Dendritic Cell-Based Immune Therapy in Liver Diseases

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Abstract: The field of immune therapy is currently undergoing a shift in focus, from non antigen-specific immune modulator-based immune therapy to antigen-based vaccine therapy to more sophisticated cell-based vaccine applications. Dendritic cells (DCs) are rare leukocytes that are uniquely potent in their ability to capture, process and present antigens to T cells. By culturing DCs with viral antigens or tumor-associated antigens or different cellular products, immunogenic or tolerogenic DCs can be produced. When antigen-pulsed DCs are administered, an increase in the functional capacities of cells of innate immune system is observed. Also, patients administered with antigen-loaded DCs exhibit an augmentation of helper T cells, cytotoxic T cells, and plasma cells activities. Patients with liver diseases exhibit distorted immune responses to invading pathogens or cancer cells or autoantigens. On the other hand, recovery from liver diseases is usually associated with restoration of host immunity. In this review, we would discuss about rationale and strategies of immune therapy including DC-based therapy in liver diseases.

Keywords: Antigen-pulsed dendritic cells, Antigen-specific immunity, Dendritic cells, Immune therapy, Liver diseases, Non antigen-specific immunity.

THE LIVER: A REGULATOR OF HOST IMMUNITY

The liver is a vital organ that performs a variety of functions. In an adult, it weighs about 1500 g, i.e., approximately one-fortieth of total adult body weight. However, the liver is a highly vascular organ that receives one-quarter of total circulating blood flow, with oxygenated blood being delivered through the systemic circulation, a phenomenon similar to other organs. The liver also receives venous blood *via* the portal system. Thus, various noxious materials enter the liver together with gut-derived portal venous blood. In addition to synthetic, metabolic and excretory functions, it is also an immunologically active organ. The liver harbors immunocytes of both the innate and adaptive immune systems. It contains considerably higher proportions of natural killer (NK) cells, the representative cells of the innate immune system, as compared to other parenchymal tissues and lymphoid organs. Moreover, large numbers of lymphocytes reside in the liver with their frequencies elevated several fold in many pathological conditions. In addition, specialized cells with certain immune regulatory functions are also present in the liver. The microenvironment of the liver allows different immunocytes to establish close contact with vascular endothelium and hepatocytes for designing the nature of hepatic immunity.

The liver is regarded as a tolerogenic organ and has been designated the graveyard of lymphocytes [1, 2]. Several food ingredients and toxic materials that enter the liver through the portal circulation are detoxified in the liver without inducing immune responses. In addition, the liver handles a variety of metabolic products without inducing aberrant immune responses. In the context of transplantation, liver transplants are usually accepted in the recipients in spite of HLA mismatch. This is because different immune regulatory cells of the liver also bear tolerogenic properties.

Despite the dominant tolerogenic properties of the liver, its microenvironment also supports immune responses and inflammatory processes. Hepatocytes are damaged and destroyed in different pathological conditions, mainly, if not solely, by immune-mediated mechanisms [3]. Immune-mediated damage and destruction of hepatocytes may continue for decades; with the ultimate outcome being the progression of hepatic fibrosis, distortion of lobular hepatic architecture of the liver, and carcinogenesis of hepatocytes.

ANTIGEN-PRESENTING DENDRITIC CELLS (DCS)

DCs are regarded as being the most potent professional antigen-presenting cells (APCs). Although many other APCs are able to present antigens, DCs are especially efficient at presenting antigenic peptides to naïve immunocytes for the induction of primary immune responses [4, 5]. After the discovery of DCs by Steinman and Cohn in 1973, thousands of reports have been published about the ontogeny, phylogeny, subtypes, localization, and function of DCs. Some features of DCs are cited in relevant chapters of this issue, and so we will not provide a detailed description of these cells. However, relevant points relating to DCs that are important in the development of DC-based therapy will be discussed here. DCs are bone-marrow-derived immunocytes widely distributed in almost all tissues of the body. DCs are also a member of the innate immune system. They migrate quickly to tissues that are infiltrated with harmful agents. Large numbers of DCs are also detected in inflamed mucosal tissues where they also produce varieties of cytokines including type-1 interferon (IFN). DCs act as bridges

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between the innate and the adaptive immune system. These cells are capable of recognizing microbial agents, harmful entities, altered cells, and malignant cells. Recognition of harmful, dangerous and non-dangerous entities by DCs is especially important for the development of DC-based therapy. After recognition, different agents are internalized and processed by the DCs. These events may cause phenotypic and functional alterations of the DCs. The levels of maturity of the DCs are dependent on the nature of the DCs, the nature of the antigens, the local microenvironment and several other factors; some of which are yet to be explored. DCs with antigenic peptides usually migrate to lymphoid tissues and interact with clonally selected immunocytes to induce antigen-specific immune responses or immune tolerance. The efficacy of DC-based therapy is dependent on (1) the efficacy of antigen capture by the DCs, (2) cleaving of the antigen into epitopes within the DCs, (3) intracellular migration of antigenic epitopes, (4) expression of antigenic epitopes on the DCs, (5) migration of the DCs to lymphoid tissues, and (6) functional abilities of the DCs to induce immune responses and immune tolerance. In addition, proper functioning of effecter immunocytes in a purposeful manner also determines the therapeutic capacities of DC-based immune therapy. Induction of immune tolerance seems to be the main function of DCs in normal conditions. DCs are capable of maintaining homeostasis in normal conditions and they also respond to danger signals in the case of pathological conditions. In fact, DCs regulate host immunity in both health and disease.

DC-BASED THERAPY IN LIVER DISEASES

The pathogenesis, clinical presentations, therapy, and prognosis of liver diseases are variable. Autoimmune mechanisms seem to be related with the pathogenesis of autoimmune hepatitis (AIH), although the exact nature of these is yet to be clarified. Immune-mediated mechanisms are responsible for liver damage and persistence of viral replication in patients with chronic hepatitis B (CHB) and chronic hepatitis C (CHC) [3]. Like most cancers, improper host immunity may underlie the pathogenesis of hepatocellular carcinoma (HCC). Taken together, various pathological processes underlie the pathogenesis of different liver diseases. However, it is expected that critical questions would arise if immune therapy were considered a practical and rational scientific approach to treat these diseases. To address this basic query, we will discuss the genesis of immune therapy in liver diseases. Finally, the concept of DC-based therapy will be described with recent progress in this field.

Limited Therapeutic Efficacies of Traditional Therapeutic Regimens Against Chronic Liver Diseases and the Emergence of the Concept of Immune Therapy as an Alternate Therapeutic Approach

Present concepts of immune therapy against liver disease involve either upregulating or down regulating host immunity with immune therapy because studies have shown that immune responses of these patients are decreased, impaired or distorted. The exact nature of the immune response in patients with AIH is not completely understood at this point; however, circumstantial evidence indicates that the magnitudes of several parameters of immune responses are exacerbated in these patients. Although immune suppressor agents down regulate the progression of liver disease in AIH, these drugs are usually given over a prolonged period of time or even throughout entire life [6]. However, prolonged use of immune suppressor drugs in AIH is endowed with severe side effects. In other words, non antigen-specific immune therapy by immune suppressive agents seems to have a limited effect in AIH. Because little is known about target antigens in patients with AIH, antigen-specific therapeutic interventions have not been applied in these patients. In this context, the exact nature of DC-based immune therapies in patients with AIH is still elusive. Tolerogenic DCs may be used in patients with AIH, but this type of immune therapy is not expected to yield better outcomes because generalized immune suppression by tolerogenic DCs may not be a better therapeutic strategy compared to immune suppressive drugs. However, if a new set of tolerogenic DCs can be developed that target only the liver or liver-related antigens, the arena of DC-based therapy may be expanded for all patients with AIH.

The concept of DC-based therapy in patients with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections has emerged from issues different to that of DC-based therapy for AIH. Approximately 2 billion people in the world bear evidence of past or ongoing infections with HBV. However, considerable levels of liver damage are detected in only some HBV-infected subjects. The replication of HBV and HBV-induced liver damage are strictly regulated by the host immune system. In some individuals, HBV replication remains at low levels for decades without any features of progressive liver damage. In others, liver damage is not significant even in the presence of high HBV replication. However, some patients with chronic HBV infection exhibit considerable liver damage with high, moderate or low levels of HBV replication. It is usually assumed that host immune responses regulate the extent of HBV replication and liver damage. However, little has been clarified about the nature of beneficial and detrimental immune responses in patients with CHB. Despite this, several antiviral agents have been used in these patients for containment of HBV replication and for minimizing liver damage. A final assessment has shown that these drugs produce limited therapeutic efficacies and cause significant side effects [7]. Some drugs that block HBV replication by inhibiting DNA polymerase may be used over a prolonged period or for the entire life of the patient. In addition, the use of these drugs is related to the emergence of mutant HBV that has the potential to cause severe liver damage.

The scenario of therapeutic options for patients with chronic hepatitis C (CHC) is more or less similar to that of patients with CHB, yet antiviral drugs do exhibit considerable potential for treating these patients [8]. However, patients infected with certain HCV genotypes and with very high levels of HCV RNA respond poorly to antiviral drugs. In addition, maintenance of sustained therapeutic effects in HBV and HCV-infected subjects represent a major challenge for clinicians.

Patients with HCC are traditionally treated by surgical resection, medical ablation of the cancer mass of various

natures, anti-cancer drugs, and radiotherapy. However, recurrence of HCC nodules and unsatisfactory prognosis of conventional therapeutic approaches also suggests that alternative therapy should be developed for treating HCC.

Taken together, the present regimens of different therapeutic interventions seem to have limited efficacy along with considerable side effects. Considering the burden of liver diseases on national and international health care delivery systems, the need for novel and alternative therapeutic approaches is paramount for patients with liver disease. As host immunity regulates the pathological processes of different liver diseases, immune-based therapy has been applied in these patients for the last 2-3 decades. However, poor clinical outcomes of ongoing regimens of immune therapy are also not inspiring in patients with liver disease. These realities have unmasked a new type of cell-based immune therapy for patients with liver disease using DCs as an adjuvant (DC-based therapy). Before we introduce DC-based therapy for liver disease, the scope and limitation of other immune-based protocols in liver disease will first be discussed.

Non Antigen-Specific Immune Therapy in Liver Disease

Limited efficacy and considerable side effects of antiviral drugs in patients with chronic HBV and HCV infection has led to investigations into alternative therapeutic approaches. As the acquisition and progression of chronic HBV and HCV infection as well as the ultimate prognosis of HCC are mainly regulated by nature and the magnitude of the host immune response, the concept of immune-based therapy has surfaced. Initially, it was shown that host immunity is diminished in patients with chronic HBV and HCV infections. Accordingly, immune therapy was accomplished in these patients through the administration of agents that upregulate host immune responses. A group of immune modulatory agents, such as cytokines, growth factors, and immune modifying agents were applied (Table **1**). Unfortunately, it was difficult to assess the true therapeutic potential of these immune modulators in HBV and HCV-infected subjects [9-19] because most of these studies were conducted as pilot studies and the long-term effects, especially off-treatment efficacies of these agents were not properly reported. Few clinical trials have been accomplished with non antigen-specific immune modulators in patients with HCC.

Antigen-Specific Immune Therapy in Chronic Liver Diseases

It has recently become evident that antigen-specific immune modulators may be therapeutically advantageous in patients with CHB and CHC because varying magnitudes of HBV and HCV-specific immune responses have the potential to provide antiviral and liver protecting properties. The outcomes of antigen-specific immune therapy in CHB and CHC are shown in Table **2**. The utilization of antigen-specific immune responses as a therapeutic modality has been seen mostly in patients with CHB [20-25]. Clinical trials were done with a hepatitis B surface antigen (HBsAg)-based vaccine in patients with CHB. Although vaccine therapy was safe for these patients, the therapeutic outcome of this therapy was controversial. Initial studies indicate that vaccine therapy has antiviral and immune modulator potential in patients with CHB, yet other studies have not found any notable therapeutic effect in CHB. To have better therapeutic effects, antigen-specific immune therapy has also been done as combinational therapy with antiviral drugs. The outcome of combined antiviral and immune therapy seems to be better, but, off- treatment follow up data has not been published to draw a conclusion about this [26-28].

The nature of different antigens in patients with HCV infection has not been properly elucidated. Thus, there is a paucity of information about the nature of antigens in HCV

Table 1. Antigen Non-Specific Immune Therapy with Polyclonal Immune Modulators in Patients with Chronic Hepatitis Patients B and C

Immune Therapeutic Agents	Type of Study	Therapeutic Effect	References
Chronic Hepatitis B			
Interferon-gamma	Open-level trial	Less hepatic fibrosis	[9]
Interleukin-2	RCT	No effect	[10]
Interleukin-12	Phase I/II studies	No notable effects	[11]
Granulocyte-macrophage colony stimulating factor	Pilot study	Inconclusive	[12]
Thymosin-alpha 1	RCT	Moderately effective	[13]
Alpha-galactosylceramide	Phase I/II	No effect	[14]
Propagermanium	RCT	Moderate effect	[15]
Chronic Hepatitis C			
Recombinant interleukin-2	Pilot study	No added benefit	[16]
Interleukin-10	Pilot study	Dichotomy about liver damage and HCV RNA	[17]
Interleukin-11	Pilot study	Side effects: edema of lower extremities in all patients	[18]
Interleukin-12	Open level study	Poorly tolerated and lack of notable response	[19]

Immune therapy with non antigen-specific immune modulators in patients with chronic hepatitis B and chronic hepatitis C. *RCT; Randomized-controlled trial.

Table 2. Immune Therapy with Hepatitis B Vaccine and Combination of Antiviral and Immune Therapy in Patients with Chronic Hepatitis B

Type of Study	Nature of Vaccine	Therapeutic Effect	References
Vaccine Monotherapy			
Pilot study	HBsAg-based,	Moderate effect	[20]
Pilot study	HBsAg-based,	Limited effects	[21]
Pilot study	HBsAg-based	Inconclusive	[22]
Pilot study	HBsAg-based	Non-effective	[23]
Pilot study	HBcAg epitope,	Ineffective	[24]
Pilot study	DNA vaccine	Immunogenic	[25]
Combination Therapy: Vaccine Plus Antiviral or Immune Modulator			
Pilot studies	Lamivudine plus HBsAg-based vaccine	Effective during therapy	[26]
Pilot study	Clevudine plus HBsAg-based vaccine	Effective	[27]
Randomized-controlled trial	HBsAg-based vaccine plus lamivudine	Ineffective	[28]

infection that have the potential to induce therapeutic immunity in patients with CHC. However, studies have assessed the therapeutic efficacies of antigen-based or HCV DNA vaccines in patients with CHC [29-34] (Table **3**).

Patients with HCC have also been treated with HCC-related antigens. Even though these antigens are expressed in the liver of the patients, the therapeutic effects of immune responses against these antigens are not clearly known.

CONCEPT OF DC-BASED IMMUNE THERAPY IN LIVER DISEASES

Different clinical trials in patients with CHB, CHC and HCC during the last 30 years has revealed that antigen-specific immune therapies may have better therapeutic potential, yet the ongoing regimen of these therapies is still not satisfactory (Tables **2** and **3**). The positive point in antigen-specific immune therapy lies in the fact that these therapeutic modalities are safe for human use. The major limitation of these immune-based therapeutic approaches is related to their divergent effects in different clinical trials.

Finally, studies on the mechanisms of action of immune therapy and contemporary interventions targeting cellular and molecular mechanisms of immune responses have revealed that the mere administration of antigens may not be the best approach for inducing responses against tolerogenic antigens in patients with liver disease. The ongoing regimens of antigen-specific immune therapy have mainly been accomplished in patients with CHB by administrating a commercially available HBsAg-based vaccine emulsified in different adjuvants. On the other hand, the antigens that have been used in CHC and HCC are HCV-related and HCC-related. These antigens have been administered to patients with liver disease with the expectation that APC-like DCs would recognize these antigens. These antigens would then be processed and presented by DCs for the induction of antigen-specific immunity. Finally, it has been presumed that immune responses to administered vaccines and antigens would control viral replication, liver damage or liver cancer. However, several other variables relating to the induction and functioning of therapeutic immunity in these diseases have not been properly considered during the design of such antigen-specific immune therapies against liver diseases. These variables will be discussed later in the context of (1) the nature of antigens, (2) the nature of adjuvants, (3) the route of administration of antigens, (4) protective effects of immunity, and (5) the microenvironment of patients with liver disease.

In addition to these variables, several studies have shown that the antigen processing and presentation capacities of APCs, especially those of DCs, are impaired in patients with

Table 3. Antigen-Specific Immune Therapy in Patients with Chronic Hepatitis C

Type of Study	Nature of Vaccine	Therapeutic Effect	References
Vaccine Monotherapy			
Core protein-derived vaccine	Phase I study	Safe, immunogenic, insignificant virological responders	[29]
HCV 1b-derived peptide	Phase I study	Limited effect	[30]
Synthetic peptide vaccine IC41	Phase II study	Limited effect	[31]
V-5 Immunitor	Open-level trail	Limited effect	[32]
CIGB-230 DNA vaccine	Phase I clinical trial	Inspiring outcome	[33]
Envelope protein E1	Pilot study	Inspiring outcome	[34]

almost all liver diseases [4]. In some cases, the expression of costimulatory antigens are reduced, in others, the functions of DCs are impaired. Furthermore, DCs do not necessarily have potent migratory capacities, or they might undergo apoptosis. These limitations should be addressed when designing immune therapeutic protocols, the efficacy of which will not be dependent on endogenous DCs of the patients with liver disease. This can be addressed, at least to a reasonable level, by preparing antigen-pulsed DCs. In this scenario, DCs are cultured with antigens *in vitro*. DCs engulf antigens and process them during culture, an environment different to the *in vivo* environment of these patients [4, 5]. In fact, studies have shown that when immune responses are not induced after administrating antigen and adjuvant alone, they can be induced if antigens are loaded on DCs. These observations provided the rationale for implementing DC-based therapy in liver disease.

DC-BASED IMMUNE THERAPY IN LIVER DISEASE

Chronic HBV Infection

In Vitro Studies

Several investigators have prepared HBV-related antigen-pulsed DCs *in vitro* and checked their immune modulator capacities in culture. In most cases, HBsAg was used to load the DCs. Use of HBsAg was done mainly for the following reasons:

1. Human consumable forms of HBsAg are commercially available.
2. Patients with CHB exhibit impaired humoral and cellular immune responses to HBsAg.
3. Antibody to HBsAg (anti-HBs) represents a protective antibody against the HBV.
4. Development of anti-HBs in the sera of patients with chronic HBV infection, either due to therapy or spontaneously, is related to the recovery from disease.

The protective role of HBsAg-specific immune responses in HBV uninfected normal subjects is well known. However, it is not clear whether HBsAg-specific immune responses have therapeutic roles in subjects with chronic HBV infection. *In vitro* studies have documented that HBsAg-pulsed DCs induce HBsAg-specific immune responses *in vitro* when immunocytes from murine HBV carriers, HBV transgenic mice (HBV TM) or patients with CHB were cultured with HBsAg-pulsed DCs. HBsAg-pulsed DCs also cause T helper polarization and increase production of proinflammatory cytokines from lymphocytes of HBV TM and patients with CHB [35, 36].

Preclinical Trial with HBsAg-Pulsed DCs in HBV TM

Some investigators including us have conducted preclinical studies using HBsAg-pulsed DCs in HBV TM to assess the therapeutic potential of these approaches [35, 36]. A study that we reported in 2004 showed that only 2 administrations of HBsAg-pulsed DCs induced antibody to HBsAg (anti-HBs) in almost all HBV TM that expressed considerable levels of HBsAg before therapeutic manipulation [35]. The antiviral potential of HBsAg-pulsed DCs could not be properly assessed because the levels of HBV DNA in the sera were too low in most HBV TM of this series. Moreover, the role of HBsAg-pulsed DCs on liver damage could not be explored because HBV TM does not display evidence of liver damage. Administration of HBsAg-pulsed DCs was safe for all HBV TM because elevation of alanine aminotransferase (ALT) or histological evidence of liver damage was not detected in any of the HBV TM. Shimizu *et al.* also showed antiviral potential of HBsAg-pulsed DCs in HBV TM [36].

HBsAg-pulsed DCs are also potent inducers of anti-HBs in immune suppressed HBV TM, where immune suppression has been induced by daily administration of FK-506 [37]. Thus, immune modulations by HBsAg-pulsed DCs have been confirmed in HBV TM, but inherent limitations of this model do not allow extensive analyses of the therapeutic effects of HBsAg-pulsed DCs (antiviral effect and minimizing liver damage potentiality).

HBsAg-Pulsed DCs in Patients with CHB

Only few attempts have been made to use antigen-pulsed DCs in patients with CHB. Prior to using HBsAg-pulsed DCs in patients with CHB, HBsAg-pulsed DCs were prepared for human use and administered in normal volunteers to assess the safety of this approach. HBsAg-pulsed DCs were found to be safe for all normal volunteers and no generalized or liver-related adverse effects were detected in any of the subjects [38]. Following this, HBsAg-pulsed DCs were administered to patients with CHB and their safety was confirmed in these studies [39, 40]. Regarding antiviral capacity, conflicting data prevails: Chen *et al.* showed that HBV DNA was reduced in patients with CHB after immunization with HBsAg-pulsed DCs [39]. However, we did not find any significant reduction of HBV DNA from the administration of HBsAg-pulsed DCs in patients with CHB [40]. HBsAg-specific humoral and cellular immune responses have been detected in some patients with CHB due to administration of HBsAg-pulsed DCs, yet without any visible antiviral effects [40].

HBcAg-Pulsed DCs and their Immunogenicity *In Vitro* and *In Vivo*

HBcAg-pulsed DCs have also shown potent immune modulatory capacities *in vitro*. There is paucity of information about the therapeutic effects of HBcAg-pulsed DCs *in vivo*. One study recently reported that DCs pulsed with HBsAg and HBcAg are capable of inducing HBcAg-specific cellular immunity in HBV TM [41]. Human trials with DCs pulsed with peptides of HBsAg and HBcAg have been reported with some clinical efficacy in HBeAg-negative subjects [42].

HCV Related Antigen-Pulsed DCs and their Immunogenicity *In Vitro* and *In Vivo*

Murine DCs have been loaded with IFN alpha or ribavirin or both. In addition, antigen-pulsed DCs have been prepared by culturing DCs with NS3, NS5a antigen, NS5a mRNA, or core antigens of HCV. HCV antigen-pulsed DCs induced antigen-specific immunity in normal mice and in

mice with HCV infections. Furthermore, phase I clinical trials with HCV antigen-pulsed DCs in patients with CHC has been reported [43-45]. However, it is still elusive as to the nature of HCV-related antigens that should be used for loading DCs for therapeutic purposes because some HCV antigens such as core antigen and NS3 antigen have been shown to block DC maturation [46].

DC-Based Therapy in Patients with HCC

Without treatment, the 5-year survival rate of HCC is less than 5%. Treatment of HCC depends on the presence of comorbidity; tumor size, location and morphology; and the presence of metastatic diseases. Complete surgical resection followed by hepatic transplantation offers the best long-term survival, but this can be applied in few patients. All other therapies are palliative. At present, radiofrequency ablation, ethanol injection, chemoembolization, selective internal radiation therapy, and systemic administration of biological and chemotherapeutic agents are used to treat HCC patients (reviewed in reference [47]).

As a part of DC-based therapy, DCs have been loaded with HCC-related antigens by various methods to prepare HCC-related antigen-pulsed DCs [48-51]. As a source of HCC-related antigens, either HCC cell lines or HCC-related antigens have been used. Pulsing has been done by cell culture methods or by transfection techniques. In some cases, DCs have been fused with HCC cell lines or HCC lysates. *In vitro* studies have shown that HCC-pulsed DCs express various costimulatory molecules and produce proinflammatory cytokines. Moreover, these DCs induce antigen-specific immunity when administered to animal models of HCC. HCC-pulsed DCs have also been administered in patients with HCC (Table 4). The data shows that HCC-pulsed DCs induce antigen-specific immunity, cause better survival, and induce decreased size of HCC nodules [52-58]. Antigen-pulsed DCs have been administered in patients with HCC using various routes. These include parental as well as intratumoral administration of antigen-pulsed DCs in patients with HCC.

DC-Based Therapy in Autoimmune Liver Diseases

DC-based therapy has been accomplished in animal models of autoimmune liver diseases. In a preliminary study, we prepared an animal model of primary biliary cirrhosis (PBC). Pyruvate dehydrogenase complex (PDC)-pulsed regulatory DCs were prepared by culturing bone marrow-derived DCs with PDC and interleukin-10. These DCs were administered to a mouse model of PBC. Administration of PDC-pulsed DCs caused decreased concentration of antimitochondrial antibody (personal communication), and the extent of the PBC lesion in the liver of the mouse showed variable outcomes due to the administration of the PDC-pulsed DCs.

DC-BASED THERAPY IN LIVER DISEASE: PAST, PRESENT AND FUTURE

The concept of DC-based immune therapy in patients with liver disease is logical because distorted immune responses are seen in most of these patients. However, proper design of DC-based immune therapy is time-consuming and requires further understanding of the host immunity in liver disease. For the last three decades, the general assumption has been that immune responses are decreased in patients with CHB, CHC and HCC, and exacerbated in patients with AIH. However, this is an oversimplification of a very complex issue. In fact, not all parameters of immune responses are decreased in patients with CHB, CHC and HCC. Furthermore, not all parameters of immune responses are upregulated in patients with AIH. Patients with CHB and CHC tend to harbor an inflamed mucosal microenvironment, and proinflammatory cytokines are elevated in liver tissue in many of these patients. Patients with HCC are known to have high levels of tumor-necrosis factor (TNF), a potent proinflammatory cytokine. Regarding antigen-specific immunity, all patients with CHB express humoral immunity to hepatitis B core antigen (anti-HBc) and many patients also express antibody to hepatitis B e antigen (anti-HBe). HBsAg-specific immune responses are also detected in patients with CHB. Antibody to HBsAg (anti-

Table 4. Dendritic Cells-Based Immune Therapy in Patients with Hepatocellular Carcinoma

DC-Based Therapy	Type of Study	Therapeutic Effect	References
Chronic Hepatitis B			
HBsAg-pulsed DC	Pilot study	Antiviral potential	[39]
HBsAg-pulsed DC	Pilot study	Safe, partially immunogenic, no antiviral efficacy	[40]
DC pulsed with epitopes of surface and core antigen of HBV	Clinical trial	Moderately effective	[42]
Hepatocellular Carcinoma			
Autologous tumor-pulsed DC	Pilot study	Moderately effective	[52]
Alpha-fetoprotein pulsed DC	Phase I/II trial	Immune modulation	[53]
Pulsing of DC in cancer nodule in situ	Pilot study	Safe	[54]
Tumor lysate-pulsed DC	Pilot study	Safe and better 1-year survival	[55]
Autologous DC after conformal radiotherapy	Open level study	Safe and immunogenic	[56]
Tumor lysate-pulsed DC	Phase II study	Antitumor effects	[57]
Transcatheter embolization plus DC	Pilot study	Insufficient efficacy	[58]

HBs), although present in most patients with CHB, may not be detected by conventional techniques. Anti-HCV is so common in patients with CHC that it has been used for a long time to diagnose HCV infection. Thus, decreased non antigen-specific or antigen-specific immune responses are neither a valid concept nor a scientific logic in patients with CHB, CHC and HCC. The present scientific know how indicates that patients with CHB, CHC and HCC harbor a distorted immune response. It is really difficult to make a distinction between decreased and distorted immune responses. Extensive studies are required to assess the type of immune responses that would be necessary for containment of HBV, HCV and cancer cells in HCC, and also to minimize liver damage in these diseases. Insights into different HCV and HCC-related antigens are yet to be established. Thus, to develop an effective immune therapeutic strategy would require extensive basic information about these diseases. However, the DCs as regulators of immunity are capable of inducing both immune responses and immune tolerance. Thus, DCs can restore the necessary immunity in therapeutic applications. Concern does remain about strategy development.

DEVELOPMENT OF EVIDENCE-BASED THERAPY FOR LIVER DISEASES USING DCS

DC-based therapy is in its infancy. This is relevant for different pathological conditions that include liver disease. DC-based therapy may be an effective therapeutic strategy if the following variables are addressed properly and scientifically.

Antigens

Investigators have shown that HBV is contained in the liver of patients with CHB due to the immune modulatory capacities of HBcAg-specific cytotoxic T lymphocytes (CTL). On the other hand, anti-HBs are required to neutralize circulating HBV. Thus, both HBcAg- and HBsAg-specific immune responses including antigen-specific CTL are necessary for controlling HBV replication in such patients. Thus, DC-based immune therapy should incorporate both HBcAg and HBsAg. In addition, other HBV-related antigens, such as HBxAg and polymerase antigens may be needed to prepare antigen-pulsed DCs for developing better therapeutic immune strategies against HBV. Current data on the immune-mediated control of HCV is unclear. Further basic studies are essential for developing antigen-pulsed DCs against HCV infection. It remains elusive as to the nature of protective antigens in HCV infection. Although various antigens are detected after HCC progression, the precise nature of these antigens must be exclusively analyzed before using them for DC-based therapy against HCC. On the other hand, clinical trials should be conducted with different antigens and their immune modulatory capacities be elucidated to establish information on the most effective antigens applicable for treating HCC.

Method of Production of Antigen-Pulsed DCs

It is generally assumed that the culture of DCs and antigens would lead to the production of a suitable DC population. This is not only ambitious, but also counter productive. Both immunogenic and tolerogenic antigen-pulsed DCs are prepared by culturing DCs with antigens. However, as immunogenic antigen-pulsed DCs would induce antigen-specific immunity, tolerogenic antigen-pulsed DCs would cause immune tolerance. Culture conditions should be optimized for preparing antigen-pulsed DCs for specific purposes. Proper attention should be given regarding the (1) nature of the DCs, (2) source of the DCs, (3) dose of the antigen, (4) nature of the antigen, and (5) incubation time for preparing antigen-pulsed DCs. When antigen-pulsed DCs are prepared, they should not contain soluble antigens, and the antigen-pulsed DCs should be checked for viability, expression of costimulatory antigens, production of cytokines, and capacity to induce proliferation of antigen-specific lymphocytes *in vitro*.

In addition, the source of DC is one of important variables for preparation of antigen-pulsed-DC. DC has been isolated from almost all lymphoid organs and tissues. However, mostly monocyte-derived DC has been used for DC-based therapy in human diseases. Also, different subtypes of DC have been detected. These include myeloid DC, lymphoid DC, and plasmacytoid DC. Antigen-pulsed DC has mainly been prepared from myeloid DC or a bulk population of DC. However, both myeloid and palsmacytoid DC may be used in future for development of better regimens of DC-based therapy.

METHOD OF ADMINISTRATION OF ANTIGEN-PULSED DCS

Studies have shown that immunogenicity of antigen-pulsed DCs depends on routes of administration. This needs to be confirmed for different types of liver disease. Although the parental route is normally used to administer antigen-pulsed DCs, a pilot study should be performed to assess comparative therapeutic benefits from intradermal and subcutaneous, and intravenous or tissue administration. Mucosal routes should also be explored because the mucosal immune system is extensively distributed throughout the body.

Dose and Duration of Therapy

Dose and duration of DC-based therapy is an important aspect in liver disease. Antigen-pulsed DCs induce antigen-specific CTLs, helper T cells and antibodies. These CTLs may later kill the activated DCs that are delivered with subsequent injections and so this type of administration should be carefully planned.

DC-BASED THERAPY AS A PART OF COMBINATION THERAPY

Combination therapy might be needed for treating patients with CHB, CHC and HCC with antigen-pulsed DCs. Patients with CHB harbor abundant amounts of HBV and HBV-related antigens, especially HBsAg in the sera and in the liver. Similar conditions are also seen in patients with CHC. Patients with HCC also develop millions of cancer cells. The presence of these cells may hinder the induction and maintenance of antigen-specific immunity from antigen-pulsed DCs. Thus, antiviral drugs may be necessary for

lowering HBV or HCV load prior to administering DC-based therapy. It may also be imperative to reduce the cancer through operation or ablation methods before administrating antigen-pulsed DCs. In fact, combination of antiviral- and immune-based therapies has been used for different liver diseases, with polyclonal immune modulators or antigens given as immune modulatory agents. Thus, antigen-pulsed DCs may be incorporated as a part of such combination therapies in liver disease.

CONCLUDING REMARKS

The concept of immune therapy for treating different liver diseases is a comparatively new one whereby various practical points have yet to be optimized. In addition, immune therapy in liver disease is yet to receive a general consensus among physicians and hepatologists. Most immune-based therapies targeting liver disease have been conducted in pilot studies or clinical trials. In fact, DC-based therapy is in its infancy and few clinical trials have been conducted in patients with liver disease. This is also the case for other pathological conditions, except cancer. DC-based therapies are not intended to upregulate or down regulate host immunity in liver disease. Instead, they should be aimed at fixing the distorted immunity of patients with various liver diseases. The challenge lies in its application in the clinical setting. As discussed earlier in this chapter, we must first gain extensive insight into the pathogenesis of different liver diseases and then evaluate whether immune therapy would be advantageous in that specific condition. Careful attention should also be given to the nature of the antigens, the method of DC preparation, the technique of production of antigen-pulsed DCs *in vitro*, and the route of administration. Moreover, this should all be accomplished with due consideration of the safety of the patients. It is a challenging job. However, if DC-based therapy can be optimized for different liver diseases, it would then be easy to develop DC-based therapies for other pathological conditions. Thus, it is imperative that immunologists, hepatologists and cellular biologists initiate collaborative approaches in order to address these challenges to better improve future immune-based therapies.

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ORIGINAL ARTICLE

Suppression of Inflammatory Mucosal Milieu by Administration of Regulatory Dendritic Cells in an Animal Model of Primary Biliary Cirrhosis

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ABSTRACT

Background/Aim: Primary biliary cirrhosis (PBC) is an autoimmune disease of the liver for which a curative therapy is still lacking. The aim of this preclinical study was to assess if down regulation of host immunity by regulatory dendritic cells (DC) bears therapeutic implications in a murine model of PBC.

Methods: An animal model of PBC was established by injecting 5 mg/kg polyinosinic polycytidylic acid (poly I:C) twice a week in female C57BL/6 mice. Regulatory DC were produced by culturing bone marrow DC with interleukin-10 and lipopolysaccharides without or with pyruvate dehydrogenase complex (PDC, 1 μg/ml). Regulatory DC and PDC-pulsed regulatory DC were injected intraperitoneally trice (8, 12 and 20 weeks after starting of poly I:C administration) to PBC model mice. Antimitochondrial antibody (AMA) was checked in the sera and liver histology was assessed to evaluate the effect of regulatory DC on inflammatory hepatic mucosal milieu.

Results: AMA in the sera and progressive infiltration of mononuclear cells were detected in all C57BL/6 due to administration of poly I:C. Injection of regulatory DC or PDC-pulsed regulatory DC for 3 times caused significant reduction of infiltrating mononuclear cells in 4 of 5 PBC model mice. However, the effect of regulatory DC on AMA negativity was not documented in murine model of PBC.

Conclusion: This pilot study inspires optimisms that regulatory DC may be an immune therapeutic approach for treating PBC, however, further study about nature of antigens, dose of antigens, duration of therapy and protocol of administration of regulatory DC need further analyses.

Abbreviations: PBC: Primary biliary cirrhosis; PDC: Pyruvate dehydrogenase complex; DC: Dendritic cells; Poly I:C: polyinosinic-polycytidylic acid; AMA: Antimitochondrial antibody; UDCA: Ursodeoxycholic acid; PBS: Phosphate-buffered saline; GM-CSF: Granulocyte-macrophage colony stimulating factor; ANA: Antinuclear antibody; BM: Bone marrow; LPS: lipopolysaccharides; IL: Interleukin.

Keywords: Primary biliary cirrhosis, Regulatory dendritic cells, Murine model, AMA

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic progressive inflammatory liver disease that leads to the immune-mediated destruction of small and medium-size intrahepatic bile duct, progressive cholestasis and eventually, fibrosis and cirrhosis of the liver.[1-3] The serologic hallmark of PBC is the presence of high-titer serum antimitochondrial autoantibodies (AMA), although the role of AMA in the pathogenesis and progression of PBC is still elusive.[4] There is no curative therapy for PBC, although ursodeoxycholic acid (UDCA) is now widely used to treat patients with PBC.[5,6] A long-term follow-up study in a cohort of 225 patients reported 10-year survival without liver transplantation to be significantly higher in UDCA-treated patients compared with survival predicted by the Mayo model.[7] However, a meta-analysis of 11 randomized trials could not confirm a significant effect of UDCA on survival and incidence of liver transplantation.[8] The peroxisome proliferator-activated receptor α-agonist, bezafibrate, has been reported to improve serum liver tests in PBC and this drug should undergo more extensive evaluation in patients with PBC.[9] In addition, corticosteroids and other immunosuppressive agents have been evaluated for therapeutic use in PBC, however, these drugs have not been shown to markedly improve the natural course of the disease or were associated with significant toxicity during long-term treatment.[3] Taken together, new and innovative therapy should be developed for PBC.

In this regard, we provided attention to immune therapy as PBC is an immune-mediated disease. Dendritic cells (DC), the most potent antigen-presenting cell, have recently used either to upregulate or down regulate host immunity in various pathological conditions and some of these approaches seem to have therapeutic potential.[10] As an inflammatory microenvironment prevails in the liver of PBC patients, we assumed that regulatory DC that down regulate host immunity may be a choice of therapy for PBC. In fact, regulatory DC has been used in different animal models of human diseases with potential therapeutic promises.[11-18]

In this study, we checked the therapeutic efficacy of regulatory DC and antigen-specific regulatory DC in an animal model of PBC. The outcome of this pilot study may be used for development of better therapeutic regimen for PBC and other autoimmune diseases.

MATERIALS AND METHODS

Murine Model of PBC

An animal model of PBC was prepared by injecting 5 mg/kg of polyinosinic-polycytidylic acid (poly I:C, Sigma, St. Lous, MO, USA) twice a week in female C57BL/6 mice, exactly as described by us in a previous communication.[19] Control mice received 200 µl of phosphate-buffered saline (PBS). They were maintained separately at the animal house of Ehime University, School of Medicine, Ehime, Japan, under controlled conditions (22°C, 55% humidity, and 12-hour day/night), and were fed freely supplied standard laboratory chow and water. All animals received adequate humane care according to good laboratory practice guidelines. The Committee of Animal Experimentation, Ehime University School of Medicine, Japan approved this study protocol.

Histopathology and Immunohistochemical Examination

To assess the extent of infiltration of inflammatory mononuclear cells, formalin-fixed, paraffin-embedded liver tissues were used for histological evaluation. The area of the liver specimens, the number of portal tracts, and the extent of cell infiltration were estimated using a combination of a digital camera and imaging tools, as described.[19]

Detection of Autoantibodies

The existence of autoantibodies was examined in HEp-2 cells using the commercially available fluoro HEPANA Test system (MBL, Nagoya, JAPAN), exactly as described.[20] After reacting HEp-2 cells with sera, Alexa Fluor 555-conjugated polyclonal goat antimouse immunoglobulin (Molecular Probes Inc. Eugene, OR) was used as a secondary antibody. Based on staining pattern, antinuclear antibody (ANA), AMA, and both ANA and AMA were detected in various sera from mice.

Production of Regulatory DC

Immature bone marrow (BM)-derived DC were prepared by culturing bone marrow cells with murine granulocyte-macrophage colony stimulating factor (GM-CSF, 5 ng/ml, R&D systems Inc, Minneapolis, MN, USA) and murine interleukin (IL)-4 (5 ng/ml, R&D systems Inc) for 7 days.[11] To prepare regulatory DC, bone marrow DC were cultured with lipopolisaccharides (LPS, 1 µg/ml, InvitroGen, Carlasbad, CA, USA) and IL-10 (20 ng/ml, R&D systems Inc) in RPMI 1640 plus 10% fetal calf serum for 48 hours. In some studies, PDC-pulsed regulatory DC were prepared by culturing bone marrow DC with LPS, IL-10 and PDC (1 µg/ml, Sigma). The cells retrieved and washed in PBS before administration to mice.

Therapeutic Protocol

Production of animal model of PBC and therapeutic regimen have been shown in Figure 1. Several female C57BL/6 were injected with poly I:C, twice in a week for 28 weeks. Some of the poly I:C-injected mice received regulatory DC or PDC-pulsed regulatory DC for 3 times (8, 12 and 20 weeks after commencement of poly I:C administration). Sera were collected from all mice at 16 and 28 weeks. Mice were also killed at 16 and 28 weeks after starting poly I:C administration to assess the extent of liver damages.

RESULTS

Mice Model of PBC

A mice model of PBC was developed due to administration of poly I:C for 8 weeks and the extent of liver damages progressed along with time. As shown in Figures 2A to C, infiltration of mononuclear cells were observed around portal area (Fig. 2A), around biliary epithelia (Fig. 2B) and in hepatic parenchyma (Fig. 2C) at 16 weeks after commencing administration of poly I:C in female C57BL/6 mice. In addition, AMA and ANA were found in the sera of all mice injected with poly I:C (Figs 3A to C).

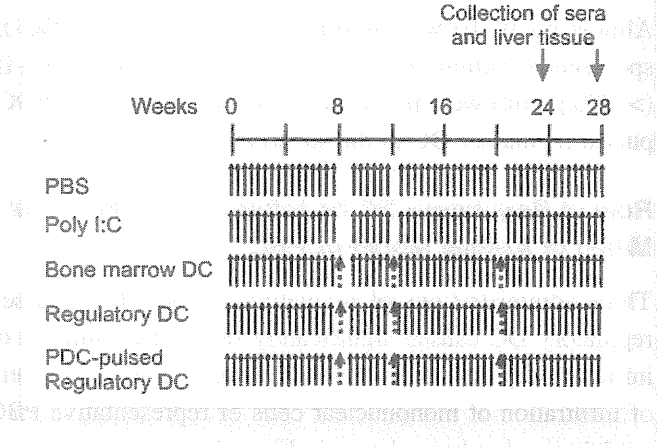
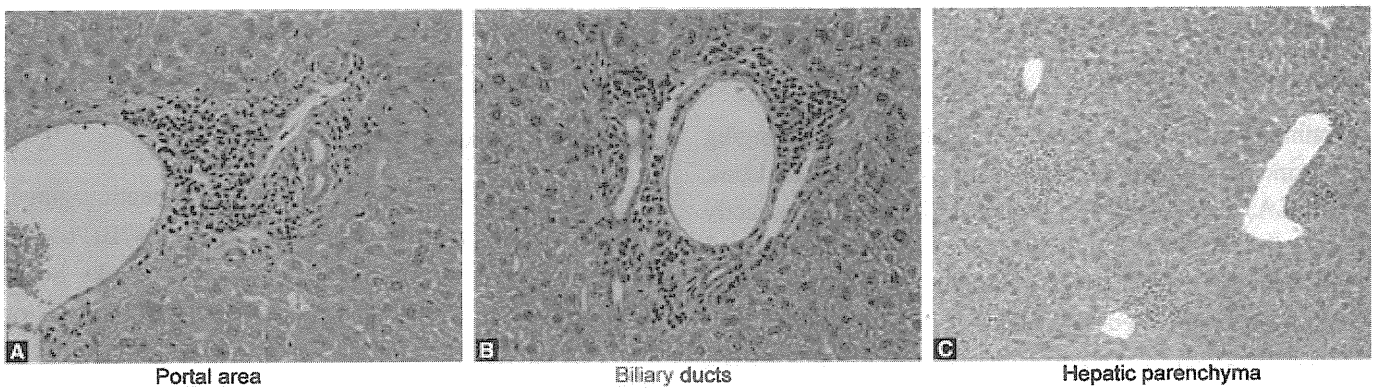
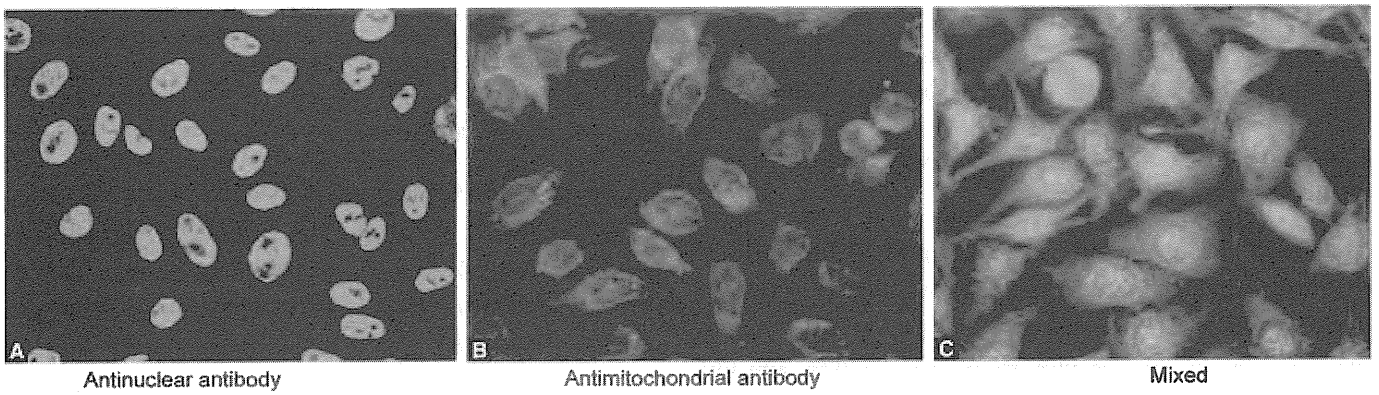


Fig. 1: Experimental strategy for production of mice model of primary biliary cirrhosis (PBC) and treatment with regulatory dendritic cells. Female C57BL/6 mice were injected with phosphate-buffered saline (PBS, control) or 5 mg/kg polyinosinic-polycytidylic acid (poly I:C), twice a week, for 28 weeks (upper panel). The mice of the 3 lower panels also received poly I:C, as mentioned for the mice of upper panel. In addition, they were given bone marrow dendritic cells (DC), regulatory DC or pyruvate dehydrogenase complex (PDC)-pulsed DC (panel 2nd, 3rd and 4th respectively) at points indicated by blue arrows. Mice were killed at different time points and assessed for PBC-like lesions and serological markers of PBC



Figs 2A to C: Features of PBC mice model due to injection with poly I:C for 16 weeks. Moderate-to-severe degrees of infiltration of mononuclear cells were seen in portal area, (A) around biliary ducts, (B) and in liver parenchyma, (C) due to injection with poly I:C



Figs 3A to C: Presence of autoantibodies in the sera of mice injected with poly I:C. HEp-2 cells were incubated with the sera from mice injected with poly I:C. The presence of antinuclear antibodies (A) anticytoplasmic antibodies, (B) and coexistence of both antibodies, (C) was evaluated by an immunofluorescence method using Alexa Fluor 555 conjugated polyclonal antimouse immunoglobulin antibody. A representative staining pattern of autoantibodies from the sera of female C57BL/6 mice-injected with poly I:C is shown

IL-10 Production by PDC-pulsed Regulatory DC

Almost no IL-10 was produced by bone marrow DC in spontaneous culture. However, abundant amounts of IL-10 (>1000 pg/ml) were produced by regulatory DC and PDC-pulsed regulatory DC in the supernatant of all cultures.

Role of Regulatory DC on Inflammatory Mucosal Milieu of Animal Model of PBC

Three administrations of regulatory DC and PDC-pulsed regulatory DC caused significantly reduced infiltration of inflammatory cells in 4 of 5 PBC model mice. The extent of infiltration of mononuclear cells of representative PBC model mice has been shown in Figure 4.

We did not mark any significant difference about cell infiltration in PBC mice due to administration of regulatory DC or PDC-pulsed regulatory DC (data not shown).

Role of Regulatory DC on Serum AMA Levels

AMA was seen in PBC model mice due to when these were examined on 16 and 28 weeks after start of poly I:C administration. Injection with regulatory DC and PDC-pulsed regulatory DC could not cause negativity of AMA in any mice.

DISCUSSION

As present understandings about pathogenesis of chronic liver diseases are broadening, it seems that new and innovative types of therapeutic approaches would be required for containment of these pathological conditions. Immune therapy represents a novel and evidence-based type therapy in this context. This is especially true for autoimmune liver diseases because there is no curative therapy for these lesions. Almost of types of therapy for PBC is endowed with limited clinical efficacy.

In this study, we have shown that regulatory DC that is capable of inducing immune tolerance *in vivo* may be an alternate therapeutic approach against PBC. In fact, we have already found that DC are involved in variable manners in the pathogenesis of PBC. Yamamoto et al from our laboratory have shown that DC in PBC patients show impaired functional capacity that may be related to breakdown of self tolerance in PBC.[21] On the other hand, study from our laboratory has also shown that the anti-

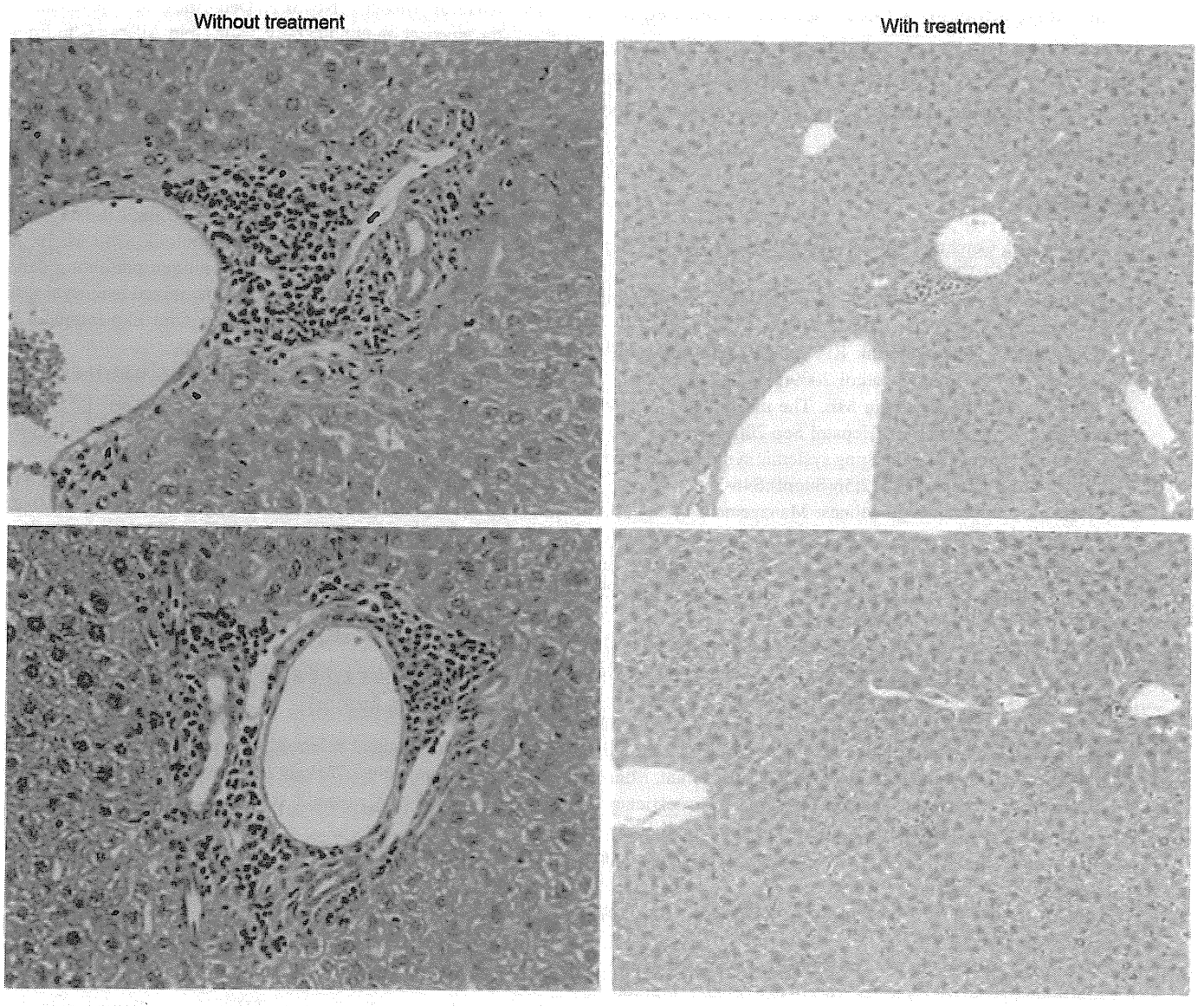


Fig. 4: Anti-inflammatory effects of regulatory dendritic cells in mice model of PBC. Accumulation of inflammatory cells in the portal area and around biliary tracts in PBC murine model of PBC. Significant down regulation of inflammatory cells due to treatment with regulatory DC. Without therapy: Receiving poly I:C according to the protocol in Figure 1. After therapy: 28 weeks receiving poly I:C according to protocol of Figure 1 with three administrations of regulatory DC

inflammatory property of bezafibrate in PBC is mediated through impaired anti-inflammatory action of DC.[22]

Administration of regulatory DC and PDC-pulsed regulatory DC but not BM DC caused reduced accumulation of inflammatory cells in the portal area and hepatic parenchyma. However, a significant improved capacity of PDC-pulsed DC over unpulsed regulatory DC could not be substantiated in this study. There may be several reasons for this. PDC represents a PBC-related antigen because almost all patients with PBC show antibody to PDC or its epitopes,[23] even if they are AMA-negative. PDC-specific antibody has shown diagnostic utility in PBC. But, PDC may not be a target antigen of immune-mediated damages of PBC. Thus, future study using other PBC-related antigen pulsed regulatory DC may be a better therapeutic choice. In addition, we have used only one dose of PDC and kinetics of DC numbers for therapeutic effects in murine PBC model has not been explored. Studies would be required to assess other PBC-related antigens and different doses of PDC and DC to prepare antigen-specific regulatory DC for therapeutic purposes. However, treatment with regulatory DC clearly showed their anti-inflammatory activities, but, the levels of AMA negativity could not be attained by DC-based immune therapy. Although little is known about genesis of AMA and inflammatory microenvironment in PBC, it seems that they may be independently regulated. This is also supported from clinical data that have shown that AMA titers have no role in disease progression in PBC patients. DC-based immune therapy has been applied in several clinical conditions, either to upregulate host immunity or to down regulate that to achieve therapeutic efficacy.[10] However, a clinically acceptable protocol is yet to be developed. The

present study, although a pilot one, has unmasked some important information for containment of hepatic damages in animal model of PBC. Further study using human regulatory DC would unveil the real implications of these observations.

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Biochemical, Virological, Immunological and Histopathological Features of 702 Incidentally Detected Chronic Hepatitis B Virus Carriers in Bangladesh

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Key Words
Incidentally detected hepatitis B virus carrier · Developing country · Liver damages · Public health problem · Hepatitis B virus control

Abstract
Background/Aims: Simultaneous assessment of biochemical, virological, and histological parameters of incidentally detected chronic hepatitis B virus (HBV)-infected subjects in Bangladesh were done to develop strategies for containment of HBV and management of liver diseases of these patients. ***Methods:*** A total of 702 chronic HBV carriers detected incidentally were enrolled in the study. Levels of HBV DNA and alanine aminotransferase (ALT) in sera were measured. The extent of hepatic inflammation and liver fibrosis was evaluated in all patients by examining liver biopsy specimens. ***Results:*** Of the 702 patients, 358 (50.7%) exhibited HBV DNA levels $>10^5$ copies/ml. ALT levels were above the upper limit of normal (ULN; >42 U/l) in more than 50% of the patients. High levels of HBV DNA ($>10^5$ copies/ml), increased ALT (>1.0 × ULN), moderate hepatic inflammation (HAI-NI ≥7) and severe hepatic fibrosis (HAI-F ≥3) were detected in 60 patients. ***Conclusion:*** As considerable numbers of apparently healthy subjects are unaware of the fact that they are chronically infected by HBV, many of whom have already developed progressive liver damage, emergency strategies would be needed for the containment and management of HBV infection in developing countries.

Introduction

In the last three decades, important advances in our understanding of the natural course of hepatitis B virus (HBV) infection have been made. It has been shown that chronic HBV infection can be divided into different phases, which may not be sequential. Patients may present (1) in a state of immune tolerance, (2) with hepatitis B e antigen (HBeAg)-positive chronic HBV, (3) with HBeAg-negative chronic HBV, or (4) as an inactive hepatitis B surface antigen (HBsAg) carrier [1, 2]. Patients with chronic HBV (both HBeAg-positive and HBeAg-negative) are assumed to be at greater risk of developing complications such as liver cirrhosis, hepatic decompensation and hepatocellular carcinoma [3]. Accordingly, major liver organizations, such as the American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of the Liver (EASL), and

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the Asian-Pacific Association for the Study of the Liver (APASL), consistently recommend antiviral therapy for patients with liver damages and complications [4–6].

However, Kumar et al. [7] reported that a fair proportion of incidentally detected HBV carriers, who were unaware of their HBV infection, had features of progressive liver damages. We also have detected severe liver damages in some HBeAg-negative patients with low HBV DNA and normal alanine aminotransferase (ALT) levels [8]. Studies in Egypt and Pakistan have revealed similar findings [9, 10]. Chu and Liaw [11] stated that asymptomatic HBV should not be viewed as an innocent long-lasting condition with a good prognosis because a considerable number of such patients develop liver cirrhosis.

We have encountered several hundred HBsAg-positive individuals in Bangladesh during routine clinical practice. All of them were apparently healthy, had never complained of liver diseases, and had not been vaccinated. All patients assumed that they did not need any antiviral treatment or active management because they were asymptomatic and apparently healthy. Also, they were unaware that they were potential source of HBV infection. This study was conducted to gain an insight into the virological, immunological, biochemical and histological features of these patients. The outcome of the study provided important insights about the management of such patients in Bangladesh and other countries.

Materials and Methods

Patients

All subjects were apparently healthy without any features of liver-related diseases when they first visited the hospital. They came to the hospital for testing of serum HBsAg (1) before traveling to a foreign country for work (compulsory requirement to obtain a working visa for most developed countries), (2) on the advice of their physician because of pregnancy, (3) before receiving HBV vaccine, or (4) before donating blood for transfusion.

When HBsAg was detected in the serum, the patient was requested to follow different public health measures. Patients were also advised to seek HBsAg testing after 6 months and to contact our clinical monitor if they experienced any physical anomaly. When HBsAg was detected 6 months after the first test, the patients were regarded as being chronically infected with HBV.

A total of 1,072 HBsAg-positive subjects were diagnosed as being chronic HBV carriers. Inclusion criteria for the study included: (1) positive for HBsAg, (2) negative for serological markers of hepatitis C virus, IgM antibodies to hepatitis A virus and hepatitis E virus, (3) having an alcohol consumption of <20 g/day, and (4) had no evidence of pregnancy. None of the patients had received any antiviral drugs (treatment-naïve) or immune modulators for treatment of HBV infection. A review board of the 'Viral Hepatitis Foundation, Bangladesh' provided permission to accomplish the study. A total of 743 patients gave informed written consent for a liver biopsy. The nature and purpose of the study were explained to all patients. Patients were excluded from further analyses if adequate amounts of liver tissue were not available during liver biopsy, as recommended [12]. Finally, a total of 702 chronic HBV carriers were available for final analyses.

Biochemical and Serological Tests

Levels of ALT in serum were measured commercially. The cutoff for the upper limit of normal (ULN) was ALT >42 U/l. HBsAg was assessed using a commercial ELISA kit (Diasorin, Fallugia, Italy). HBeAg was checked using an ELISA kit (Abbott Labs, Chicago, Ill., USA).

Quantification of Serum HBV DNA Levels

Serum HBV DNA was quantified using an RT-PCR kit (Amplicon HBV Monitor Assay, Roche Molecular Systems, Calif., USA). The lower limit of detection was 250 copies of HBV DNA/ml.

Liver Biopsy

A percutaneous liver biopsy was performed in all patients with prior, voluntary, informed written consent. In the case of minors, consent was obtained from a legal guardian. Biopsies were performed under local anesthesia using a 16G Tru-cut biopsy needle (Cardinal Health, McGaw Park, Ill., USA). A biopsy specimen of more than 1.0 cm in length with five to six portal tracts was evaluated. Histology was graded according to the histologic activity index (HAI) using the criteria of Knodell et al. [13]. The total HAI score comprises necroinflammation (HAI-NI) and fibrosis (HAI-F) scores. The HAI-NI scale includes three components (0–10, piecemeal necrosis; 0–4, lobular necrosis and inflammation; 0–4, portal inflammation). HAI-F was graded according to severity: 0, absence of fibrosis; 1, fibrous portal expansion; 3, bridging fibrosis; 4, cirrhosis.

Statistical Analyses

Data are expressed as mean ± SD. Data were analyzed by the unpaired t test if they were normally distributed and by the Mann-Whitney rank-sum test if they were skewed. Differences were considered significant at $p < 0.05$.

Results

Baseline Information of All Patients

The baseline data of 702 patients with chronic HBV infection are given in table 1. The mean age of the patients was 27.3 years (SD 8.8 years); 534 were male and the other 168 were female. Of a total of 702 patients, 248 were expressing HBeAg in the sera (HBeAg-positive) (35.3%) and the remaining 454 (64.7%) were HBeAg-negative. The mean level of serum ALT was 52 U/l (range 4–948 U/l). The levels of HBV DNA varied considerably among patients ranging from 300 to 1×10^{12} copies/ml (median value 1.2×10^5 copies/ml).

Virological, Biochemical and Histopathological Data of the Patients with Chronic HBV Infection

As shown in table 2, comparatively higher levels of HBV DNA (1×10^5 copies/ml) were seen in increased numbers of HBeAg-positive patients compared to HBeAg-negative patients (92.3 vs. 28.4%, $p < 0.05$). The levels of ALT above ULN (>42 U/l) were detected in 141 of 248 (56.8%) HBeAg-positive patients and 206 of 454 (45.4%) HBeAg-negative patients. Thirty-seven HBeAg-positive patients and 34 HBeAg-negative patients had ALT levels more than twice the ULN (>84 U/l) (table 2). ALT levels of >126 U/l (3 ULN) were detected in 12 HBeAg-positive and 11 HBeAg-negative asymptomatic HBV carriers (table 2). The magnitude of necroinflammation (assessed by HAI-NI score) was HAI-NI ≥7 in 22% (56 of 248) of the HBeAg-positive and 16% (71 of 454) of the HBeAg-negative chronic HBV carriers. In addition, severe hepatic fibrosis (HAI-F ≥3) was detected in 25% (62 of 248) of the HBeAg-positive and 20% (90 of 454) of the HBeAg-negative patients.

Comprehensive Analyses of Virological, Biochemical and Histopathological Parameters of Incidentally Detected Chronic HBV Carriers

The AASLD, EASL, and APASL guidelines recommend antiviral treatment for patients with chronic HBV infection with high HBV load and features of liver damages [4–6]. To evaluate the need of antiviral therapy in this cohort, we made a comprehensive assessment of their biochemical, virological and histopathological features. As shown in table 3, among 248 HBeAg-positive patients, 229 had HBV DNA of $>10^5$ copies/ml. Some 126 patients had both HBV DNA $>10^5$ copies/ml and serum ALT >42 U/l. Among them, 32 patients had HBV DNA $>10^5$ copies/ml, serum ALT >42 U/l, moderate levels of hepatic necroinflammation (HAI-NI ≥7) and severe hepatic fibrosis (HAI-F ≥3).

Among HBeAg-negative patients, 67 had both HBV DNA $\geq 10^5$ copies/ml and serum ALT >42 U/l. Among them, 28 patients had HBV DNA $\geq 10^5$ copies/ml, serum ALT >42 U/l, moderate hepatic necroinflammation (HAI N1≥7) and severe hepatic fibrosis (HAI-F ≥3) (table 3).

Discussion

Considerable numbers of chronic HBV carriers in Bangladesh were not aware of their HBV infection. Similar situations in terms of knowledge, attitude and practice regarding HBV prevail in most developing countries of

Table 1. Baseline data of the 702 patients

Number of patients	702
Age, years	27.3 ± 8.8
Male, n	534
Female, n	168
HBeAg-positive, n	248
HBeAg-negative, n	454
ALT levels	52 ± 55 U/l (range 4–948 U/l)
HBV DNA levels	300–5.3 $\times 10^{12}$ copies/ml

Table 2. Serum HBV DNA, ALT, and liver histology of study population

	HBeAg-positive (n = 248)	HBeAg-negative (n = 454)
HBV DNA		
HBV DNA <1 × 10^5 copies/ml	19 (7.7%)	325 (71.6%)
HBV DNA >1 × 10^5 copies/ml	229 (92.3%)	129 (28.4%)
ALT		
≤42 U/l	107 (43.1%)	248 (54.6%)
43–84 U/l (twice of ULN)	104 (41.9%)	172 (37.9%)
85–126 U/l (thrice of ULN)	25 (10.1%)	23 (5%)
>126 U/l	12 (4.8%)	11 (2.4%)
Liver histology		
Extent of hepatitis		
HAI-N1 ≤3	76 (30.6%)	216 (47.6%)
HAI-N1 4–6	116 (46.8%)	167 (36.8%)
HAI-N1 ≥7	56 (22.6%)	71 (15.6%)
Extent of fibrosis		
HAI-F0	20 (8.1%)	49 (10.8%)
HAI-F1	166 (66.9%)	315 (69.3%)
HAI-F3	54 (21.8%)	79 (17.4%)
HAI-F4	8 (3.2%)	11 (2.4%)

ALT: normal range <42 U/l.

Table 3. Incidentally detected chronic HBV carriers with high levels of HBV DNA, increased ALT, moderate necroinflammation and significant fibrosis

	HBeAg-positive	HBeAg-negative
Number of patients	248	454
HBV DNA >10^5 copies/ml	229 (92.3%)	129 (28.4%)
HBV DNA >10^5 copies/ml plus ALT >42 U/l	126 (50.8%)	67 (14.8%)
HBV DNA >10^5 copies/ml plus ALT >42 U/l plus HAI-NI ≥7 plus HAI-F ≥3	32 (12.9%)	28 (6.2%)

Asia and Africa. As they are asymptomatic and unaware of their HBV infection, they act as a permanent reservoir of HBV infection and transmit the virus to healthy persons in developing countries.

Although all patients in this study cohort were unaware of their HBV infectivity, many of them are on the way to developing severe complications like liver cirrhosis. A total of 60 patients (~8.5%) had high levels of HBV DNA, ALT above the ULN, a moderate degree of hepatic inflammation and severe liver fibrosis. All professional liver organizations recommend treatment for these patients to block further progression of liver damage [4–6]. Similar studies have been conducted in different countries to assess the real extent of problem regarding management of chronic HBV carriers. Kumar et al. [7] from India assessed virological, biochemical and histological parameters of liver functions in 1,387 incidentally detected chronic HBV carriers. They showed that more than 50% of these patients had evidence of progressive liver diseases for which treatment may be indicated. A similar outcome has also been reported from Pakistan, Egypt, and other countries [9, 10, 14–17].

However, these patients are not considered for antiviral therapy owing to complex socio-economic problems and the medical delivery system in developing countries. The majority of HBV-infected subjects have not been identified in developing countries owing to lack of an effective HBV monitoring system. In addition, both patients and physicians are reluctant to further assess the virological, biochemical and histological status because they assume that no curative therapy can be offered to patients. Finally, because of a lack of health insurance in these countries, patients are usually incapable of bearing the costs of expensive investigations and therapy.

Although there is a paucity of information about data of a nationwide survey regarding HBV prevalence in Bangladesh, published data show that about 5–6% apparently healthy individuals seem to be infected with HBV. Rudra et al. [18] have shown that about 6.2% of apparently health blood donors of Mymensingh, Bangladesh were infected with HBV. Ashraf et al. [19] have reported that 5.8% of the population of a semiurban area of Dhaka was expressing HBsAg. Mahtab et al. [20] also found almost similar data by checking HBsAg among 1,018 apparently healthy individuals. Taken together, there may be about 6–8 million chronic HBV carriers in Bangladesh (population of Bangladesh 160 million) and most of them are unaware of their infective state with the HBV. Although Bangladesh harbors millions of chronic HBV carriers, biochemical (ALT levels), immunological (HBeAg status), virological (HBV DNA levels), and histopathological (liver biopsy assessment) tests for such patients have not been conducted. In fact, this is the first study to assess biochemical, virological, and histopathological parameters of liver functions in considerable numbers of chronic HBV carriers in Bangladesh. This study clearly states that mechanisms should be developed to diagnose and manage these patients for containment of HBV and HBV-related complications.

One of the apparent limitations of this study is that the subjects were checked for HBV DNA, ALT and liver histology only once. Serial assessment of virological, biochemical and histological parameters would provide more insight into the natural disease course in these patients. However, a national policy would be required to accomplish this. Our main aim was to gain an insight into the pathogenesis of these patients to design a strategy for their management. We found that considerable numbers of asymptomatic HBV carriers have been harboring severe liver damage, so our study should convince physicians and policy makers in Bangladesh and other developing countries to develop a management strategy for such patients.

Disclosure Statement

All authors do hereby declare that there is no conflict of interest about this article.

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Inter-Genotypic Recombinant Hepatitis C Virus Strains in Japan Noted by Discrepancies Between Immunoassay and Sequencing

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Genetic recombination plays a significant role in the survival and evolution of hepatitis C virus (HCV), but methodological limitations have hindered the exploration of genetic recombination. HCV serotypes were evaluated in 104 patients with chronic hepatitis C when they initially presented in hospitals. Subsequently, HCV genotypes were analyzed using primers for core gene and NS5B gene. Near-complete nucleotide sequences of eight HCV isolates from two suspected patients with 2b/1b recombinant HCV were analyzed by amplification of nine overlapping regions of HCV-specific oligonucleotide primers at different time points: (i) at the first admission; (ii) before and (iii) after interferon therapy; and (iv) after development of hepatocellular carcinoma. The nucleotide sequence of eight HCV isolates obtained was 9,321–9,471 nucleotides in length, comprising a single ORF (polyprotein of 3,014 amino acids.) and segregated into discordant genotypes of 2b and 1b HCV with a recombination junction in NS2. This study highlights the need for more precise characterization of HCV in clinical samples where there is a discrepancy between immunoassays and sequencing. It also demonstrates the circulation of novel inter-genotypic recombinant HCV in Japan, because the cross over point of 2b/1b recombinant HCV in eight clinical isolates of these two patients differed from previously reported HCV recombinant from the Philippines and Japan. ***J. Med. Virol. 84: 1018–1024, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HCV recombination; Japan; serogroup; genotype

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and it is a major cause agent of post-transfusion hepatitis and parenterally transmitted, sporadic non-A, non-B hepatitis throughout the world [Moradpour et al., 2007]. Considerable advances have been made in the fields of epidemiology and virology during the last two decades, and there have been many investigations on the mode of HCV transmission and therapeutic approaches [Neumann et al., 1998; Di Bisceglie and Hoofnagle, 2002; Seeff, 2002]. However, major concerns remain about the development of prophylactic vaccines and the treatment of non-responder patients, which may be attributable to limited understanding of HCV genetic diversity.

HCV is an RNA virus and it exploits all the known mechanisms of genetic variation to ensure its survival [Choo et al., 1991; Enomoto et al., 1996; Holmes et al., 1999; Chung et al., 2010]. The polymerase enzyme of most RNA viruses, including HCV, lacks proofreading ability, so it is unable to correct errors during viral replication and this leads to genomic mutations. In addition, HCV exists in a host as a heterogeneous population of viruses with closely related genomes, a condition referred to as a quasispecies. These alterations in the HCV genome allow the virus to escape the host immune response and these changes in the viral

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