inflammation and fibrosis have been reported in some, but not all, chronic hepatitis B patients after treatment with antiviral drugs. In spite of improvements in virological, biochemical, immunological, and histological parameters with antiviral therapy, few studies show that antiviral drugs significantly block or delay hepatic decompensation, the occurrence of liver cirrhosis and HCC, and liver-related death. It is controversial whether the HBV DNA load, HBeAg, HBsAg, anti-HBe, anti-HBs, and liver histology can be regarded as true surrogate markers of an efficient therapeutic outcome of antiviral therapy in chronic hepatitis B patients [24–26]. A surrogate marker should be strong enough to predict a final outcome. However, because chronic hepatitis B progresses slowly, it will take considerable time to determine viable surrogate markers of therapeutic efficacy. Studies must be conducted for decades, and serial observations should be done regarding the kinetics of different virological, biochemical, histological, and immunological markers. As a result, one or more surrogate markers may be determined, or, in contrast, studies may determine that no surrogate marker is available. There is a paucity of studies that assess the final outcome of antiviral therapy in chronic hepatitis B patients. One study showed that the occurrence of HCC and liver cancer was decreased in antiviral-treated chronic hepatitis B patients. However, the study was not designed to provide the kinetics of all surrogate markers of treatment outcome [14]. It seems that HBV DNA levels and HBeAg seroconversion may not be true surrogate markers of the clinical outcome of antiviral therapy for chronic hepatitis B [24, 26]. However, assessment should be made regarding the surrogacy of levels of HBsAg or covalently closed circular DNA (cccDNA) in RCTs, because few reports have examined the kinetics of these markers. The value of a surrogate marker of efficacy has considerable influence on antiviral therapy in a global context, and especially in developing countries. Assessment of questionable surrogate markers may shatter the ultimate purpose of good medical practice for treating chronic hepatitis B in developing countries. In these countries, improvement of any surrogate marker of treatment outcome may be defined as a better therapeutic outcome. The idea of using elusive surrogate markers is counterproductive, and, in the long run, compromises confidence in the health systems of developing countries. If surrogate markers are improperly defined, this can negatively regulate different preventive measures against HBV infection.

Significance of HBV reduction due to antiviral therapy

The development of evidence-based therapeutic approaches against HBV infection is dependent on the proper understanding of different cellular and molecular events that control HBV replication and liver damage. To develop insights into these factors, we examined differences in HBV control that occurs naturally versus that caused by antiviral drugs. Almost all epidemiologic data have shown that a high HBV load in patients with chronic hepatitis B has been consistently associated with a poorer clinical outcome. This is particularly relevant when patients with chronic HBV infection with very low or undetectable levels of HBV DNA are compared with patients with 10^5 or 10^6 copies/ml of HBV DNA [27, 28]. However, considerable numbers of HBV-infected patients do not develop complications and exhibit natural control of HBV DNA replication without any therapy [1, 3]. Progressive downregulation and negativity of HBV during natural infection is usually followed by the resolution of hepatitis, disappearance of HBV-related antigens, and expression of protective antibody like anti-HBs. The picture is quite different in patients with chronic hepatitis B treated with antiviral drugs. Although most patients with chronic hepatitis B receiving antiviral drugs show HBV DNA reduction, this is not usually followed by the disappearance of all HBV-related antigens, appearance of anti-HBs, or resolution of the disease. Although the mechanisms underlying these differences have not been properly elucidated, during the natural course of resolved HBV infection, downregulation of HBV DNA may be accomplished by the restoration of inherent host immunity. Thus, HBV DNA reduction is usually accompanied by the loss of HBeAg and HBsAg and the expression of anti-HBe and ant-HBs in the sera. On the other hand, in patients in whom antiviral therapy is used, downregulation of HBV DNA is a result of the interplay between drugs and the HBV replication system. This may be followed by the restoration of host immunity in some patients [29–31], but questions remain about the nature, magnitude, and sustainability of host immunity following antiviral drug treatment in chronic hepatitis B patients.

Future aspects of therapy for HBV via modulation of host immunity

HBV therapy faces two major challenges in future. First, more potent or alternative therapeutic approaches should be developed to combat HBV infection. Second, therapeutic regimens should be applicable to people in developing and resource-constrained countries. This latter challenge requires some type of meeting between science and social factors.

Treatment of HBV infection would be drastically altered if an antiviral drug capable of eradicating all forms of HBV; replicating HBV DNA, cccDNA, and extrahepatic HBV DNA, emerged. However, it is unknown whether such a drug will ever be available.

In this context, future attention should focus on developing alternative therapeutic approaches for HBV infection. Insights into prospective therapies have been gained from studies of HBV replication and liver damage after natural infection and after antiviral therapy. HBV is not a cytotoxic virus, and liver damage and HBV-related complications are mediated by the host immune responses. The immune responses of chronic hepatitis B patients may be broadly categorized into two types: one is protective immunity that controls the virus and liver damage and the other is of pathogenic nature, which induces liver damage and may have not a role in virus control. It has been shown that HBV-specific immune responses are usually protective in chronic hepatitis B patients, whereas non-HBV-specific immune responses cause liver damage [32–35]. Although these facts provide a rationale for immune therapy as an alternative therapeutic approach to chronic hepatitis B, the limited efficacy of polyclonal immune modulators in chronic hepatitis B patients is understandable because non-HBV-specific immune therapy may not have proper therapeutic efficacy. However, it is possible that HBV-specific immune responses may be induced later by polyclonal immune modulators, and this hypothesis needs to be assessed in chronic hepatitis B patients [35]. Immune therapy targeting natural killer cells, natural killer T cells, cells of innate immunity, and other immunocytes could provide important insights about the role of immune modulation in patients with chronic hepatitis B. In addition, cell-based immune therapy with bone marrow cells, dendritic cells, stem cells, and mesenchymal cells has shown some promise in chronic hepatitis B, but there is limited information about these studies. HBV-specific immune therapy that uses antigenic epitopes of HBV, HBV-related antigens, and HBV DNA may be effective. An HBsAg-based vaccine has been widely used as an antigen-specific therapy in chronic hepatitis B patients around the world. However, the efficacy of this vaccine is not clear [36]. An HBsAg-based vaccine has also been used in combination with antiviral agents. Unfortunately, significantly better efficacy of a combination of antiviral therapy and HBsAg-based immune therapy versus monotherapy with antiviral drugs could not be substantiated in an RCT [37].

In the meantime, it has become evident that hepatitis B core antigen (HBcAg)-specific cytotoxic T lymphocytes (CTL) are associated with the control of HBV replication and minimizing liver damage in chronic hepatitis B [33, 34]. However, there is a paucity of information about HBcAg-based therapeutic vaccine in chronic hepatitis B, although investigators have highlighted its use [37]. Recently, preliminary data about the therapeutic efficacy of a combination of HBsAg and HBcAg-based vaccine in chronic hepatitis B patients, administered by mucosal (nasal) and parenteral routes has been shown to lead to the sustained control of HBV and ALT levels 1 year after the discontinuation of therapy [38]; however, the authors of this study enrolled only 18 patients with chronic hepatitis B, and a larger study and prolonged follow up is warranted before any claims can be made. Other strategies of immune therapy using different immune modulators should also be tried in chronic hepatitis B patients.

The next challenge is to implement a regimen of anti-HBV therapy in developing countries. Antiviral agents such as NAs are now widely used in developing countries, but proper use of these agents cannot be expected due to the inherent social and economic limitations of these countries. As chronic hepatitis B progresses slowly, its negative impact on a healthcare delivery system will take time, and realization of the adverse effects of misusing current therapies may not be noted until it is too late to reverse them. In this context, attention should focus on innovative and alternative therapeutic regimens against chronic hepatitis B. As antigen-based vaccines are safe and inexpensive, clinical trials with these vaccines may be accomplished in developing countries with the active collaboration of developed countries. There are millions of treatment-naïve chronic hepatitis B patients in developing countries. At the same time, physicians in developed countries are highly experienced at conducting clinical trials. If a credible collaboration can be developed among physicians and hepatologists in developed and developing countries, it may be possible to develop a better regimen of HBV-specific immune therapy to treat chronic hepatitis B.

Conclusion

Future aspects of therapy for chronic hepatitis B virus infection may show considerable heterogeneity in different parts of the world if the present trends continue. More potent NA drugs may be available in the near future, but these drugs cannot be expected to improve clinical outcomes drastically. In advanced countries, an atmosphere of stagnation regarding treatment for chronic hepatitis B may develop if we remain fully dependent on and solely committed to NAs. At the same time, developing countries may become be filled with non-responders and partial responders to NAs, with only a small group of patients benefiting from therapy. Patients with mutant HBV would constitute a major burden to the healthcare delivery systems of resource-constrained countries in the future. Similarly, indiscriminant use of NAs in chronic hepatitis B patients co-infected with other viruses, such as human immune-deficiency virus (HIV), would create a more complex situation for controlling both HBV and HIV. Free drugs are available for HIV infection, but not for chronic hepatitis B, although many HIV/HBV coinfected patients

die due to HBV-related liver diseases as opposed to the deaths being due to HIV. In addition, different doses of NAs are prescribed for HIV and chronic hepatitis B, and these drugs are not appropriately used in developing countries. Alternative and innovative therapies constitute some hope for a breakthrough in the treatment of chronic hepatitis B patients. But several steps are needed to get any real benefit from these therapeutic regimens. Vaccines containing antigens, epitopes, and DNA or immune therapy targeting different cells offer hope as alternative therapeutic strategies for chronic hepatitis B. The safety and efficacy of alternative therapies in chronic hepatitis B patients, determined by RCTs, could provide insights into whether these therapies would be able to stand the test of time. Finally, strong collaboration between developed and developing countries would be required to obtain benefits from new and innovative therapies.

Conflict of interest The authors declare that they have no conflicts of interest.

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Safety and immunogenicity of hepatitis B surface antigen-pulsed dendritic cells in patients with chronic hepatitis B

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SUMMARY. The immune modulator capacity of antigen-pulsed dendritic cells (DC) has been documented in patients with cancers and in animal models of chronic viral infections. Cancer antigen-pulsed DC are now used for treating patients with cancer. But viral antigen-pulsed DC are not used in chronic viral-infected patients because safety of antigen-pulsed DC has not been evaluated in these patients. DC were isolated from human peripheral blood mononuclear cells by culturing with human-grade granulocyte-macrophage colony stimulating factor and interleukin-4. Human blood DC were cultured with hepatitis B surface antigen (HBsAg) for 8 h to prepare HBsAg-pulsed DC. After immunogenicity assessment of HBsAg-pulsed DC *in vitro*, five million HBsAg-pulsed DC were administered intradermally to five patients with chronic hepatitis B (CHB) 1–3 times. HBsAg-pulsed DC were immunogenic in nature because they produced significantly higher levels of interleukin-12 and interferon-γ compared to unpulsed DC ($P < 0.05$). Also, HBsAg-pulsed DC induced proliferation of HBsAg-specific T lymphocytes *in vitro*. CHB patients injected with HBsAg-pulsed DC did not exhibit generalized inflammation, exacerbation of liver damage, abnormal kidney function, or features of autoimmunity. Administration of HBsAg-pulsed DC induced anti-HBs in two patients and HBsAg-specific cellular immunity in 1 patient. This is the first study about preparation of antigen-pulsed DC using human consumable materials for treating patients with CHB. Because HBsAg-pulsed DC were safe for all patients with CHB and had immune modulation capacity in some patients, phase I and phase II clinical trials with antigen-pulsed DC in CHB and other chronic infections are warranted.

Keywords: antigen-pulsed dendritic cells, chronic hepatitis B, dendritic cells, hepatitis B surface antigen, therapeutic vaccine.

INTRODUCTION

There is no curative therapy for patients with chronic hepatitis B (CHB). Antiviral drugs are recommended to patients with CHB to attain sustained control of replication of the hepatitis B virus (HBV) and minimize liver damage [1]. However, the therapeutic efficacy of antiviral agents in patients with CHB is not complete, as most studies have reported only intermediate outcomes. A well-designed study for a National Institutes of Health consensus development conference analysed all randomized clinical trials with antiviral drugs in patients with CHB from 1989 to 2008 [2]. Results showed that no single drug treatment improved clinical outcomes or all intermediate outcomes of CHB [2], although improvements of some intermediate outcomes have been seen. However, adverse events during antiviral treatment occurred in about 50% patients. These facts support the need for a new and innovative therapeutic strategy against CHB.

Chronic HBV infection represents a viral-mediated immunological disease. Although HBV is a noncytopathic virus, patients with CHB exhibit features of liver damage and associated complications. HBV-specific immune responses are narrowly focused and weak in most patients with CHB [3]. Recent studies have also shown that impaired HBV-specific immunity and exacerbated polyclonal immune responses are related to viral persistence and liver damage in patients with CHB [4]. On the other hand, effective control

Abbreviations: ALT, alanine aminotransferase; CHB, chronic hepatitis B; CHB, chronic hepatitis B; DC, dendritic cells; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PBMC, Peripheral blood mononuclear cells; PBS, phosphate-buffered saline.

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of HBV replication and liver damage are associated with strong HBV-specific immunity in these patients [5].

Taken together, restoration of HBV-specific immunity in patients with CHB may have some therapeutic potential. In fact, a new field of clinical application of vaccines containing hepatitis B surface antigen (HBsAg) has been initiated to restore HBsAg-specific immune responses in patients with CHB [6,7]. Although safer and cheaper than commercially available antiviral drugs, restoration of HBsAg-specific immunity was not attained in patients with CHB by vaccine therapy [8].

To develop a more potent regimen of antigen-specific immune therapy, the role of antigen-presenting dendritic cells (DC) in adaptive immunity has been examined. DC are responsible for processing and presenting antigens for induction of antigen-specific immune responses in normal conditions as well as in the immune tolerance state [9,10]. Studies have shown that the phenotypes and functions of DC are distorted in patients with CHB [11,12]. Accordingly, during vaccine therapy, HBsAg may not be properly processed by DC in patients with CHB to induce HBsAg-specific immune responses.

One way to circumvent this problem is to produce antigen-pulsed DC and use them as a vaccine [9,10]. In fact, cancer antigen-pulsed DC are widely used to induce cancer-specific immunity in patients with cancer [13]. However, antigen-pulsed DC have not been produced for human use in other patient groups, and almost nothing is known about their therapeutic use outside cancer.

In our study, human blood DC were cultured with human consumable HBsAg to prepare human-grade HBsAg-pulsed DC. The functions of HBsAg-pulsed DC were assessed *in vitro*. Finally, a pilot study was carried out in patients with CHB to evaluate the safety of HBsAg-pulsed DC. The induction of HBsAg-specific immunity by HBsAg-pulsed DC was also assessed in these patients.

MATERIALS AND METHODS

Clinical trial design and study population

The study was an open-label, phase-1 safety trial in patients with CHB. Five patients with CHB were enrolled in this study. Informed written consent has been obtained from each patient. The study has been performed according to the World Medical Association declaration of Helsinki, and the procedures have been approved by the Ethical Committee of Ehime University Graduate School of Medicine, Japan. Enrolled patients did not have serological markers of hepatitis A virus, hepatitis C virus, hepatitis E virus, or human immune deficiency virus at that time of trial start. The diagnosis of CHB was made from data on clinical and serological parameters. All subjects were positive for HBsAg, HBV DNA, and antibody to hepatitis core antibody in the sera. Two subjects (patients 1 and 2) were positive for hepatitis B e antigen, whereas three subjects (patients 3, 4, and 5) were positive for antibody to hepatitis B e antigen. Anti-HBs antibodies were not detected in any patient. In four patients, liver biopsy specimens were available to make a histological diagnosis (patients 1, 2, 3, and 5). All of them had moderate levels of hepatitis. Levels of fibrosis were mild in patients 1 and 2 and severe in patients 3 and 5. These patients were attending our university hospital for regular follow-up and treatment. The clinical profiles of patients before administration of HBsAg-pulsed DC are shown in Table 1. Levels of alanine aminotransferase (ALT) in the sera were elevated in three patients (patients 1, 2, and 5) and within normal limits in patients 3 and 4. No patient showed any feature of general inflammation (assessed from serum levels of C-reactive protein) or abnormal kidney function.

Isolation of DC from peripheral blood and loading of HBsAg in vitro

Isolation of human blood DC and production of HBsAg-pulsed DC were carried out as reported previously [14]. A special room was assigned for cell cultures, and DC were isolated from one person at a time. All reagents used for cell culture studies were free from endotoxin and toxoplasma. Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn heparinized whole blood, washed three times, and resuspended in RPMI 1640 (Nipro, Osaka, Japan) plus 10% autologous serum. DC were enriched from an adherent population of PBMC, as described previously [14]. PBMC were cultured in RPMI 1640 plus 10% autologous sera and human-grade granulocyte-macrophage stimulating factor (800 U/mL) and interleukin (IL)-4 (400 U/ml) (Pepro Tech EC Ltd., London, UK) for 7 days. DC were retrieved from the culture and washed three times with phosphate-buffered saline (PBS). The expressions of DC-related markers on human DC were checked by direct flow cytometry.

To produce HBsAg-pulsed DC, blood DC were cultured with a commercially available HB vaccine containing 10 μg of HBsAg (Heptavax-II, subtype adw, Banyu Pharmaceutical Co., Tokyo, Japan) for 8 hours. After the end of culture, DC were pelleted and washed five times in PBS. After the last wash, the final solutions were collected and preserved at −20 °C to assess if there was any free HBsAg in HBsAg-pulsed DC. As a control, human blood DC were cultured in RPMI 1640 plus autologous sera for 8 h.

Analyses of phenotype and functions of DC

The expression of HLA DR and CD86 on unpulsed DC and HBsAg-pulsed DC were assessed by direct flow cytometry using fluorescein isothiocyanate-conjugated monoclonal antibody to human HLA DR (Clone L243) and phycoerythrin-conjugated monoclonal antibody to human CD86 (clone 2331 [FUN-1]) (all from BD Pharmingen, San Jose,

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Table 1 Clinical profiles of patients with chronic hepatitis B before administration of HBsAg-pulsed DC

	Patient no.				
	1	2	3	4	5
Age (years)	36	30	35	48	57
Sex	Male	Male	Male	Female	Male
Alanine aminotransferase (5–48 IU/L)*	169	83	35	31	95
Asparate aminotransferase (6–45 IU/L)	76	52	30	40	80
Prothrombin time (80–120%)	112.6	100	84	85.3	108.6
Creatinine (0.61–1.04 mg/dL)	0.6	0.7	0.6	0.6	0.9
Blood urea nitrogen (6–20 mg/dL)	8.0	12.0	11.0	11.0	14.0
C-reactive protein (<0.30 mg/dL)	0.04	0.05	0.03	0.03	0.04
Antinuclear antibody (<40)	–	–	–	–	–
Histology					
Activity	A2	A1	A2	ND	A2
Fibrosis	F1	F1	F3	ND	F3
HBV DNA (Log genomic equivalent)	7.2	7.5	<3.7	4.8	7.5
Hepatitis B surface antigen (IU/ml)	+	+	+	+	+
Hepatitis B e antigen (S/CO)	+	+	–	–	–
Antibody to hepatitis B e antigen	–	–	+	+	+
Antibody to hepatitis B core antigen	+	+	+	+	+
Antibody to hepatitis B surface antigen	–	–	–	–	–

HBV, hepatitis B virus.
*Levels in parenthesis indicate normal range.

CA, USA). Data acquisition and analysis were performed on fluorescein-activated cell sorter (Becton Dickinson Biosciences, San Jose, CA, USA) [14].

T-cell stimulatory capacity and HBsAg-specific proliferative capacity of DC were assessed in allogenic mixed leucocyte reaction and antigen-specific lymphoproliferative assays. Human blood DC were cultured with allogenic T cells, or autologous T cells were cultured with unpulsed or HBsAg-pulsed DC for 104 h, and then [^{3}H]-thymidine was added to the cultures. The cultures were done for an additional 16 h. The cultures were then harvested using a semiautomatic harvester, and the level of incorporation of [^{3}H]-thymidine was shown as counts per minute in a scintillation counter (Beckman LS 6500, Beckman Instruments, Inc., Fullerton, CA, USA) [14]. Data were also expressed as counts per minute or a stimulation index that was calculated by dividing the counts per minute in culture containing HBsAg-pulsed DC with that of unpulsed DC. A stimulation index >2.0 was considered a significant proliferation.

Levels of IL-12 and interferon (IFN)-γ in samples were measured by an enzyme-linked immunosorbent assay (ELISA) using commercial kit (BD Pharmingen).

Estimation of HBsAg and anti-HBs

To estimate levels of HBsAg and anti-HBs, samples were sent to commercial companies (Special Reference Laboratory, Osaka, Japan). The estimation was performed using the chemiluminescence enzyme immunoassay method. Detection limits of HBsAg and anti-HBs were 0.2 ng/mL and 3.0 mIU/mL, respectively. Some samples were sent to a second commercial company (Shikoku Chuken Co, Matsuyama, Japan) for further confirmation.

Immunization of CHB patients with HBsAg-pulsed DC

HBsAg-pulsed DC were suspended in PBS and placed in two syringes. In one syringe, 20 μL of PBS containing 2×10^5 HBsAg-pulsed DC was suspended. In a second syringe, 5 million HBsAg-pulsed DC were diluted in 250 μL of PBS. First, 2×10^5 HBsAg-pulsed DC in 20 μL of PBS were injected at the anterior part of forelimb of patients with CHB to see if there was any hypersensitivity reaction. After 15 minutes, patients were injected intradermally in the deltoid region with 5 million HBsAg-pulsed DC. The patients with CHB were allowed to rest for 30 min. They were then monitored periodically for temperature, pulse rate, blood pressure, and respiratory rate for the first 24 h. Blood was collected from all patients before immunization and at different times after immunization with HBsAg-pulsed DC. Parameters of generalized inflammation, liver function test, kidney function test, and autoantibodies were checked in all patients at different times after the administration of HBsAg-pulsed DC. Patients received 1, 2, or 3 injections over 4 months. The immunization schedule for each patient is shown in Fig. 1.

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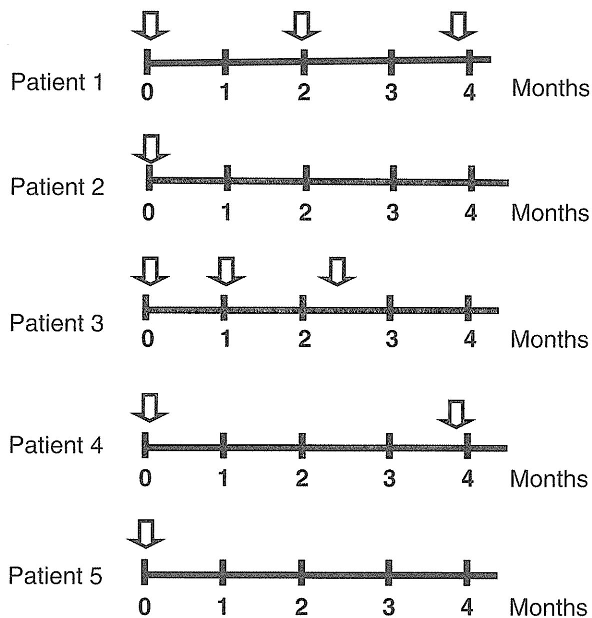


Fig. 1 Immunization schedule with HBsAg-pulsed DC in patients with chronic hepatitis B (CHB). HBsAg-pulsed DC were administered 1–3 times (arrows).

Statistical analysis

Values are represented as mean ± standard deviation (SD). Data were analysed by unpaired t tests if data were normally distributed and by Mann–Whitney rank-sum test if they were skewed. Differences were considered significant if $P < 0.05$.

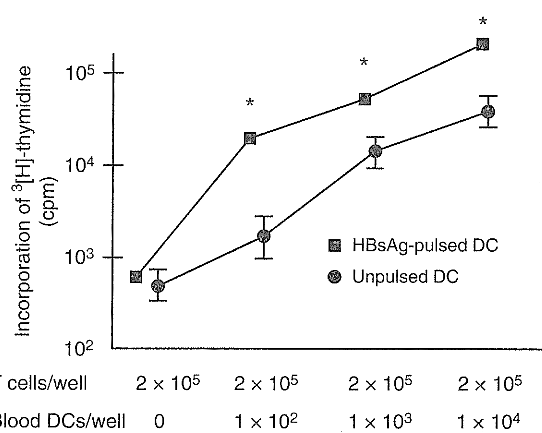
RESULTS

Features of human blood DC

As a preliminary study, we first isolated DC from patients with CHB and pulsed with HBsAg, as described previously [15]. Flow cytometry analysis revealed that the frequencies of contaminating T lymphocytes (CD3-positive cells), B lymphocytes (CD19, 20, 21-positive cells), monocytes (CD14-positive cells), and natural killer cells (CD56-positive cells) were less than 5% of the total DC (data not shown). DC from patients with CHB expressed DC-related antigens, such as HLA-A, B, C, HLA DR, CD86, and CD40. A functional study showed that DC from patients with CHB stimulated allogenic T cells in a dose-dependent manner (Fig. 2).

Characterization of HBsAg-pulsed DC from patients with CHB

During preliminary experiments, we checked two features of HBsAg-pulsed DC for this clinical trial: (i) there should be no free HBsAg in HBsAg-pulsed DC and (ii) HBsAg-pulsed DC should be immunogenic in nature so that it can induce



T cells/well	2×10^5	2×10^5	2×10^5	2×10^5
Blood DCs/well	0	1×10^2	1×10^3	1×10^4

Fig. 2 DC from patients with chronic hepatitis B (CHB) stimulated allogenic T cells in a dose-dependent manner. However, HBsAg-pulsed DC had significantly higher capacities to induce blastogenesis of T cells compared with unpulsed DC. Data from 5 separate experiments are shown. cpm, counts per minute. *$P < 0.05$.

HBsAg-specific immune responses *in vivo*. No HBsAg was detected in the final wash solution of HBsAg-pulsed DC. Levels of IL-12 were significantly increased in culture containing HBsAg-pulsed DC from patients with CHB (156.2 ± 20.7 pg/mL) compared to unpulsed DC (29.2 ± 9.8 pg/mL) from patients with CHB ($P < 0.05$). Similarly, levels of IFN-γ were significantly increased in culture containing HBsAg-pulsed DC (232.4 ± 24.1 pg/mL) from patients with CHB compared to unpulsed DC (80.5 ± 12.9 pg/mL) from patients with CHB ($P < 0.05$). HBsAg-pulsed DC also induced significantly higher levels of blastogenesis of T cells compared to unpulsed DC (Fig. 2). Also, HBsAg-pulsed DC induced significant proliferation of HBsAg-specific T lymphocytes *in vitro* (data not shown). After confirming these points, we prepared HBsAg-pulsed DC for administration to patients with CHB.

Safety of HBsAg-pulsed DC in patients with CHB

No immediate or delayed inflammatory or allergic reaction was detected at the injection site of HBsAg-pulsed DC in any patient. No patient complained of any allergic reaction or fever after immunization with HBsAg-pulsed DC. Different parameters of blood biochemistry, liver and kidney functions, and immunological statuses of all patients with CHB were checked periodically, and data during follow-up period after the end of administration of HBsAg-pulsed DC in these patients have been shown in Table 2. There was no significant change in levels of C-reactive protein or other parameters of general features of inflammation because of administration of HBsAg-pulsed DC. In addition, parameters of kidney function were within normal ranges in all patients after the administration of HBsAg-pulsed DC. Features of autoimmunity or autoantibodies were not detected in any patient.

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Table 2 Clinical profiles of patients with chronic hepatitis B during follow-up after completion of administration of HBsAg-pulsed DC

	Patient no. 1		Patient no. 2		Patient no. 3		Patient no. 4		Patient no. 5	
	1M*	3M	1M	3M	1M	3M	1M	3M	1M	3M
Alanine aminotransferase (5–48 IU/L)	171	147	57	36	37	30	43	41	109	72
Asparate aminotransferase (6–45 IU/L)	89	73	37	28	32	38	33	35	88	58
Prothrombin time (80–120%)	113.8	99.5	80.3	98.9	97	95	81.5	84.5	103.4	12.1
Creatinine (0.61–1.04 mg/dL)	0.7	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.9	1.0
Blood urea nitro- gen (6–20 mg/dL)	10	7	8	9	10	8	9	9	13	10
C-reactive protein (<0.30 mg/dL)	0.03	0.03	0.03	0.03	0.02	0.03	0.03	0.04	0.04	0.03
Antinuclear antibody (<40)	–	–	–	–	–	–	–	–	–	–
HBV DNA (Log genomic equivalent)	7.1	7.0	6.3	5.5	<3.7	<3.7	3.8	4.0	6.8	6.5

HBV, hepatitis B virus.
*Indicates month after end of last administration with hepatitis B surface antigen (HBsAg)-pulsed dendritic cells (DC). 1M; 1 month after completion of administration of HBsAg-pulsed DC, 3M; 3 months after completion of administration of HBsAg-pulsed DC.

Effect of HBsAg-pulsed DC on serum ALT levels

ALT levels just before the administration of HBsAg-pulsed DC are shown in Table 1. Because of the CHB, patients exhibited fluctuating ALT levels. The highest ALT level was <300 IU/L in all patients. There was no significant alteration in serum ALT levels because of administration of HBsAg-pulsed DC (Table 2).

Impact of HBsAg-pulsed DC as a therapeutic vaccine

The main aim of this study was to evaluate whether immunogenic HBsAg-pulsed DC can be prepared from DC of patients with CHB. The next goal was to assess whether administration of HBsAg-pulsed DC was safe. Although this study was not designed to assess the therapeutic potential of HBsAg-pulsed DC in patients with CHB, we checked levels of HBsAg, HBeAg negativity, and anti-HBe seroconversion in all patients. Administration of HBsAg-pulsed DC did not cause any major change in these parameters (data not shown). As shown in Table 2, the levels of HBV DNA were decreased slightly, but not significantly, because of administration of HBsAg-pulsed DC in these patients. Adverse effects were not detected in any patient. For example, three patients were anti-HBe positive, and the administration of HBsAg-pulsed DC did not cause reappearance of HBeAg in any patient.

Increased cytokine production by immunocytes of patients with CHB because of administration of HBsAg-pulsed DC

DC and T cells of patients with CHB were collected before and after the administration of HBsAg-pulsed DC and cultured in autologous mixed leucocyte reaction. Levels of IL-12 (before, 32.3 ± 5.2 pg/mL; after, 145 ± 9.2 pg/mL) and INF-γ (before, 21.7 ± 3.2 pg/mL; after, 154 ± 11.8 pg/mL) were significantly higher in culture supernatants after the administration of HBsAg-pulsed DC compared to before administration ($P < 0.05$).

HBsAg-specific immunity because of administration of HBsAg-pulsed DC

Administration of HBsAg-pulsed DC induced anti-HBs in two patients (patients 1 and 3). Anti-HBs were detected 1 month after HBsAg-pulsed DC administration in patient 1 and 2 months after HBsAg-pulsed DC administration in patient 3. Levels of anti-HBs increased progressively for 5 months in patient 1 and then started to decline. In contrast, levels of anti-HBs remained similar over time in patient 3 (Fig. 3).

HBsAg-specific cellular immunity in patient 1

HBsAg-specific T cells proliferation was not detected in any patient before study commencement. However, HBsAg-specific cellular immune responses 2 months after the third injection of HBsAg-pulsed DC were detected in patient 1 (Fig. 4).

DISCUSSION

Potent antiviral and antitumour effects of antigen-pulsed DC have been documented in animal models of human diseases since the 1980s. The first clinical trial of cancer antigen-pulsed DC was conducted in patients with cancer in 1996 [15]. Several clinical trials with cancer antigen-pulsed DC are ongoing worldwide [13]. It is expected that viral antigen-pulsed DC may be an effective immune therapy in patients with chronic viral infections, but such approaches have not yet been attempted clinically.

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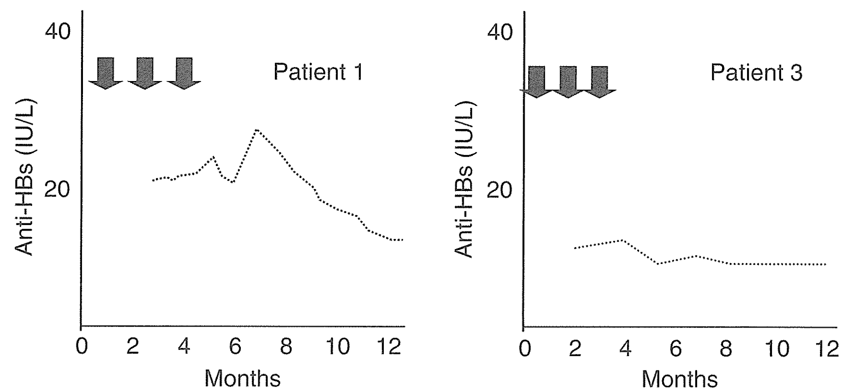

Fig. 3 Anti-HBs in 2 patients with chronic hepatitis B (CHB) after administration (arrows) of HBsAg-pulsed DC.

To treat patients with cancer, DC have been loaded with tumour-associated antigens, crude tumour products, tumour RNA, and other tumour-derived products *in vitro* [13,16]. The resultant cancer antigen-pulsed DC have been injected into patients with cancer, especially in those with advanced cancer. Many of these patients were immune compromised because they were in terminal stage of cancer. However, almost all patients with chronic viral infections are immune competent. Thus, a systemic and cautious approach is needed to prepare viral antigen-pulsed DC and initiate a clinical trial.

In this study, we used HBsAg, a well-known protein of HBV, to load human blood DC. As the safest source of HBsAg, we used a prophylactic hepatitis B vaccine. We showed that there was no free HBsAg in HBsAg-pulsed DC. This is important because if free antigen is present in HBsAg-pulsed DC, it will be difficult to assess the real effects of antigen-pulsed DC *in vivo* because free HBsAg may also induce immune responses *in vivo*. We also found that HBsAg-pulsed DC produced significantly higher levels of IL-12 and IFN-γ, two immune stimulatory cytokines compared to unpulsed DC. Furthermore, we checked for any contamination in HBsAg-pulsed DC. When we reproducibly produced immunogenic HBsAg-pulsed DC without any contamination, we moved forward to conduct this pilot study.

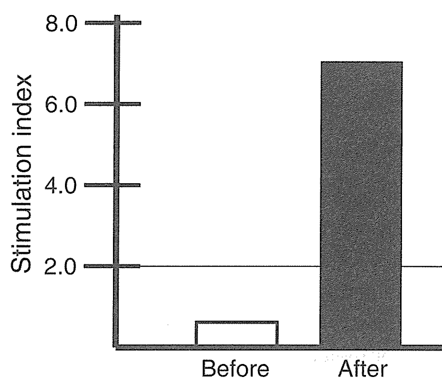

Fig. 4 HBsAg-specific cellular immune responses were seen after the third immunization of HBsAg-pulsed DC in patient 1.

Antigen-specific humoral and cellular immune responses were detected in 2 and 1 patients, respectively, because of administration of HBsAg-pulsed DC. It is premature to comment about the therapeutic efficacy of HBsAg-pulsed DC in this study because this is a pilot study. Indeed, there are several opportunities to improve our protocol. As the safest form of HBsAg, we used HBsAg in an HB vaccine. This allowed us to use a maximum of 10 μg of HBsAg to maximize DC viability during preparation of HBsAg-pulsed DC. In the future, human consumable recombinant HBsAg in larger doses may be used for preparing antigen-pulsed DC. We used only 5 million HBsAg-pulsed DC, as this was the first clinical trial of this nature. In the future, a dose escalation study with HBsAg-pulsed DC should be conducted. In addition, the number of patients needs to be increased.

Our study represents an initial effort to develop antigen-specific immune therapy for patients with CHB. We have systematically prepared HBsAg-pulsed DC and used them in normal volunteers [17] and hepatitis B vaccine nonresponders [14]. HBsAg-pulsed DC were completely safe and immunogenic in normal volunteers and hepatitis B vaccine nonresponders, who have been followed for 5 years after the administration of HBsAg-pulsed DC [14,17]. Finally, we used HBsAg-pulsed DC in patients with CHB and also confirmed the safety of this treatment. However, this is a small study, and there are several limitations that affect the interpretation of the findings. First, we only included five patients. In addition, patients were immunized 1–3 times with HBsAg-pulsed DC. However, this was a pilot study to demonstrate the use of DC-based therapy in immune competent persons with a chronic viral infection. We are now planning to prepare other HBV-related antigen-pulsed DC for patients with CHB because only HBsAg-pulsed DC may not be the best therapeutic option. Hepatitis B core antigen-pulsed DC may be required to restore therapeutic HBV-specific immunity. Also, antigen-pulsed DC may be used as a part of combination therapy with antiviral drugs in patients with CHB [18]. Finally, the concept and techniques used in this

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study may be used to prepare antigen-pulsed DC for other chronic infections.

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Mechanism of restoration of immune responses of patients with chronic hepatitis B during lamivudine therapy: increased antigen processing and presentation by dendritic cells

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SUMMARY. Restoration of host immunity has been reported in patients with chronic hepatitis B (CHB) after treatment with lamivudine; however, the underlying mechanisms of this treatment have not been determined. This study examined the role of antigen-presenting dendritic cells (DC) in restoration of host immunity. Circulating DC were isolated from peripheral blood of 23 patients with CHB before and 1, 3, and 12 months after starting lamivudine therapy. The non-antigen-specific proliferation of DC was assessed in allogenic mixed leucocyte reaction. Dendritic cells were cultured with hepatitis B surface antigen (HBsAg) to prepare HBsAg-pulsed DC. Proliferative capacity and production of interleukin (IL)-12 and interferon (IFN)-γ of HBsAg-pulsed DC were evaluated. Circulating unpulsed DC and HBsAg-pulsed DC showed significantly higher levels of T-cell proliferation capacities 1 month after lamivudine therapy compared to proliferation levels before therapy ($P < 0.05$). HBsAg-pulsed DC also produced significantly higher levels of IL-12 and IFN-γ with lamivudine therapy compared to levels before therapy ($P < 0.05$). HBsAg-pulsed DC from lamivudine-treated patients induced proliferation of T cells of patients with CHB in an antigen-specific manner ($P < 0.05$). However, T-cell stimulatory capacity of DC did not increase significantly 3 and 12 months after lamivudine therapy compared to 1 month after lamivudine therapy. Immune restoration as a result of lamivudine therapy is regulated at least in part by activation of DC. However, progressive activation of DC was not seen as treatment duration progressed, indicating the limitations of this mechanism of viral clearance.

Keywords: chronic hepatitis B, dendritic cells, immune restoration, lamivudine.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major cause of morbidity and mortality worldwide. Of 350 million people chronically infected with HBV globally, considerable numbers develop chronic hepatitis B (CHB) and its complications, such as liver cirrhosis, liver failure, and hepatocellular carcinoma. Approximately, 0.5–1.2 million people die annually because of HBV-related diseases [1]. A study by Perz *et al.* has shown that chronic HBV infection likely accounts for the majority of both cirrhosis and hepatocellular carcinoma globally and in all regions of the world [2]. Treatment is recommended for patients with CHB so that complications can be minimized or delayed. The main goal of antiviral therapy is to suppress HBV replication and induce remission of liver disease. The ultimate goal of therapy is to prevent cirrhosis, hepatic failure, and hepatocellular carcinoma [3].

The number of medications that can treat CHB is increasing. Based on their mechanism of action, two types of antiviral agents have been approved or are in clinical development: (i) immune modulators (interferon alpha-2b, pegylated interferon-2a and interferon alpha-2a) and (ii) viral polymerase inhibitors that belong to the nucleoside and nucleotide analogue family.

Nucleoside analogues are well tolerated, less expensive than interferon, and can be administered orally. Accordingly, nucleoside analogues are now used widely around the world, especially in developing countries, where more than 90% of patients infected with chronic HBV reside. Nucleoside

Abbreviations: CHB, chronic hepatitis B; CPM, counts per minute; DC, dendritic cells; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MLR, mixed leucocyte reaction; PBMC, Peripheral blood mononuclear cells.

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analogues are potent inhibitors of HBV replication in CHB. However, several studies have shown that these agents have immune modulator capacities in patients with CHB. Boni *et al.* showed that lamivudine treatment restores T-cell responsiveness [4]. Lamivudine has also been reported to overcome cytotoxic T-cell hyporesponsiveness in CHB [5]. Recently, Cooksley *et al.* showed that adefovir dipivoxil treatment caused increased $CD4^+$ -cells responses in patients with CHB compared to placebo [6]. Restoration of T lymphocyte subpopulation has been detected in patients with CHB treated with entecavir [7]. A study by Evans *et al.* has shown immune modulator capacities of telbivudine in patients with CHB [8].

These findings support the idea that in addition to having potent antiviral capacity, nucleoside analogues can also modulate host immunity in patients with CHB. However, almost nothing is known about the mechanism of immune restoration or immune induction capacities of nucleoside analogues. Proper insights about different cellular and molecular events regarding immune modulator capacities of nucleoside analogues may contribute to development of more potent therapeutic regimens for CHB.

The primary aim of this study was to dissect the mechanisms underlying the immune modulator capacities of lamivudine, the most commonly used nucleoside analogue. We focused on the functions of antigen-presenting dendritic cells (DC), initiators, and regulators of immune responses [9–12]. This was carried out for two principal reasons. First, DC regulate both innate and antigen-specific immunity in situ. DC infiltrate to the site of viral localization, produce critical mediators of innate immunity, and ultimately recognize, process, and present viral antigens for induction of antigenic-specific immunity [9–12]. Second, DC function has been reported to be decreased and impaired in patients with CHB mainly because of increased HBV load, high levels of HBV-related antigens, and localization of HBV DNA in DC [13–15]. Thus, it is possible that immune restoration of patients with CHB during lamivudine therapy may be mediated through DC. Accordingly, we examined DC function in patients with CHB before and after lamivudine therapy.

MATERIALS AND METHODS

Twenty-three patients with lamivudine-naïve CHB were enrolled in this study. Clinical profiles of the patients are shown in Table 1. All patients had chronic liver disease and attended our university hospital for periodic monitoring and therapy. The diagnosis of CHB was made based on clinical symptoms and liver function tests. In 20 patients, the final diagnosis of CHB was confirmed by histologic evaluation of the liver biopsy specimen. All patients were positive for HBsAg and HBV DNA in the sera. They also exhibited exacerbation and remission of liver disease during the last 6 months. Concomitant infection and super-infection with hepatitis A virus, hepatitis C virus, cytomegalovirus, Epstein–Barr virus, or herpes simplex virus were ruled out serologically or molecularly. All patients with CHB were provided with oral lamivudine at a dose of 100 mg once daily. The nature and possible consequences of this study were explained to all patients, and all of them gave written informed consent to receive lamivudine therapy. The studies have been performed according to the World Medical Association Declaration of Helsinki, and the procedures have been approved by the local Ethics Committee of Ehime University, Japan.

Table 1 Clinical profiles of patients

Parameters	
Number of patients	23
Age (mean and range)	42 ± 12.2 (range: 23–70 years)
Sex (Male : Female)	16:7
Alanine aminotransferase (U/L)	157 ± 52
HBV DNA (log genomic equivalent)	6.8 ± 1.3
HBeAg + patients	13
Anti-HBe + patient	10
Liver histology	
Hepatitis	
Mild	6
Moderate	10
Severe	4
Level of fibrosis	
F1	6
F2	2
F3	7
F4	5

Data are mean and standard deviation. Liver biopsy specimens were available in 20 patients with chronic hepatitis B.

Study design

Whole blood was collected from patients with CHB just before the start of lamivudine therapy. In addition, whole blood was collected 1, 3, and 12 months after therapy began. The functions of T lymphocytes, monocyte-derived DC, and circulating DC were assessed. In some patients, immunocyte function was evaluated serially.

Estimation of HBV markers

The presence of HBsAg and antibody to HBsAg (anti-HBs) in the sera was determined using chemiluminescent immunoassay kits (Architect™ HBsAg and Architect™ anti-HBs, Dainabot, Tokyo, Japan). Levels of HBeAg and anti-HBe were determined using enzyme immunoassay kits (AxSYM™ HBeAg Assay and AxSYM™ HBeAg Assay, Dainabot). HBV DNA in sera was assayed with a commercial kit (DNA probe

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'Chugai-HBV', Chugai Diagnostic Science Co., Ltd., Tokyo, Japan), and levels were expressed as log genomic equivalent (LGE/mL) (limit of HBV DNA detection >3.7 LGE/mL).

Isolation of T lymphocytes and DC from peripheral blood

Peripheral blood mononuclear cells (PBMC) from patients with CHB were isolated by density gradient separation using Ficoll-Conray (Pharmacia, San Jose, CA, USA) and were resuspended in RPMI 1640 (Iwaki, Chiba, Japan) plus 10% heat-inactivated foetal calf serum (Filtron Pty., Ltd., Brooklyn, Australia) containing L-glutamine and gentamycin. Cell viability was checked by the trypan blue exclusion (0.1% trypan blue) test.

Circulating DC were isolated from PBMC by two-step immunomagnetic cell sorting using a commercial kit (DC Isolation Kit, Miltenyi Biotech GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions and as described previously [16,17]. Briefly, T cells, monocytes, and natural killer cells were depleted from PBMC using magnetic beads coated with monoclonal antibodies against CD3 (clone BW264/56), CD11b (clone M1/70.15.11.5), and CD16 (clone VEP-13) by Auto MACS (Miltenyi Biotech Gmbh). Purified populations of circulating DC were isolated from the depleted cell fractions by a positive selection step using a monoclonal antibody against CD4 (clone M-T321, Miltenyi Biotech Gmbh). Flow cytometric analysis revealed <1.0% contaminating T lymphocytes, B lymphocytes, and natural killer cells in circulating DC.

In some experiments, monocyte-derived DC were prepared by culturing PBMC with granulocyte-macrophages stimulating factor (800 U/mL) and interleukin (IL)-4 (400 U/mL) (Pepro Tech EC Ltd., London, UK) for 7 days. DC were retrieved from the culture and washed for three times with phosphate-buffered saline (PBS) as described elsewhere [18].

T lymphocytes were isolated from PBMC of one normal volunteer or patient with CHB using an affinity column (Collect™, Biotex Laboratories INC, Edmonton, Canada) in which B cells were depleted from PBMC during their passage through the affinity column containing polyclonal goat anti-human IgG (H + L) [16]. Flow cytometric analysis revealed that the purity of T-cell populations was >95%.

Expression of MHC/HLA antigen and costimulatory antigens on circulating DC

The expressions of MHC class II (HLA DR), MHC class I (HLA A, B, C), CD86, and CD40 on circulating DC were assessed by direct flow cytometry using fluorescein isothiocyanate-conjugated monoclonal antibody to human HLA DR (MHC class II, Clone L243), HLA A, B, C (MHC class I), and phycoerythrin-conjugated monoclonal antibody to human CD86 (clone 2331 [FUN-1]) and CD40 (all from BD Pharmingen, San Jose, CA, USA). Data acquisition and analysis were performed on a fluorescein-activated cell sorter (Becton Dickinson Biosciences, San Jose, CA, USA).

Production of HBsAg-pulsed DC

To produce HBsAg-pulsed DC, blood DC were cultured with a recombinant HBsAg (Tokyo Institute of Immunology, Tokyo, Japan) for 48 h. After the end of culture, DC were pelleted and washed five times in PBS. After the last wash, the final wash solutions were collected and preserved at −20 °C to assess whether there was any free HBsAg in HBsAg-pulsed DC [18].

Lymphoproliferative assays

We optimized the culture condition of allogenic mixed leucocyte reaction (MLR) and antigen-specific lymphoproliferation by conducting a series of preliminary experiments as described in previous studies [17]. T cells (2×10^5) from allogenic normal controls or patients with CHB were cultured with γ-irradiated (40 Gy, HILTEX Co., Ltd., HW-150, Osaka, Japan) DC or HBsAg-pulsed DC (1×10^4) for 5 days at 37 °C in a humidified incubator containing 5% CO_2 in air. The levels of incorporation of [^{3}H]-thymidine during the last 12 h of the 5-day culture were determined in a liquid scintillation counter (Beckman LS 6500; Beckman Instruments, Inc., Fullerton, CA, USA) as counts per minute (CPM). All assays were performed in 96-well plates and the mean CPM of at least 12 wells calculated. Stimulation index was measured by dividing the levels of CPM in culture containing stimulants with the levels of CPM in control cultures.

Cytokine production assays

The allogenic MLR was performed for 5 days without adding [^{3}H]-thymidine to the cultures. HBsAg-pulsed DC were cultured for 2 days. The supernatants were collected and centrifuged. Levels of IL-12 and IFN-γ in the supernatants were measured by an enzyme-linked immunosorbent assay using a commercial kit (BD Pharmingen) [17].

Statistical analysis

Values are as mean ± standard deviation (SD). Data were analysed by unpaired *t* tests if data were normally distributed and by Mann–Whitney rank-sum test if they were skewed. Differences were considered significant if $P < 0.05$.

RESULTS

Circulating DC expressed DC-related markers such as HLA A, B, C, HLA DR, and CD86. The proportion of contaminating lymphocytes, natural killer cells, and monocytes were <5% in circulating DC. The functionality of DC was assessed in

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allogenic MLR. DC induced proliferation of allogenic T cells in a dose-dependent manner (data not shown).

Increased proliferation of T lymphocytes from patients with CHB because of lamivudine therapy

Mean levels of Con A-induced T-cell proliferation were significantly higher in samples collected 1 month after lamivudine therapy (stimulation index; 147.8 ± 22.1) compared to before treatment (stimulation index; 51.2 ± 11.5) ($P < 0.05$). However, the proliferative capacity of T lymphocytes did not show any further increases at 3 months after therapy started (stimulation index; 130.9 ± 25.6) ($P > 0.05$). We also checked Con A-induced T-cell proliferation 12 months after the start of lamivudine therapy. Levels of proliferation of T cells (stimulation index; 127.3 ± 37.3) did not show any significant changes compared to levels after 1 month of lamivudine therapy (stimulation index; 147.8 ± 22.1) ($P > 0.05$).

Increased T-cell proliferation capacities of circulating DC from patients with CHB during lamivudine therapy

The proliferative capacities of DC were increased in patients with CHB because of lamivudine therapy. T-cell stimulatory capacities of circulating DC of all patients with CHB were estimated 1 and 3 months after therapy started. Levels of blastogenesis (assessed from stimulation index values) were significantly higher ($P < 0.05$) in cultures containing DC from patients with CHB 1 month after therapy (stimulation index; 90.3 ± 15.6) started compared to before treatment (stimulation index; 45.3 ± 10.0). Similarly, circulating DC from patients with CHB 3 months after therapy started showed significantly higher levels of T-cell proliferation compared to DC before therapy (stimulation index; 90.4 ± 14.3) (Fig. 1). However, the proliferative capacity of DC did not show any significant difference between samples at 1 and 3 months after therapy started (Fig. 1).

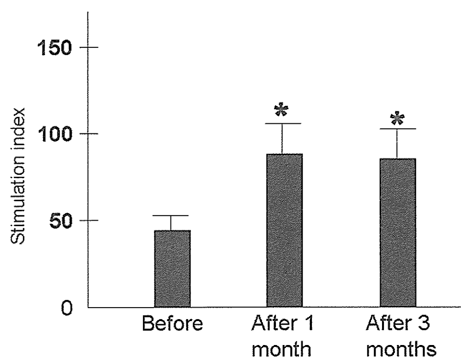

Fig. 1 Increased proliferation of allogenic T cells by DC from lamivudine-treated patients with CHB. Data are mean and standard deviation. $P < 0.05$ compared to before therapy.

Kinetics of proliferation capacities of DC because of lamivudine therapy

We checked the proliferation capacities of DC serially before, and 1, 3, and 12 months after therapy started in six patients. The proliferative capacity of DC increased significantly (before therapy versus 1 month after therapy; stimulation index 44.2 ± 123 versus stimulation index, 105.2 ± 18.2) ($P < 0.05$) 1 month after lamivudine therapy started, but did not show any significant increase during the next 11 months, although patients were receiving lamivudine on a daily basis (Fig. 2).

Increased production of IL-12 and IFN-γ in allogenic MLR culture containing DC from patients with CHB 1 month after therapy started

Levels of IL-12 and IFN-γ were significantly higher in allogenic MLR culture supernatants containing DC from lamivudine-treated CHB 1 month after start of therapy compared to cultures containing DC from pretreated patients ($P < 0.05$) (Table 2).

Increased cytokine production and antigen processing and presentation capacity of DC because of lamivudine therapy

DC from patients with CHB were pulsed with HBsAg before and 1 and 3 months after lamivudine therapy. These DC were cultured for 48 h to assess their production of two critical cytokines required for immune responses: IL-12 and IFN-γ. As shown in Fig. 3, levels of IFN-γ were significantly increased in culture containing HBsAg-pulsed DC from patients with CHB 1 month (76.4 ± 8.3 pg/mL) and 3 months after lamivudine therapy (73.9 ± 8.8 pg/mL) compared to HBsAg-pulsed DC before therapy (36.5 ± 13.7 pg/mL) ($P < 0.05$). Similarly, levels of IL-12 were significantly increased in culture containing HBsAg-pulsed DC from patients with CHB 1 month (181.8 ± 32.7 pg/mL) and 3 months after lamivudine therapy (187.7 ± 33.1 pg/mL) compared to HBsAg-pulsed DC before therapy (111.4 ± 25.4 pg/mL) ($P < 0.05$). The levels of these cytokines were

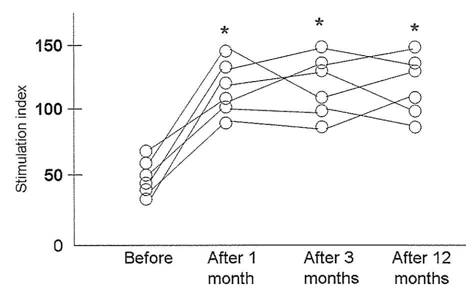

Fig. 2 Kinetics of DC function before and after lamivudine therapy. Data are shown as stimulation index of six individual cases. $P < 0.05$ compared to before therapy.

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Table 2 Increased cytokine production by DC in patients with chronic hepatitis B before and after treatment with lamivudine

	Interleukin-12 (pg/mL)	Interferon-γ (pg/mL)
Before lamivudine therapy	45 ± 6	32 ± 5
1 month after lamivudine therapy	354 ± 45*	167 ± 29*

Data are mean and standard deviation.
*$P < 0.05$, compared to culture containing DC before therapy.

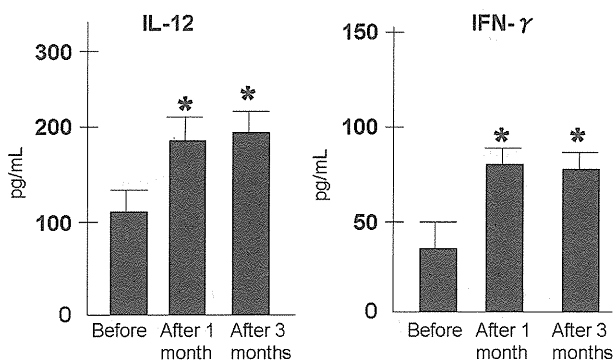

Fig. 3 Increased IL-12 and INF-γ production by HBsAg-pulsed DC from patients with CHB after lamivudine therapy. Data are mean and standard deviation. $P < 0.05$ compared to before therapy.

almost comparable in culture containing HBsAg-pulsed DC from patients with CHB 1 and 3 months after lamivudine therapy (Fig. 3).

Increased T-cell activation capacity of HBsAg-pulsed DC from lamivudine-treated patients with CHB

DC were isolated before and 1 month after the start of lamivudine therapy. These DC were cultured with HBsAg to produce HBsAg-pulsed DC. As shown in Fig. 4, HBsAg-pulsed DC from patients with CHB before lamivudine therapy could not induce proliferation of autologous T cells *in vitro*. However, HBsAg-pulsed DC from lamivudine-treated patients with CHB induced proliferation of T cells from patients with CHB (Fig. 4).

DISCUSSION

Restoring T-cell responsiveness and overcoming cytotoxic T-cell (CTL) hyporesponsiveness have been reported in patients with CHB treated with lamivudine therapy [4,5]. In addition, restoration of immune responses by adefovir, entecavir, and telbivudine has also been reported [6–8]. However, the mechanism underlying this immune restoration by nucleoside analogues in patients with CHB is not understood, although these drugs mainly block or reduce replication of the HBV. Reduction of HBV DNA and downregulation of HBV-related proteins with lamivudine treatment may be one of the factors that lead to restoration of immune responses in patients with CHB. HBV DNA and HBV-related antigens may have a dominant role in impaired HBV-specific immunity in patients with CHB. However, other factors may be related to immune restoration because of nucleoside treatment, and further characterization of these events is essential.

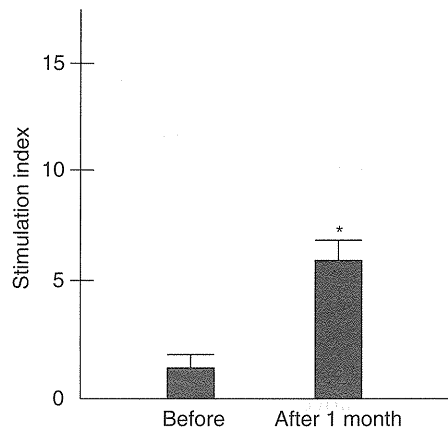

Fig. 4 Increased T-cell proliferation capacity of HBsAg-pulsed DC before and 1 month after start of lamivudine therapy. $P < 0.05$ compared to before therapy.

In this study, we showed increased proliferative capacities of DC in a non-antigen-specific (allogenic MLR stimulation) manner as well as in an antigen-specific manner (HBsAg-pulsed DC activation). In addition, lamivudine treatment induced increased cytokine production from DC. However, the functional capacity of DC was not increased with time because DC from lamivudine-treated patients 3 or 12 months after lamivudine intake did not show increased activity compared to 1 month after treatment.

These findings may have important clinical implications. Boni *et al.* found transient restoration of immune responses with lamivudine [4,5]. Their study showed a transient increase in HBV-specific CD4 and CD8 responses, but these effects were not sustained over time. We found similar results regarding DC activation. It is probable that further activation of DC may be required to attain sustained antiviral activity with lamivudine. Also, DC function should be monitored in HBsAg-positive, HBV DNA-negative, and treatment-naïve patients in future to develop insights about role of HBV replication on DC function.

Understanding the biochemical and molecular targets of lamivudine and other nucleoside analogues may lead to new therapeutic strategies against chronic HBV infection. Lamivudine treatment caused transient increases in DC function, but not progressive activation of DC. This effect can be

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achieved by adding an immune modulator. The combination of lamivudine with an immune modulator has shown efficient antiviral potential, but, their role in modulation of ultimate clinical outcome is not so promising [19–22]. In large randomized phase III studies comparing lamivudine and peginterferon monotherapy and the combination of peginterferon and lamivudine in HBeAg-positive and HBeAg-negative patients, combination therapy was associated with a more profound decrease in viral load, compared with either monotherapy. However, no significant difference was observed in treatment end points such as viral suppression, HBeAg seroconversion, and HBsAg clearance between peginterferon monotherapy and combination therapy.

In addition to interferon, lamivudine has been used with HBsAg-based prophylactic vaccine or IL-12 to improve the clinical outcome in patients with CHB. However, the capacities of these drugs to restore immune responses, especially HBV-specific immune responses, have not been properly elucidated. Although we found that lamivudine caused transient increased HBsAg-specific immunity in patients with CHB, the persistence of this effect needs to be examined in future studies. Addition of appropriate immune modulators with lamivudine may induce progressive stimulation of DC and potent restoration of antigen-specific immunity in patients with CHB. Also, the function of capacity of DC should be assessed when these patients switch to another antiviral in future.

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REVIEW ARTICLE

Immune Interventional Strategies against Chronic Infection Diseases and Cancers: Present Challenges and Road Map to Solution

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ABSTRACT

The therapeutic efficacy of antiviral therapy against chronic viral infections and anticancer strategy against cancers is not satisfactory. Most of the antiviral drugs cause reduction of viral replication in chronic virus-infected subjects, however, recovery or cure from diseases does not occur in most cases. Various types of therapeutic approaches, such as ablation of cancer tissues, use of anticancer drugs and radiation therapy, are applied to treat patients with cancers. But, recurrence of cancer is a formidable problem in clinics. Taken together, sustained control of virus replication in chronic viral carriers and control of cancer recurrence in cancer patients are two major challenges. It is now evident that although different factors are responsible for pathogenesis of chronic viral infections and cancers, almost all patients exhibit distorted antiviral and anticancer immune responses. Thus, a new field of treatment of these diseases by immune intervention has been emerged. Immune therapy against chronic viral infections and cancers are in their infancy and facing several challenges. These challenges will be discussed to provide a road map for development of clinically-acceptable and potent immune therapeutic approaches against these diseases, especially in the context of liver diseases.

Abbreviations: HBV—Hepatitis B virus; HCV—Hepatitis C virus; HIV—Human immunedeficiency virus; DC—Dendritic cells.

Keywords: Chronic viral infection, Cancer, Antiviral and anticancer drugs, Immune therapy.

Host Immunity is a Critical Regulator of Pathogenesis of Chronic Viral Infections and Cancers

Some viruses, such as influenza virus, measle virus, hepatitis A virus and hepatitis E virus, usually causes acute and self-limiting infections. On the contrary, hepatitis B virus (HBV), hepatitis C virus (HCV), herpes viruses and human immunodeficiency virus (HIV) are notorious for causing chronic infections. Patients with chronic viral infections exhibit ongoing replications of viruses without (asymptomatic) or with features of inflammations and tissue damages (symptomatic). The mechanisms that regulate the pathogenesis and clinical courses of chronic viral infections are not well understood, however, both viral-derived factors and host-related factors play critical roles in this regard. The nature of viruses, amounts of viruses, genotypes of viruses and routes of infection may be related to viral persistency, however, the role of individual viral-derived factor during establishment of chronic viral infections could not be substantiated. For example, if immune-competent and healthy persons are infected with same sources of HBV, some of them develop acute or self-limiting HBV infection, whereas, others are chronically-infected with the virus. However, once a chronic viral infection is established in a host, either immune-competent or immune-comprised, the hosts usually exhibit distorted immune responses to various viral antigens.[1-3]

The etiological factors, cellular events and molecular mechanisms underlying carcinogenesis are also highly variable. Interestingly, the immune statuses of the cancer-bearing hosts are comparable with those of chronic virus-infected persons. In spite of harboring abundant amounts of cancer cells in all patients with cancers, they show diminished, impaired and distorted anticancer immunities.[4]

The limited therapeutic efficacies of antiviral drugs and anticancer therapeutic approaches against chronic viral infections and cancers, and presence of distorted antiviral and anticancer immunity in these patients have exposed a new field of therapeutic intervention, immune therapy, against these diseases. However, there is no standard or universal regimen of immune therapy because different viruses employ different mechanisms to establish chronic

infections, and similar diversities are prevailing regarding pathogenesis of cancers. The efficacies of antiviral drugs and anticancer therapeutic approaches are also highly variable. Naturally, the challenges of immune therapies against chronic viral infections and cancers are also diverse. Unfortunately, it is not possible to provide any universal road map to address these challenges. In this article, we would provide a general discussion about present challenges of immune therapy. Also, a road map will be given to address these challenges on the basis of present realities and scientific developments. Specific examples about some gastrointestinal diseases, more specifically of liver diseases would be given for better understanding of these factors.

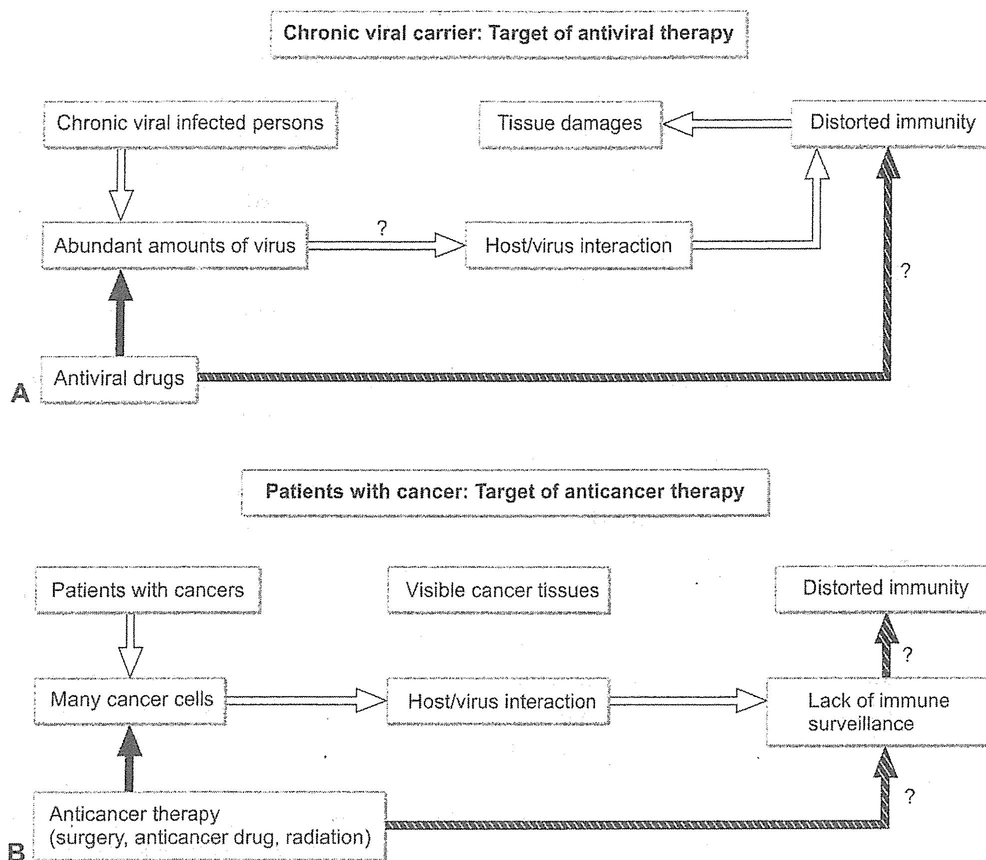
Limitations of Therapeutic Options against Chronic Viral Infections and Cancers

Patients with chronic viral infections are treated by antiviral drugs. These drugs usually reduce the levels of virus replication, but they cannot eradicate the virus completely from chronic viral carriers. Before prescribing antiviral drugs, it is assessed whether (1) these patients exhibit subjective, biochemical or histological features of tissues damages, (2) the diseases are progressive or not and (3) the patients are going to develop complications. At present, treatment is usually recommended for chronic viral carriers with tissue damage, progressive diseases or complications. In fact, antiviral drugs are not effective against asymptomatic chronic viral carriers, and are not capable of eradicating the viruses completely from chronic viral carriers.[5]

The principle of treatment of chronic viral carriers by antiviral drugs has been summarized in Figures 1A and B. Antiviral drugs are capable of reduction of viral replication in most patients with chronic viral infection (shown by black arrow). It is expected that reduction of virus replication will be followed by restoration of antiviral immunity and control of tissue damages. However, antiviral drugs induce sustained control of virus in only few patients. Accordingly, restoration of host immunity and control of tissue damages are not accomplished by antiviral therapy in most chronic viral carriers (shown by hatched arrow). Highly potent antiviral drugs are available due to better understandings of viral life cycle and tremendous development of medicinal chemistry. For example, type 1 interferon has been used for treating chronic HBV and HCV infections for more than two decades. Interferon induces antiviral microenvironments in liver tissues, however, their direct antiviral potentialities are not clear. At present, true antiviral drugs are available against various viruses. Patients with chronic HBV infection are now treated by nucleoside analogs. Some of these drugs are phosphorylated to the triphosphate and competes with dCTP for incorporation into growing DNA chain causing chain termination of the replicating HBV. This may occur during reverse transcription of the first strand of HBV DNA, and during synthesis of second strand of HBV DNA.[6,7] For treating HIV-infected persons, most therapeutic regimens are combinations of inhibitors of viral enzymes—reverse transcriptase and protease. In addition, newer drugs that target viral entry into the cells have been developed as antiviral drugs against HIV.[8] However, administration of nucleoside analogs or enzyme inhibitors usually fails to induce sustained control of viral replication in majority of patients.

The presently-available therapies against cancers are directed to (1) destruction of cancer tissues by surgery or other ablation techniques, and (2) destruction of cancer cells by anticancer drugs or radiation therapies.[9,10] The therapeutic efficacy of these approaches is highly variable and depends on: (1) The nature of cancer, (2) the type of cancerous tissue, (3) presence or absence of metastasis and (4) the nutrition statuses of the patients. If a cancer is detected in its early stage, the cancer tissues can be destroyed almost completely by these therapeutic approaches. However, after successful removal of cancer tissues by surgery or ablation techniques or destruction of cancer tissues by anticancer agents or radiations, the cancer may relapse at the original site of cancer or in another site. As shown in Figure 1B, it is expected that if cancer burden of a cancer-bearing host is diminished by anticancer therapeutic approaches, the persons will restore the immune surveillance system against cancer cells. However, it is not guaranteed, rather restoration of immune surveillance is an unexpected event in most cancer patients. Accordingly, recurrence of cancer is a common feature of cancer patients treated by conventional anticancer therapies.

Commercially-available antiviral drugs and conventional anticancer therapeutic approaches may be regarded as first line of therapeutic approaches against chronic viral infections and cancers respectively. These therapeutic approaches are endowed with capacities to transient reduction of viruses and reduction or eradication of cancer tissues. However, control of tissue damage and sustained control of viral replication is not achieved by antiviral drugs in most patients with chronic viral infections. Again, immune surveillance mechanism is not properly induced and maintained in most patients with cancers by conventional anticancer therapeutic approaches. Thus, second line of therapeutic approaches is needed to induce and maintain sustained control of virus in chronic viral infections and proper induction and maintenance of



Figs 1A and B: The limitations of antiviral and anticancer therapies in patients with chronic viral infections (A) and cancers (B) are shown. (A) All patients with chronic viral infections harbor abundant amounts of virus and also exhibit distorted antiviral immunity. Antiviral drugs usually reduce viral replication but restoration of immunity (shaded lines) may or may not be achieved. (B) Anticancer approaches reduce cancer burden, but anticancer immune surveillance system is not usually induced by conventional anticancer therapeutic approaches

anticancer immune surveillance system in patients with cancers. One way to accomplish this is dependent on further developments of medicinal chemistry because if more potent antiviral drugs can eradicate the virus completely from chronic viral carriers, sustained antiviral responses will be attained by antiviral drugs. Regarding treatment of cancers, the possibility of control of cancer recurrence is not so encouraging. Relapse of cancer cells may start even after complete eradication of cancer cells from the hosts because several factors induce carcinogenesis and many of these factors are not controllable *in situ*.

In this context, immune therapy may represent an alternative therapeutic approach or a second line of therapeutic option against chronic viral infections and cancers.

Immune Therapy against Chronic Viral Infections and Cancers

Major immune interventional approaches against patients with chronic viral infections and cancers have been shown in Figure 2. Initially, cytokines, such as interleukin-2, interleukin-12 and interferon-gamma, have been administered to patients with chronic viral infections to upregulate host immunity. Various growth factors have also been used as immune therapeutic agents. Most of these studies were done as pilot study or open clinical trials. Some studies have shown that administration of polyclonal immune modulators caused subjective improvements of

Immune interventional strategies against chronic viral infection and cancers

Nonantigen-specific immune modulators:
1. Cytokines
2. Growth factors

Antigen-specific immune intervention:
1. Antigen-based vaccine therapy
2. Epitope-based vaccine therapy
3. DNA-based vaccine therapy

Cell-based immune therapy:
1. T cell-based immune therapy
2. Dendritic cell-based vaccine therapy

Fig. 2: Cytokines, growth factors and antigen-specific immune interventional strategies are now used for treating patients with chronic viral infections and cancers