safety and efficacy tests in chimpanzees. *Amer. J. Med. Sci.* **270**, 395–399.
30. Szmuness, W., Stevens, C. E., Harley, E. J., Zang, E. A., Oleszko, W. R., William, D. C., Sadovsky, R., Morrison, J. M., and Kellner, A. (1980) Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high-risk population in the United States. *N. Engl. J. Med.* **303**, 833–841.
31. Centers for Disease Control (1982) Inter-Agency Group to monitor for vaccine development, production, and usage. Hepatitis B virus vaccine safety: report of an inter-agency group. *Morbid. Mortal. Weekly Rep.* **31**, 465–467.
32. Szmuness, W., Stevens, C. E., Zang, E. A., Harley, E. J., and Kellner, A. (1981) A controlled clinical trial of the efficacy of hepatitis B vaccine (Hepatavax B): a final report. *Hepatology* **1**, 377–385.
33. Francis, D. P., Hadler, S. C., Thompson, S. E., et al. (1982) The prevention of hepatitis B with a vaccine: report of the Centers for Disease Control Multi-Center Efficacy Trial among homosexual men. *Ann. Int. Med.* **97**, 362–366.
34. Poiesz, B., Tomar, R., Lehr, B., and Moore, J. (1984) Hepatitis B vaccine: evidence confirming lack of AIDS transmission. *Morbid. Mortal. Weekly Rep.* **33**, 685–687.
35. Sitrin, R. D., Wampler, D. E., and Ellis, R. W. (1993) Survey of hepatitis B vaccines and their production processes, in *Hepatitis B Vaccines in Clinical Practice* (Ellis, R. W., ed.), Marcel Dekker, New York, pp. 83–101.
36. Lemon, S. M. and Thomas, D. L. (1997) Vaccines to prevent viral hepatitis, *N. Engl. J. Med.* **336**, 196–204.
37. Aristegui, J., Muniz, J., Perez Legorburu, A., Imaz, M., Arrate, J. P., Suarez, M. D., and Goiri, M. D. (1995) Newborn universal immunisation against hepatitis B: immunogenicity and reactogenicity of simultaneous administration of diphtheria/tetanus/pertussis (DTP) and oral polio vaccines with hepatitis B at 0, 2 and 6 months of age. *Vaccine* **13**, 973–977.
38. Da Villa, G., Picciottoci, L., Elia, S., Peluso, F., Montanaro, F., and Maisto, T. (1995) Hepatitis B vaccination: universal vaccination of newborn babies and children at 12 years or age versus high risk groups. A comparison in the field. *Vaccine* **13**, 1240–1243.
39. Tong, M. J., Poovorawan, Y., and Coursaget, P. (1991) Immunoprophylaxis of neonates against hepatitis B, in *Viral Hepatitis and Liver Disease* (Pine, J., ed.), Williams and Wilkins, Baltimore, MD, pp. 849–855.
40. Rustigi, V. K., Schuleupner, C. J., and Krause, D. S. (1995) Comparative study of the immunogenicity and safety of Energix-B administered at 0, 1, 2 and 12 months and Recombivax HB administered at 0, 1 and 6 months in healthy adults. *Vaccine* **13**, 1665–1668.
41. Centers for Disease Control. (1991) The hepatitis B virus: a comprehensive strategy for eliminating transmission in the United States through universal childhood vaccination: recommendations of the Immunization Practices Advisory Committee. *Morbid. Mortal. Weekly Rep.* **40(RR–13)**, 1–19.
42. Centers for Disease Control (1994) General recommendations on immunization: recommendations of the Advisory Committee on Immunization Practices (ACIP). *Morbid. Mortal. Weekly Rep.* **43(RR–1)**, 1–38.
43. American Liver Foundation website: http://gi.ucsf.edu/alf/alffinal/infohepbvac.html
44. Mahoney, F. J., Stewart, K., Hu, H., Coleman , P., and Alter, M. J. (1997) Progress

toward the elimination of hepatitis B virus transmission among health care workers in the United States. *Arch. Int. Med.* **157**, 2601–2605.
45. Centers for Disease Control. (1987) Recommendations of the Immunization Advisory Committee. Update on hepatitis B prevention. *Morbid. Mortal. Weekly Rep.* **36**, 353–355.
46. Krugman, S. and Davidson, M. (1987) Hepatitis B vaccine: prospects for duration of immunity. *Yale J. Biol. Med.* **60**, 333–339
47. Wainwright, R. B., McMahon, B. J., Bulkow, L. R., Hall, D. B., Fitzgerald, M. A., Harpster, A. P., Hadler, S. C., Lanier, A. P., and Heyward, W. L. (1989) Duration of immunogenicity and efficacy of hepatitis B vaccine in a Yupik Eskimo population. *J. Am. Med. Assoc.* **261**, 2362–2366.
48. Moyes, C. D., Milne, A., and Waldon, J. (1990) Very low dose hepatitis B vaccination in the newborn: anamnestic response to a booster at four years. *J. Med. Virol.* **30**, 216–218.
49. West, D. J., Watson, B., Lichtman, J., Hesley, T. M., and Hedberg, K. (1994) Persistence of immunologic memory for twelve years in children given hepatitis B vaccine in infancy. *Pediat. Infect. Dis. J.* **13**, 745–747.
50. Jilg, W., Schmidt, M., and Deinhardt, F. (1988) Immune response to hepatitis B vaccine. *J. Med. Virol.* **24**, 377–384.
51. Pillot, J., Poynard, T., Elias, A., Maillard, J., Lazizi, Y., Brancer, M., Dubreuil, P., Budkowska, A., and Chaput, J. C. (1995) Weak immunogenicity of the preS2 sequence and lack of circumventing effect on the unresponsiveness to the hepatitis B vaccine. *Vaccine* **13**, 289–294.
52. West, D. J., Kersh, L., and Ioli, V. (1990) Anti-HBs responses to a booster dose of hepatitis B vaccine in healthy adult nonresponders and hyporesponders to primary vaccination. *1990 Intern. Symp. Viral Hep. Liver Dis.* Houston, TX, Abstract 304.
53. Jilg, W., Schmidt, M., and Deinhardt, F. (1988) Prolonged immunity after latet booster doses of hepatitis B vaccine. *J. Infect. Dis.* **157**, 1267–1269.
54. Weissman, J. Y., Tsuchiyose, M. M., Tong, M. J., Co, R., Chin, K., and Ettenger, R. B. (1988) Lack of response to recombinant hepatitis B vaccine in nonresponders to the plasma vaccine. *J. Am. Med. Assoc.* **260,** 1734–1738.
55. Wismans, P., van Hattum, J., Stelling, T., Poel, J., and de Gast, G. C. (1988) Effect of supplementary vaccination in healthy non-responders to hepatitis B vaccination. *Hepato-Gastroenterology* **35**, 78–79.
56. Chedid, M. G., Deulofeut, H., Yunis, D. E., Lara-Marquez, M. L, . Salazar, M., Deulofeut, R., Awdeh, Z., Alper, C. A., and Yunis, E. J. (1997) Defect in Th1-like cells of nonresponders to hepatitis B vaccine. *Hum. Immunol.* **58**, 42–51.
57. Tanner, N. K., Schaff, S., Thill, G., Petit-Koskas, E., Crain-Denoyelle, A. M., and Westhof, E. (1994) A three-dimensional model of hepatitis delta virus ribozyme based on biochemical and mutational analyses. *Curr. Biol.* **4**, 488–489.
58. Caredda, F., Antinori, S. Pastecchia, C., Coppin, P., Ponzetto, A., Rizzetto, M., and Moroni, M. (1989) Presence and incidence of hepatitis delta virus infection in acute HBsAg-negative hepatitis. *J. Infect. Dis.* **159**, 977–979.
59. Saracco, G., Maccagno, S., Caredda, F., Antinori, S., and Rizzetto, M. (1988) Serologic markers with fulminant hepatitis in persons positive for hepatitis B surface antigen. A worldwide epidemiologic and clinical survey. *Ann. Int. Med.* **106**, 380–384.
60. Smedile, A., Niro, G. A., and Rizetto, M. (1997) Hepatitis D virus, in *Clinical Virology* (Richman, D. D., Whitley, R. J., and Hayden, F. G., eds.), Churchill Livingstone, New York, pp. 1273–1284.

61. Dreesman, G. R. (1984) Polypeptide and synthetic peptide vaccines for hepatitis B virus, in *Advances in Hepatitis Research* (Chisari, F. V., ed.), Masson Pub., New York, pp. 216–222.
62. Meleon, R. H., Casal, J. I., Dalsgaard, K., and Langeveld, J. P. M. (1995) Synthetic peptide vaccines: success at last. *Vaccine* **13**, 885–886.
63. Milich, D. R. (1993) Application of synthetic peptide technology to experimental HBV vaccine design, in *Hepatitis B Vaccines in Clinical Practice* (Ellis, R. W., ed.), Marcel Dekker, New York, pp. 351–381.
64. Dreesman, G. R. (1984) Polypeptide and synthetic peptide vaccines for hepatitis B virus, in *Advances in Hepatitis Research* (Chisari, F. V., ed.), Masson Pub., New York, pp. 216–222.
65. Rajadhyaksha, M., Yang, Y.-F., and Thanavala, Y. M. (1995) Immunological evaluation of three generations of anti-idiotype vaccine: study of B and T cell responses following priming with anti-idiotype, anti-idiotype peptide and its MAP structure. *Vaccine* **13**, 1421–1426.
66. Rutgers, T., Hauser, P., and De Wilde, M. (1993) Potential future recombinant vaccines, in *Hepatitis B Vaccines in Clinical Practice* (Ellis, R. W., ed.), Marcel Dekker, New York, pp. 383–407.
67. Stoute, J. A., Slaoui, M., Heppner, D. G., Momin, P., Kester, K. E., Desmons, P., Wellde, B. T., Garcon, N., Krzych, U., Marchand, M., Ballou, W. R., and Cohen, J. D. for the RTS, S malaria Vaccine Evaluation Group. (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. *N. Engl. J. Med.* **336**, 86–91.
68. Tamura, M., Yoo, Y. C., Yoshimatsu, K., Yoshida, R., Oka, T., Ohkuma, K., Arikawa, J., and Azuma, I. (1995) Effects of muramyl dipeptide derivatives as adjuvants on the induction of antibody response to recombinant hepatitis B surface antigen. *Vaccine* **13**, 77–82.
69. Boulter, N. R., Glass, E. J., Knight, P. A., Bell-Sakyi, L., Duncan Brown, C. G., and Hall, R. (1995) *Theileria annulata* sporozoite antigen fused to hepatitis B core antigen used in a vaccination trial. *Vaccine* **13**, 1152–1160.
70. Cheng-hua, S., Cheng, C., Jing-Sheng, Z., Jiezhi, L., and Qing-jun, M. (1995) Gene fusion of cholera toxin B subunit and HBV preS2 epitope and the antigenicity of fusion protein. *Vaccine* **13**, 933–937.
71. Raz, R., Dagan, R., Gallil, A., Brill, G., Kassis, I., and Koren, R. (1996) Safety and immunogenicity of a novel mammalian cell-derived recombinant hepatitis B vaccine containing Pre-S1 and Pre-S2 antigens in children. *Vaccine* **14**, 207–211.
72. Schodel, F., Kelly, S., Tinge, S., Hopkins, S., Peterson, D., Milich, D., and Curtiss R., III (1996) Hybrid hepatitis B virus core antigen as a vaccine carrier moiety. II. Expression in avirulent *Salmonella spp.* For mucosal immunization, in *Novel Strategies in Design and Production of Vaccines* (Cohen, S. and Shafferman, A., eds.), Plenum, New York, pp. 15–21.
73. Kruskall, M. S., Alper, C. A., Awdeh, Z., Yunis, Z., and Marcus-Bagley, D. (1992) The immune response to hepatitis B vaccine in humans: inheritance patterns in families. *J. Exp. Med.* **175**, 495–502.
74. Pignatelli, M., Waters, J., Lever, A., Iwarson, S., Gerety, R., and Thomas, H. C. (1987) Cytotoxic T-cell responses to the nucleocapsid proteins on HBV in chronic hepatitis-evidence that antibody modulation may cause protracted infection. *J. Hepatol.* **4**, 15–21.

75. Grob, P. J., Joller-Jemelka, H. I., Binswanger, U., Zaruba, K., Descoeudres, C. and Fernex, M. (1984) Interferon as an adjuvant for hepatitis B vaccination in non- and low-responder populations. *Eur. J. Clin. Microbiol.* **3**, 195–198.
76. Mauri, J. M. and Valles, M. (1997) Effects of recombinant interleukin-2 and revaccination for hepatitis B in previously vaccinated, non-responder, chronic uraemic patients. Collaborative Group of Girona. *Nephrol. Dial. Transplant.* **12**, 729–732.
77. Tang , Y. W. and Graham, B. S. (1995) Interleukin–12 treatment during immunization elicits a T helper cell type 1-like immune response in mice challenged with respiratory syncytial virus and improves vaccine immunogenicity. *J. Infect. Dis.* **172**, 734–738.
78. Wynn, T. A. and Sher, A. (1995) IL12 as an adjuvant for vaccines designed to prevent infection and immunopathology by schistosomes. *Res. Immunol.* **146**, 582–590.
79. Harford, N., Cabezon, T., Crabeel, M., Simeon, E., Rutgers, A., and De Wilde, M. (1983) Expression of hepatitis B surface antigen in yeast. *Nature* **54**, 125–130.
80. Valenzuela, P., Medina, A., Rutter, J., Ammerer, G., and Hall, B. D. (1982) Synthesis and assembly of hepatitis B surface antigen particles in yeast. *Develop. Biol. Stand.* **298**, 347–350.
81. Halliday, M. L., Rankin, J. G., Bristow, N. J., Coates, R. A., Corey, P. N. J., and Strickler, A. C. (1990) A randomized double-blind clinical trial of a mammalian cell-derived recombinant DNA hepatitis B vaccine compared with a plasma-derived vaccine. *Arch. Int. Med.* **150**, 1195–1200.
82. Patzer, E. J., Nakamura, G. R., Hershberg, R. D., Gregory, T. J., Crowley, C., Levinson, A. D., and Eichberg, J. W. (1986) Cell culture derived recombinant HBsAg is highly immunogenic and protects chimpanzees from infection with hepatitis B virus. *Biotechnology* **4**, 630–636.
83. Yerushalmi, B., Raz, R., Blondheim, O., Shumov, E., Koren, R., and Dagan, R. (1997) Safety and immunogenicity of a novel mammalian cell-derived recombinant hepatitis B vaccine containing Pre-S1 and Pre-S2 antigens in neonates. *Ped. Infect. Dis. J.* **16,** 587–592.
84. Hruby, D. E. (1990) Vaccinia virus vectors: new strategies for producing recombinant vaccines. *Clin. Micro. Rev.* **3**, 153–170.
85. Morin, J. E., Lubeck, M. D., Barton, J. E., Conley, A. J., Davis, A. R., and Hung, P. P. (1987) Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamsters. *Proc. Natl. Acad. Sci. USA* **84**, 4626–4630.
86. Charbit, A., Sobczak, E., Michel, M.-L., Molla, A., Tiollais, P., and Hofnung, M. (1987) Presentation of two epitopes of the presS2 region of hepatitis B virus on live recombinant bacteria. *J. Immunol.* **139**, 1658–1664.
87. Wu, J. Y., Newton, S., Judd, A., Stocker, B., and Robinson, W. S. (1989) Expression of immunogenic epitopes of hepatitis B surface antigen with hybrid flagellin proteins. *Proc. Natl. Acad. Sci. USA* **86**, 4726–4730.
88. Davis, H. L. (1998) DNA-based immunization against hepatitis B: experience with animal models. *Curr. Topics Micro. Immunol.* **226**, 57–68.
89. Mason, H. S., Lam, D. M. K., and Arntzen, C. J. (1992) Expression of hepatitis B surface antigen in transgenic plants. *Proc. Natl. Acad. Sci. USA* **89**, 11,745–11,749.
90. Thanavala, Y., Yang, Y.-F., Lyons, P., Mason, H. S., and Arntzen, C. J. (1995) Immunogenicity of transgenic plant-derived hepatitis B surface antigen. *Proc. Natl. Acad. Sci. USA* **92**, 3358–3361.

Index

About the Editor

Dr. Specter received his BA in Biology (1969) and Ph.D. in Microbiology and Immunology (1975) from Temple University, Philadelphia, PA. He was a post-doctoral fellow at the Albert Einstein Medical Center, Philadelphia, in Experimental Immunology and then served as Director of the Clinical Virology Diagnostic Laboratory at the same institution. Dr. Specter joined the faculty of the University of South Florida College of Medicine in 1979 as Assistant Professor of Medical Microbiology and Immunology. He became a Professor in 1991 and was appointed Associate Dean for Preclinical Medical Education in 1998 after one year as Co-Director of Curriculum and Medical Education. He established the Annual Clinical Virology Symposium in 1985 and continues to Chair this internationally renowned conference. He has served as Chair of the Clinical Immunology Division of the American Society for Microbiology, was Editor-in-Chief of the Cumitech series for the American Society for Microbiology, was President of the Pan American Society for Clinical Virology, and is President-elect of the Florida Branch of the American Society for Microbiology.

Dr. Specter serves on numerous journal editorial boards and professional councils. His research spans the fields of retrovirus induced immune deficiency and effects of abused drugs on the immune system. With a research grant funded by the National Institutes of Health, Dr. Specter is examining combination therapy approaches for treating an AIDS model in mice. He is the author of more than 150 original scientific publications, review articles, and book chapters. In recognition of his work, Dr. Specter was awarded the Theodore and Vanette Askonas-Ashford Distinguished Scholar Award by the University of South Florida in 1997.