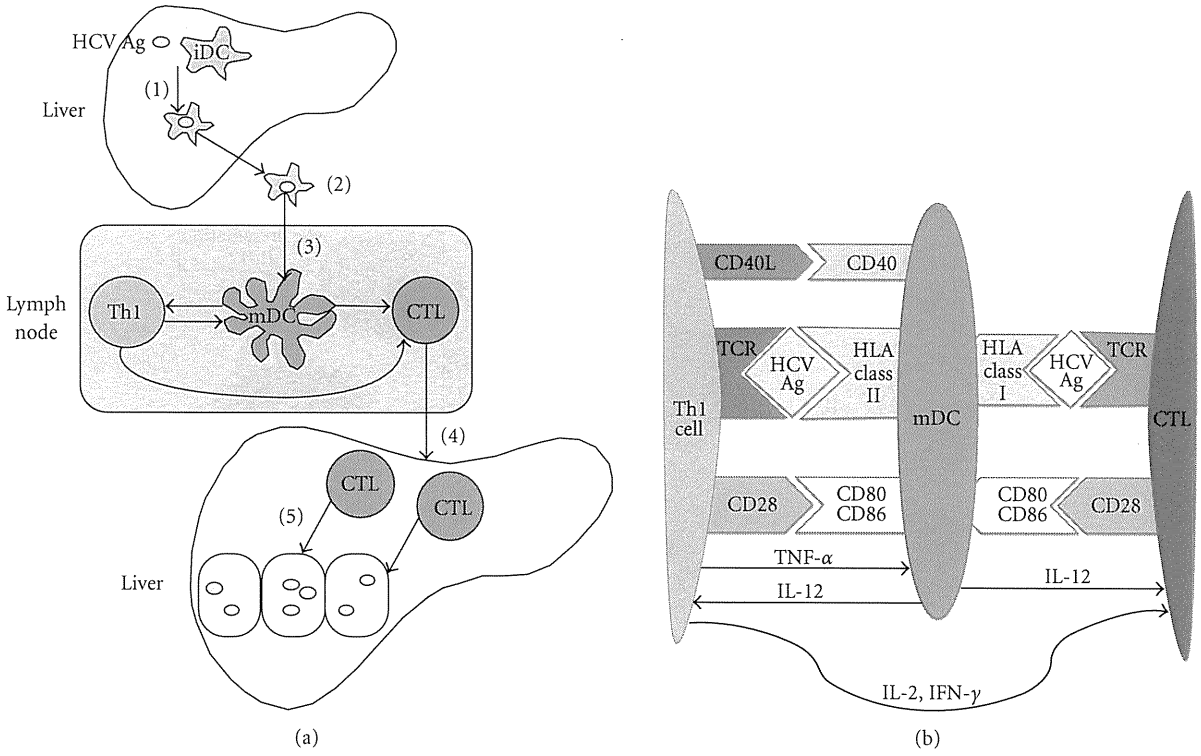

FIGURE 2: Destruction of HCV-infected hepatocytes by CTLs. (1) Immature myeloid dendritic cells (iDC) take up hepatitis C virus antigens (HCV Ag) in the liver. (2) The DCs move to a draining lymph node. (3) Matured DCs activate naïve helper T (Th) cells efficiently through stimulation with HLA class II, costimulatory molecules (CD80 and CD86), and cytokines such as IL-12. The stimulated Th cells, in turn, activate DCs by expressing CD40 ligand and secreting TNF-α. IL-12 produced by myeloid DCs differentiates these stimulated Th cells towards Th1 cells. Naïve cytotoxic T lymphocytes (CTLs) recognize the HCV Ag presented on the DCs. IL-2 and IFN-γ secreted by activated Th1 cells induce the activation and proliferation of the HCV-specific CTLs. (4) The stimulated HCV-specific CTLs leave the lymph nodes and move toward the liver. (5) They recognize HCV antigens together with HLA class I on the surface of HCV-infected hepatocytes, and try to eradicate HCV by killing the infected hepatocytes. Abbreviated terms: Th1 cell, type 1 helper T cell; mDC, myeloid dendritic cells; CTL, Cytotoxic T lymphocyte; CD, cluster of differentiation; CD40L, CD40 ligand; TCR, T-cell receptor; HLA, human leukocyte antigen; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon.

other hand, markedly potent immune responses would lead to severe hepatitis and fulminant hepatitis as proven in a hepatitis B virus (HBV) model [12], although this is a rare event in HCV infection.

We evaluated the relation between HCV-specific CTL responses and the clinical course of acute HCV infection and found that HCV eradication cannot be predicted on the basis of a strong $CD8^+$ T-cell response [13]. However, Lauer et al. reported that potent and broad CTL responses against HCV peptides were observed in patients with resolved infection but not in those with persistent infection [14]. Another report indicated that patients with complete resolution of HCV infection exhibited broader CTL responses with higher functional avidity and wider cross-recognition ability than patients with persistent HCV infection [15]. The opposite observations can be attributed to the differences in the monitoring methods of the CTL responses. Race and HCV genotype might also affect the contradiction of the results. Further investigation is needed to clarify this issue.

We analyzed the immune response of chronic HCV patients by studying their HLA-B44-restricted CTLs that recognized the HCV core amino acid residues 88–96; the CTL response and viral load were found to be inversely correlated [16]. The findings of this study suggested that HCV-specific CTLs may inhibit HCV replication. Otherwise, as many reports have suggested that HCV protein impairs the CTL responses by several mechanisms (see Section 3), HCV infection with a high titer of HCV RNA may suppress the HCV-specific CTLs by an excess of HCV antigens. No relation between other CTL responses recognizing other HCV epitopes and the HCV status was found in the study. From the data, it was supposed that the HLA-B44-restricted CTLs recognizing HCV core amino acid residues 88–96 were immunodominant.

Hence, there is a need to investigate HCV-specific CTL responses and clarify some issues. First, HCV exists as quasispecies in hosts and it has a high replicative ability and low fidelity RNA polymerase [17]. Thus, many HCVs with mutations in different amino acid sequences in the epitopes may be present in the host. Other issue is that most HCV-specific CTLs may infiltrate and compartmentalize in the host liver where inflammation occurs, and thus, only a few circulating HCV-specific CTLs can be detected. Although it is very crucial to investigate liver-infiltrating CTLs, the difficulty associated with obtaining liver specimen limits such study.

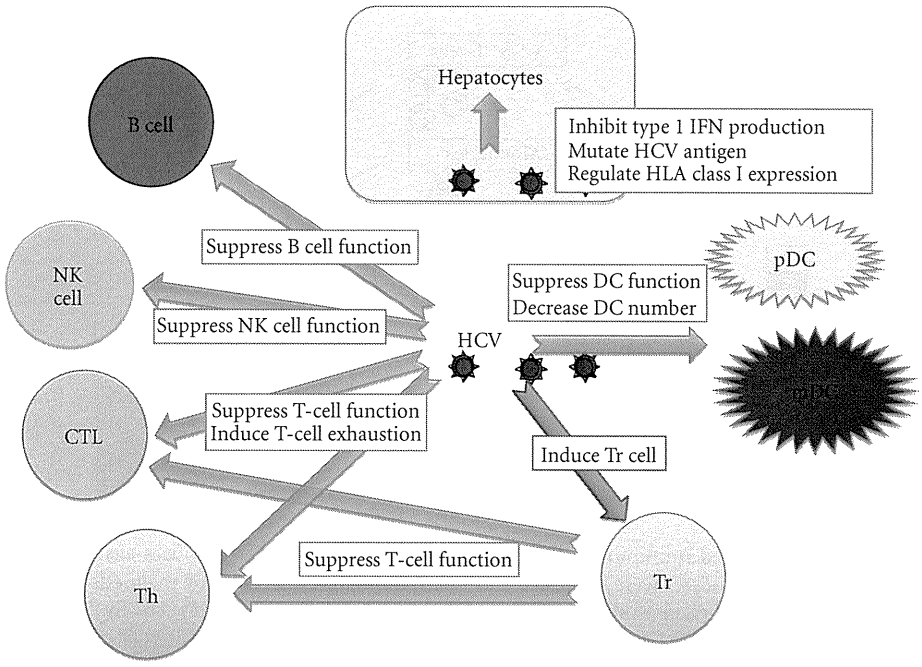

FIGURE 3: Immune suppressive mechanisms in HCV infection. HCV mutates its amino acid sequence to escape from immune surveillance, inhibits type 1 IFN production, and suppress NK cell function, T-cell function, and DC function. In addition, HCV induces Tr cells, which suppress T-cell function. Abbreviated terms: CTL, Cytotoxic T lymphocyte; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; Th cell, helper T cell; NK cell, natural killer cell; IFN, interferon.

3. Immunosuppression in HCV Infection

3.1. Escape from Immune Surveillance of Cellular Immune Responses. It was reported that amino acid mutations have been detected in the immunodominant regions of HCV in all patients with acute HCV infection, and mutations by which HCV escapes from CTL surveillance have been observed only in patients with viral persistence [18]. Hughes et al. investigated the variable intensity of purifying selection on CTL epitopes, and reported that the purifying selection of CTL epitopes on nonenvelop proteins was strong, particularly when the epitope was matched [19]. Since a variety of CTLs are induced in the early stage of HCV infection, a single amino acid mutation within a CTL epitope does not appear to contribute to persistent infection. It is supposed that escape mutation is a result rather than a cause of persistent HCV infection.

3.2. Impaired Function of CTL in HCV Infection. HCV inhibits cellular immune responses in the host by several ways; immune suppressive mechanisms in HCV infection are summarized in Figure 3.

In our study, the stimulation of peripheral blood lymphocytes of HCV-infected patients with synthetic peptides corresponding to CTL epitopes revealed that patients who were infected with HCV within the past 3 years exhibited CTL responses, while those infected with HCV more than 10 years ago did not exhibit this response. There are some reasons why HCV persistence is so common although a variety of HCV-specific $CD8^+$ T-cells can be detected in the liver and peripheral blood. The impaired function of HCV-specific CTLs as effector cells is due to the reduced expression of CD3 ζ chain [20], defective IFN-γ production, low perforin content, and decreased capacity for proliferation and cytotoxicity [21]. Incomplete differentiation of the memory CTLs to effector cells in patients with acute HCV infection may be due to IL-2 deficiency during T-cell activation [22]. Programmed cell death 1 (PD-1) receptor, the ligation of which inhibits the function of effector T-cells, is upregulated on exhausted $CD8^+$ cells in patients with acute and chronic hepatitis C [23–25]. Another inhibitory receptor, namely, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), has also been reported to be upregulated on PD-1^+ T-cells in the liver of HCV patients. The blockade of both these molecules is critical for the restoration of the function of HCV-specific effector cells [26].

Accumulated data have suggested that HCV itself actively suppresses host immune responses. Although spontaneous liver disease did not occur in mice expressing liver-targeted HCV NS5A transgene, both innate and adaptive immune responses were impaired [27]. HCV core protein inhibits IL-2 and IL-2 receptor α gene transcription [28], T-cell activation and proliferation, and IFN-γ production by T cells [29, 30]. HCV NS4A/B protein blocks the expression of HLA class I molecules [31].

Impaired function of DCs, which play the crucial role of antigen-presenting cells in inducing immunity, may be responsible for the impaired immune responses. It has been reported that the HCV core, E1, and NS3 proteins inhibit DC maturation [32, 33]. HCV is thought to infect DCs through the binding of HCV E2 protein and thereby suppress

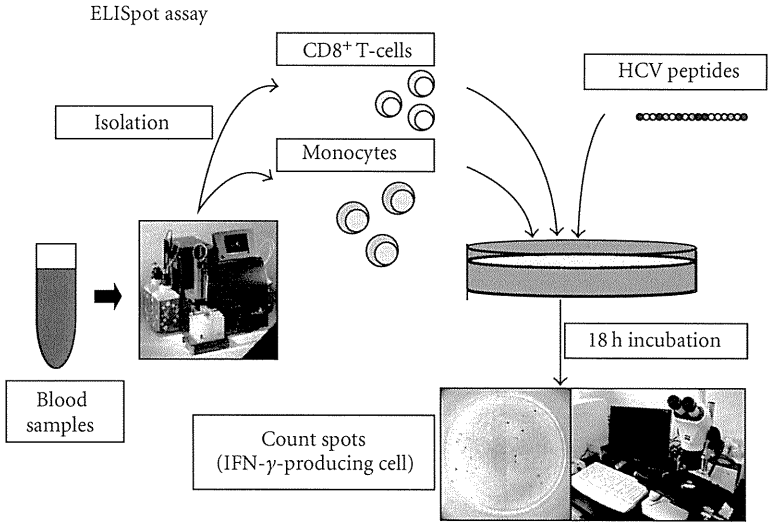

FIGURE 4: The procedure of enzyme-linked immuno-spot (ELISpot) assay. To detect CTL responses against HCV, we performed IFN-γ-based ELISpot assay. $CD8^+$ cells and monocytes were separated from peripheral blood samples by magnetic beads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and used as effector cells and antigen-presenting cells, respectively. These cells with synthetic HCV peptides were incubated for 18 h at 37°C in 5% CO_2 atmosphere. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted.

DC function [34, 35]. In addition, long-term ethanol consumption impairs CTL responses to HCV protein and subsequently alters DC function [36].

Regulatory T- (Tr) cells are also involved in HCV persistence. It has been shown that Tr cells ($CD4^+$ $CD25^+$ T cells) directly suppress T-cell function in chronic hepatitis C patients [37]. Forkhead box P3 (FOXP3)-positive Tr cells and IL-10 producing HCV-specific Tr cells infiltrate the liver of chronic HCV patients, and IL-10 mediates immune suppression in these patients [38, 39]. HCV core-specific Tr cells can be induced from the peripheral blood of patients with chronic hepatitis C [40].

4. Immunotherapy for Hepatitis C

4.1. IFN Therapy and Immune Response. Currently, chronic HCV infection can be resolved only with IFN-α-based therapy. IFN-α has been reported to have biologic effects on the immune system [41]. IFN-α upregulates HLA class I molecules on the cell surface. This cytokine appears to favor the proliferation of type 1 Th cells and activate CTLs. Ribavirin, which is used in combination with IFN-α, exerts an antiviral effect that drives the Th2 response towards a Th1 response [42]. During the primary immune response, IFN-α promotes both clonal expansion and survival of antigen-specific CTLs in vivo [43]. We also demonstrated that IFN-α prevents activation-induced cell death of CTLs [44]. A low dose of IFN-α augments cellular immune response, whereas a high dose suppresses CTL response [45]. Recently, it has been reported that although IFN-α upregulates MHC class I expression on hepatocytes, it reduces their sensitivity to CTL cytotoxicity, which may be due to the enhancement of granzyme-B inhibitor-proteinase inhibitor 9 (PI-9) expression [46]. Although it has been reported that intrahepatic and peripheral HCV-specific CTL activity was detected more often in patients with a sustained response to IFN therapy than in patients who relapsed or did not respond to the treatment [47], further study is needed to clarify the effect of IFN therapy on host immune responses in vivo.

4.2. Identification of Novel Epitopes Recognized by HCV-Specific CTLs. As described above, we first identified an HLA B44-restricted CTL epitope [48, 49]. Then, we tried to identify more novel CTL epitopes in the HCV polyprotein, and performed IFN-γ-based enzyme-linked immuno-spot (ELISpot) assay [50, 51]. The procedure of this assay is presented in Figure 4. We synthesized 297 20-mer peptides overlapping by 10 residues and spanning the entire HCV sequence based on the amino acid sequence of HCV [13]. After separation with magnetic beads, we used $CD8^+$ T-cells as effector cells and monocytes as antigen-presenting cells. After the $CD8^+$ T-cells were incubated with the monocytes and the synthetic HCV peptides for 18 hours, IFN-γ-producing cells were counted. This procedure enabled to minimize the IFN-γ production for nonspecific response. Then, we identified more than 20 CTL epitopes in the HCV protein by using the synthetic peptides (Table 1). Furthermore, our group has identified several epitopes of HCV-specific CTLs using synthetic peptides and recombinant vaccinia viruses [52].

The HLA-24 allele of HLA class I is more common among the Japanese population. Thus, CTL induction by synthetic peptides based on HLA-A24 binding motifs has been investigated mainly in Japan [53]. HCV NS5A 2132–2142 peptide corresponding to the HLA-A24 binding motif has been reported to be able to induce both cellular and humoral immune responses in most HCV-positive patients

Table 1: CTL epitopes identified by using different procedures.

(a) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV sequence of genotype 1b

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt1	A*0207,2601 B*3501,4601 CW*0102,0303	NS3	1527–1546	WYELTPAETTVRLRAYLNTP	B*3501? A*2601?
		NS5B	2591–2605	KMALYDVVSTLPQAV	A*0207?
Pt2	A*2402,3303 B*4403,5401 CW*0803,1403	E1	332–351	LVVSQLLRIPQAVVDMVAGA	B*5401?
		NS3	1638–1656	THPITKFVMACMSADLEVV	B*5401?
		NS5B	2591–2605	KMALYDVVSTLPQAV	n.d.
Pt3	A*2602,3101 B*5101,5102 Cw1402,1502	NS3	1373–1380	IPFYGKAI	B*5101? B*5102?
Pt4	A*2402 B*0702,5201 Cw*0702,1202	E2	611–618	YPYRLWHY	n.d.
Pt5	A*1101,3101 B*6701,5101 Cw*0702,1401	NS5A	2290–2298	RPDYNPPLL	B*6701? B*5101?
Pt6	A*2402,2601 B*4002 Cw*0304	NS2	957–964	RDWAHAGL	B37
		NS5A	2122–2130	FTELDGVRL	n.d.
Pt7	A*2402,3303 B*0702,3501 Cw*0303,0702	Core	91–110	LGWAGWLLSPRGSRPSWGPT	A*3303? B*3501?
Pt8	A*2402 B*4801,5201 Cw*0803,1202	NS3	1643–1656	KFVMACMSADLEVV	n.d.
Pt9	A*2402 B*5201 Cw*1202	NS4	1760–1768	FWAKHMWNF	A*2402
		NS5B	2556–2564	TIMAKNEVF	n.d.
		NS5B	2803–2811	LTRDPTTPL	n.d.
Pt10	A*0201,0301 B*4402,4601 Cw*0102,0501	NS4	1958–1977	KRLHQWINEDCSTPCSGSWL	n.d.
Pt11	A*1101,2601 B*1501,5201 Cw*0401,1202	NS4	1858–1867	GVAGALVAFK	A*1101?
Pt12	A*2402 B*3501,4002 Cw*0303,0304	NS3	1618–1626	LHGPTPLLY	A*2402?

(b) CTL epitopes identified by HCV-derived synthetic peptides with binding motif of HLA-A24 [51]

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt13	A*2402,1101 B*3902,5201 Cw*0702,1202	NS3	1375–1385	FYGKAIPIEAI	n.d.
Pt14	A*2402,2601 B*4006,5401 Cw*0801,0803	E1	284–293	VFLVSQLFTF	n.d.
		E2	790–798	LYGVWPLLL	Cw*0801
		NS4	1759–1768	AFWAKHMWNF	n.d.
		NS5A	1990–1999	DFKTWLQSKL	n.d.
		NS5A	2280–2288	KFPPALPIW	A*2402
Pt15	A*2402,2601 B*3501,4002 Cw*0303,0304	NS2	910–919	PYFVRAQGLI	Cw*0303, 0304
		NS2	947–956	TYVYDHLTPL	B*4002
		NS3	1243–1252	AYAAQGYKVL	Cw*0303, 0304
Pt16	A*0206,2402 B*5201,5901 Cw*0102,1202	NS3	1443–1451	GFTGDFDSV	A*0206
Pt17	A*2402,3101 B*4801,5101 Cw*0304,0801	E2	790–798	LYGVWPLLL	Cw*0801
Pt18	A*2601,3101 B*3501,5101 Cw*0303,1402	NS5B	2456–2466	VYSTTSRSASL	n.d.

(c) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV sequence [13]

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt19	A*2602,3101 B*5101,5102 C*1402,1502	NS3	1373–1380	IPFYGKAI	n.d.
Pt20	A*0402 B*0702,5201 C*0702,1202	E2	611–618	YPYRLWHY	n.d.
Pt21	A*1101,3101 B*6701,5101 C*0702,1402	NS5A	2290–2298	RPDYNPPLL	n.d.
Pt22	A*2402 B*5201 C*1202	NS4	1759–1768	AFWAKHMWNF	n.d.
		NS5B	2556–2564	TIMAKNEVF	n.d.
		NS5B	2803–2811	LTRDPTTPL	n.d.
Pt23	A*0201,0301 B*4402,4601 C*0102,0501	NS4	1958–1977	KRLHQWINEDCSTPCSGSWL	n.d.
Pt24	A*2402,4801 B*5201 C*0803,1202	NS3	1637–1656	LTHPITKFVMACMSADLEVV	n.d.

(d) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV core sequence

Region	Amino acid residues	Sequence	HLA restriction	Reference
core	88–96	NEG(L,M,C)GWAGW	B*4403	[49]
core	28–36	GQIVGGVYL	B60	[50]

(e) CTL epitopes identified by HCV-derived synthetic peptides with binding motif of HLA-B*4403

Region	Amino acid residues	Sequence	HLA restriction	Reference
NS5a	2095–2103	AEVTQHGSY	B*4403	[16]

(f) CTL epitopes identified by comprehensive CTL induction from PBMC of HCV patients

Region	Amino acid residues	Sequence	HLA restriction	Reference
NS3	1373–1380	IPFYGKAI	B*5603	[52]

with HLA-A24 [54]. Three novel vaccine candidate peptides capable of CTL induction and antibody production have also been identified [55]. In the study, the HCV core 30–39 peptide was shown to induce peptide-specific CTLs from peripheral blood mononuclear cells (PBMCs) of patients with HLA-A11, -A31, or -A33.

Yerly et al. [56] developed a novel "epitome" approach and analyzed its in vitro performance. This approach compresses the common immune targets of HCV-specific cellular immune response into a short immunogen sequence and may be applied to induce cellular immune responses against highly variable antigens.

The most important concern in peptide vaccine development is the selection of peptides from among the CTL epitopes because some peptides may rather induce tolerance of effector cells [57] or Tr cells, which will result in immune suppression. Hence, it is necessary to develop tailor-made therapy using appropriate peptides according to the HLA haplotypes of the patients.

4.3. Trials for the Development of HCV Vaccine. Many attempts for inducing immune responses against HCV by vaccination have been performed using animal models. Splenocytes isolated from mice pretreated with Fms-like tyrosine kinase receptor 3 ligand exhibited NS5-specific cellular immune responses after vaccination with DCs containing magnetic beads coated with HCV NS5, lipopolysaccharide, and anti-CD40 antibody [58, 59]. It has been reported that the adoptive transfer of HCV NS3 protein-pulsed mature DCs could effectively promote potent HCV-specific protective immune responses in a mouse model [60]. From the data, DC-based therapy appears to be one of the candidates for immune therapy against HCV infection.

Since HCV envelope glycoproteins are heavily glycosylated, such modification would affect immune responses in hosts. The engineering of *N*-glycosylation of HCV E2 protein enhances HCV-specific cellular immune responses [61], whereas the deletion of *N*-glycosylation sites of HCV E1 protein augmented HCV-specific cellular and humoral immune responses [62].

Gene therapy has been tried to elicit strong immune responses in vivo. It has been reported that vector-based minigene encompassing 4 domains of HCV NS3, NS4, and NS5B proteins effectively induced CTL induction in HLA-A2 transgenic mice [63]. Using replication-incompetent adenoviruses expressing HCV core and NS3 proteins, HCV-specific CTLs could be induced from PBMCs of HCV-infected patients [64]. Administration of recombinant yeast cells producing HCV NS3-core fusion protein, namely, GI-5005, induced potent antigen-specific proliferative and CTL responses in mice [65]. As described above, gene therapy would be a candidate for HCV vaccine. However, a careful survey for adverse effects induced by the therapy must be performed before clinical application.

Adjuvants may help the induction of HCV-specific CTLs, and it is important to investigate what adjuvant we should use for HCV vaccination. Protein immunization using CpG and montanide ISA 720 have been reported to enhance HCV-specific Th-1 type immune responses [66]. Cytokines such as granulocyte-monocyte colony stimulating factor and IL-23 have been also used for argument of immune responses induced by HCV core vaccination [67]. In a mouse model, HBV precore protein enhanced HCV-specific CTL responses induced by the genetic immunization of DNA encoding truncated HCV core proteins [68]. In another model, HBs antigen enhanced the induction of HCV-specific CTLs by DNA vaccine harboring HCV CTL epitopes [69].

Not only animal experiments, but also several human trials have been proceeding. Yutani has reported a phase I study of HCV vaccine in Japanese patients who were either nonresponders to IFN therapy ($n = 23$) or had refused treatment ($n = 3$). A peptide derived from the HCV core region amino acid residues 35–44 is capable of inducing cellular immune responses in many patients with different HLA class I-A alleles [70]. This peptide was used to develop a series of 6 vaccine injections that enhanced the peptide-specific peripheral CTL activity in 15 out of 25 patients and 12 vaccine injections that augmented peptide-specific IgG production [71]. Improvement in serum alanine aminotransferase (ALT) level (>30% decrease) was also observed in 7 out of 24 patients in the study. The results revealed that the selection of candidate peptides is crucial for developing a successful HCV vaccine.

In another clinical trial of a synthetic peptide vaccine, IC41 containing the 7 relevant HCV-specific Th cell and CTL epitopes and the adjuvant poly-L-arginine were used. It has been reported that IC41 can induce HCV-specific responses in both Th1 cells and CTLs in patients not responding to or relapsing from IFN therapy [72, 73]. Although this vaccination was tolerated and induced serious adverse events, HCV RNA reduction was rarely observed in the study [73]. In the phase II trial of pegylated interferon plus ribavirin therapy in combination with this vaccine, an enhanced HCV-specific T-cell response was observed in 73% of patients, and the responses could be detected more frequently in patients with sustained virologic response than in those showing relapse [74].

A recent Phase I placebo-controlled study has revealed that a prototype vaccine, which consists of HCV core protein and the adjuvant ISCOMATRIX, induces cytokine production by T-cells, but CTL responses were detected in a few healthy individuals [75]. A tableted therapeutic bivalent vaccine, which consists of heat-inactivated HCV antigens derived from HBV- and HCV-infected donors, has been applied in the treatment of chronic hepatitis C patients. Oral administration of this vaccine showed no adverse effects, and the elevated liver enzyme levels observed before the study were reduced in all patients at the end of the study.

A therapeutic DNA vaccine developed using the mixture of plasmid expressing HCV structural antigens and a recombinant HCV core protein, namely, CIGB-230, has also been used to treat chronic hepatitis C patients who did not respond to previous IFN therapy in a Phase I study [76]. This vaccination induced specific T-cell responses in 73% of the participants. Interestingly, 40% of the vaccinated patients showed reduction in liver fibrosis.

5. Conclusions and Future Directions

Since HCV was first identified, many investigations have been performed to resolve and prevent HCV infection. It has been demonstrated that HCV-specific CTLs are implicated in not only viral eradication but also the immunopathogenesis of hepatitis C. Development of IFN-based therapy in combination with ribavirin and protease/polymerase inhibitor has improved the sustained viral response rate of patients. However, there are still many nonresponders who suffer from chronic hepatitis C, cirrhosis, and hepatocellular carcinoma. Moreover, the HCV infection mechanism in many patients is still unknown. For these patients, a novel immune therapy and vaccination should be urgently established. For this purpose, we have to continue further investigation of immune responses in HCV infection.

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Journal of Medical Virology 82:2064–2072 (2010)

Reactivation of Epstein–Barr Virus in B Cells of Patients With Chronic Hepatitis C

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Hepatitis C virus (HCV) infection is associated with lymphoproliferative disorders. HCV infection of B cells is a predictive factor for lymphoproliferative disorders in patients with chronic hepatitis C, although its molecular mechanisms remain unknown. Epstein–Barr virus (EBV) is a B cell-tropic virus with the potential to cause lymphoproliferative disorders, and its reactivation is induced by several viruses and cytokines. The possibility that HCV infection triggers reactivation of EBV and induces lymphoproliferative disorders were investigated. Expression of EBV mRNAs was analyzed by RT-PCR in patients infected with HCV and control subjects, and correlations between reactivation of EBV and markers for lymphoproliferative disorders were investigated. BZLF1 mRNA, a starter molecule of reactivation, was detected in peripheral blood mononuclear cells from 12 of 52 (23%), patients infected with HCV and the frequency was higher than in healthy subjects [3 of 43 (9%), $P = 0.032$]. But the presence of the BZLF1 mRNA was not associated with an abnormality of markers for lymphoproliferative disorders. This study on BZLF1 mRNA expression among lymphoid cell subsets showed that reactivation of EBV was observed specifically in B cells. The BZLF1 mRNA disappeared following anti-viral therapy and remained negative after eradication of HCV in patients with a sustained viral response, while the EBER1 RNA, a marker for persistence of EBV, was detected throughout the therapy. Infection with HCV induces reactivation of EBV in B cells, but this reactivation was not associated directly with lymphoproliferative disorders triggered by HCV. ***J. Med. Virol. 82:2064–2072, 2010.***

KEY WORDS: hepatitis C virus; Epstein–Barr virus; reactivation; BZLF1; lymphoproliferative disorders

INTRODUCTION

Hepatitis C virus (HCV) is a causative agent for chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [Tong et al., 1995; Ikeda et al., 1998]. In addition, some patients infected with HCV develop proliferative disorders of lymphocytes, such as mixed cryoglobulinemia [Agnello et al., 1992; Frangeul et al., 1996; Donada et al., 1998] and B cell non-Hodgkin's lymphoma (NHL) [Ferri et al., 1994]. Although an epidemiological association has been noted between HCV infection and lymphoproliferative disorders, the underlying pathogenic mechanisms remain unclear. HCV is reported to infect B cells persistently, and somatic hypermutations in immunoglobulin genes have been observed in B cell lines infected with HCV as well as proto-oncogenes [Sung et al., 2003; Machida et al., 2004, 2005]. These observations indicate direct and/or indirect effects of HCV infection of B cells on the induction of lymphoproliferative disorders. In our previous study, infection of B cells and/or adsorption with HCV was observed in 64% of patients infected with HCV [Inokuchi et al., 2009]. Furthermore, the HCV RNA in B cells was an independent factor associated with the presence of markers for lymphoproliferation. HCV infection or phenomena induced by HCV may trigger the clonal proliferation of B cells, which leads to the development of lymphoproliferative disorders. The presence of monoclonal B cells circulating in patients infected with HCV was confirmed by analysis for rearrangement of the clonal immunoglobulin heavy-chain (IgH) gene, although its molecular mechanism remains unknown [Zuckerman et al., 2001].

Epstein–Barr virus (EBV) belongs to the human herpes viruses, is oncogenic and is disseminated widely. Antibodies against EBV have been confirmed in all

Potential conflicts of interest: Authors do not have a commercial or other association that might pose a conflict of interest.

Grant sponsor: KAKENHI (to T.I.); Grant number: 22590745; Grant sponsor: Research on Publicly Essential Drugs and Medical Devices (to T.I.); Grant number: KHC1012.

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Accepted 2 June 2010

DOI 10.1002/jmv.21890

Published online in Wiley Online Library (wileyonlinelibrary.com).

population groups worldwide; ~90–95% of adults are seropositive for EBV [Thorley-Lawson, 2001]. EBV transforms B cells and is associated with the development of lymphoproliferative disorders. EBV manifests two activity levels distinctly: latency, a state of limited expression of viral genes, and lytic infection, which ultimately leads to production of virions. Several viruses and cytokines have been reported to induce reactivation of EBV, even in healthy individuals, including human herpesvirus 6 (HHV-6), cytomegalovirus, and TGF β-1 [Flamand et al., 1993; Aalto et al., 1998; Fahmi et al., 2000]. The latency-to-lytic switch can be started by expression of activator genes for the lytic cycle of EBV, BZLF1, and BRLF1, which encode ZEBRA and Rta. These genes induce expression of viral and cellular genes related to replication of DNA and viral and cellular factors for reactivation [Amon and Farrell, 2005].

In order to examine the possibility that infection with HCV triggers reactivation of EBV to induce development of clonal expansion of B cells and lymphoproliferative disorders, profiles for expression of EBV mRNA associated with its reactivation were investigated in patients with chronic hepatitis C.

MATERIALS AND METHODS

Patients

From 2007 through 2008, 52 patients infected with HCV (chronic hepatitis, 41; and cirrhosis, 11) were enrolled in the study. Seven patients had complicating HCC. All patients were treated and/or followed up at Showa University Hospital. Diagnosis of infection with HCV was confirmed by detection of HCV RNA in the serum. Serving as controls, 43 healthy adults, 17 patients infected with hepatitis B virus (HBV), and 19 patients who had non-viral liver injury (alcoholism, 5; non-alcoholic steatohepatitis, 3; primary biliary cirrhosis, 9; and autoimmune hepatitis, 2) were enrolled in the study. Another 42 patents with chronic hepatitis C, who were admitted for treatment with pegylated interferon and ribavirin, were enrolled. Informed consent was obtained from all participants in the study, and the study was approved by the Ethics Committee of Showa University School of Medicine.

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from 10 to 30 ml of whole blood in LeucoSep® tubes (Greiner Bio-One, Tokyo, Japan) according to the manufacturer's instructions. Cells were frozen and stored at −80°C until use.

Isolation of Lymphoid Cell Subsets

Beads with affinity for each type of lymphoid cell, MicroBeads® (Miltenyi Biote, Bergisch Gladbach, Germany) were added to PBMCs to isolate lymphoid cell subsets ($CD8^+$, $CD4^+$, $CD19^+$, and others) in 30 patients with chronic hepatitis C. Suspension of the cells was mixed well, incubated for 15 min at 4°C and centrifuged at 900*g* for 10 min in a tube. Then the tube was placed on a magnet, and the free floating cells were transferred to another tube. The pellet containing $CD8^+$ cells was collected and stored at −80°C until use. $CD4^+$ and $CD19^+$ cells were separated from the supernatant using similar procedures. The supernatant remaining was labeled as the fraction of "others." Free B cells were isolated from PBMCs, using a system for separation of touch-free cells (MagCellect B cell isolation kit II®; R&D Systems, Minneapolis, MN), of 12 patients with chronic hepatitis C.

Detection of EBV mRNA by Nested RT-PCR

Total RNA was extracted from PBMCs or B cells using the RNeasy Mini Kit® (Qiagen, Tokyo, Japan). A portion was reverse transcribed by AMV® RT (Roche, Munich, Germany) using random primers under the conditions recommended by the manufacturer. The mRNAs of *Bam*HI Z leftward reading frame 1 (BZLF1), latent membrane protein 1 (LMP1), and Epstein–Barr virus-encoded small RNAs 1 (EBER1) were amplified by nested RT-PCR. GAPDH mRNA was amplified by RT-PCR as an internal control in each sample. First, PCR was performed using 2.5 μl of cDNA in 2.5 ml of mixture for RT reaction containing 0.25 μl of sense and anti-sense primers, 2.0 μl of dNTP mixture, and 0.5 U of Taq polymerase in reaction buffer. The PCR program was 94°C for 3 min for denaturation, 35 cycles of 94°C for 30 sec, 58°C for 1 min and 72°C for 1 min and 72°C for 7 min for extension. The outer sense primer for BZLF1 mRNA was 5′-ATT GCA CTT TGC CGC CAC CTT TG-3′, the outer anti-sense primer was 5′-CGG CAT TTT CTG GAA GCC ACC CGA-3′, the inner sense primer was 5′-GAC CAA GCT ACC AGA GTC TAT-3′, and the inner anti-sense primer was 5′-CAG AAT CGC ATT CCT CCA GCG A-3′. The outer sense primer for EBER1 RNA was 5′-AGG ACC TAC GCT GCC CTA GA-3′, the outer anti-sense primer was 5′-AAA ACA TGC GGA CCA GC-3′, the inner sense primer was 5′-GGT TTT GCT AGG GAG GAG AC-3′, and the inner anti-sense primer was 5′-GGT ACT TGA CCG AAG ACG GC-3′. The outer sense primer for LMP1 mRNA was 5′-TCC TCC TCT TGG CGC TAC TG-3′, the outer anti-sense primer was 5′-TCA TCA CTG TGT CGT TGT CC-3′, the inner sense primer was 5′-CTT GTC CTC TAT TCC TTT GC-3′, and the inner anti-sense primer was 5′-CAC AAT TCC AAG GAA CAA TGC C-3′. The sense primer for GAPDH mRNA was 5′-GCC TCC TGC ACC ACC AAC TG-3′, and the anti-sense primer was 5′-CGA CGC CTG CTT CAC CAC CTT CT-3′.

Serum Markers of Lymphoproliferative Disorders

Cryoglobulinemia was detected using a semi-quantitative method by centrifugation, as described previously [Inokuchi et al., 2009]. Rheumatoid factor was determined by the latex turbidimetric assay, and complement 4 and 50% hemolytic complement activity (CH50) were determined by nephelometry and Mayer's method,

respectively. Markers of LPD were determined in 52 patients with chronic hepatitis C.

PCR Amplification for Rearrangement of IgH Genes

Genomic DNA was extracted from PBMCs containing B cells by QIAamp DNA mini (Qiagen). To detect monoclonal rearrangement of IgH genes, the DNA was amplified by PCR using a semi-nested protocol, as described previously [Ngan et al., 1989]. The upstream primer was complementary to the third framework V region (FR3) conserved in the IgH gene, and the downstream primer annealed to an outer region conserved in the IgH joining (J) region in the first round of amplification and to an inner sequence conserved in the same J region in the second round. The upstream primer for FR3 (Fr3A; 5′-ACA CGG C[C/T][G/C] TGT ATT ACT GT-3′) and the downstream primer for JH (LJH; 5′-TGA GGA GAC GGT GAC C-3′) were used in the first round of amplification. In the second round, an inner downstream primer (VLJH; 5′-GTG ACC AGG GTN CCT TGG CCC CAG-3′) was substituted for LJH. PCR products were separated by 7.5% polyacrylamide gel electrophoresis. Clonal expansion was determined by the presence of one or more discrete, dominant bands within the range 72- to 118-bp, and polyclonal populations were indicated by the presence of smears with no specific dominant bands.

Anti-EBV Antibodies

Anti-Epstein–Barr virus nuclear antigen (EBNA) and anti-viral capsid antigen (VCA) IgG antibodies were determined in sera of healthy adults, patients with chronic hepatitis C, hepatitis B, and non-viral liver diseases using the SERION ELISA classic kit® (Virion-Serion, Würzburg, Germany) according to the manufacturer's instructions.

Statistical Analysis

The median of continuous variables, with and without normal distribution, was compared by Student's *t*-test and Wilcoxon's test, respectively. Comparison of discontinuous variables was performed by a chi-squared test and Fisher's exact test. A P-value <0.05 was considered to be statistically significant. Values with a normal distribution were expressed as means ± SD.

RESULTS

Expression Profiles of EBV Genes in PBMCs of Patients Infected With HCV

Figure 1 shows the profiles of expression for EBV mRNA in PBMCs from seven patients with chronic hepatitis C. EBER1 RNA was expressed in all patients, indicating that PBMCs were infected with EBV persistently. BZLF1 mRNA, which codes early proteins after reactivation of EBV immediately, was detected in

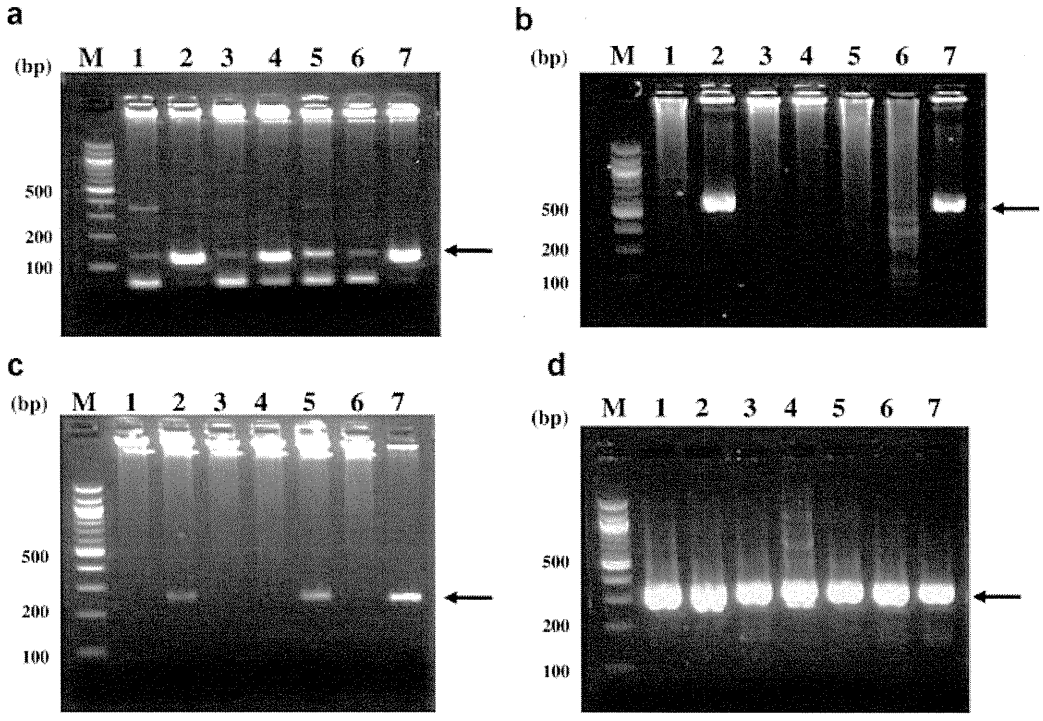

Fig. 1. Expression profiles of EBER1 RNA, and BZLF1 and LMP1 mRNAs from seven patients infected with HCV. **a**: All patients showed expression of EBER1 RNA in peripheral blood mononuclear cells (PBMCs) (lanes 1–7). Expression of BZLF1 (**b**) and LMP1 (**c**) mRNAs was observed in PBMCs from patients 2 and 7 (lanes 2 and 7), and patients 2, 5, and 7 (lanes 2, 5, and 7), respectively. **d**: GAPDH mRNA was expressed in PBMCs from all patients equally. Molecular markers are indicated in lane M. Arrows indicate appropriate sizes of the molecular markers for each RT-PCR product (EBER1: 126 bp; BZLF1: 556 bp; LMP1: 252 bp; and GAPDH: 310 bp).

TABLE I. Expression Profile of EBV mRNAs in PBMCs and Serum Antibodies Against EBV Among Patients Infected With HCV and Controls

	Healthy adults (N = 43)	HCV (N = 52)	HBV (N = 17)	Non-viral Liver diseases (N = 19)
Age	59.7 ± 19.1	66.2 ± 12.8, NS	55.9 ± 15.3, NS	59.7 ± 12.4, NS
Gender: male (%)	28/43 (65%)	22/52 (43%), $P = 0.027$*	11/17 (65%), NS	9/19 (47%), NS
EBER1 (%)	43/43 (100%)	49/52 (94%), NS	17/17 (100%), NS	19/19 (100%), NS
BZLF1 (%)	3/43 (9%)	12/52 (23%), $P = 0.032$*	1/17 (6%), NS	1/19 (5%), NS
LMP1 (%)	5/43 (12%)	9/52 (18%), NS	0/17 (0%), NS	4/19 (21%), NS
Anti-EBNA antibody (%)	29/35 (83%)	41/50 (82%), NS	17/17 (100%), NS	18/19 (95%), NS
Anti-VCA-IgG antibody (%)	33/34 (97%)	50/50(100%), NS	17/17 (100%), NS	19/19 (100%), NS

P, *P*-value; NS, not significant; PBMC, peripheral blood mononuclear cell; EBER1, Epstein–Barr virus-encoded small RNAs; BZLF1, *Bam*HI Z leftward reading frame 1; LMP1, latent membrane protein 1; EBNA, Epstein–Barr virus nuclear antigen; VCA, viral capsid antigen.
Data are no (%) or the mean ± SD.
*Significantly lower than healthy adults ($P < 0.05$).

patients 2 and 7. This suggests that reactivation of EBV occurred in these patients. LMP1, which is one of the later genes associated with transformation of host cells, was also identified in patients 2, 5, and 7. GAPDH mRNA was expressed in all studied samples equivalently.

Table I shows a summary of expression of EBV mRNA in PBMCs among patients who had liver diseases associated with and without HCV. EBER1 RNA was expressed in almost all examined patients and control subjects, indicating that most adults were infected with EBV persistently. BZLF1 mRNA was detected in PBMCs from 12 of 52 (23%) patients with chronic hepatitis C, thus suggesting that reactivation of EBV was observed in these patients. The prevalence of antibodies against EBV protein in patients was not significantly different when compared to those of healthy adults. The frequency of positivity for BZLF1 mRNA was higher than in healthy subjects [3 of 43 (9%), $P = 0.032$]. The frequency in patients with other liver diseases was lower than that in patients with chronic hepatitis C (chronic hepatitis B: [1 of 17 (6%)] and non-viral liver diseases [1 of 19 (5%)]). The mRNA of EBV LMP1, which correlates with activity for the transformation of host cells, was also detected in 9 of 52 (18%) patients with chronic hepatitis C and 5 of 43 (12%) control subjects. Five of 52 (9.6%) patients with chronic hepatitis C were positive for mRNAs of both BZLF1 and LMP1. These results confirm that reactivation of EBV had occurred in these patients with chronic hepatitis C. Anti-VCA antibody (IgG class) was positive in almost all subjects examined in this study, except for one healthy adult, indicating that most of the subjects were infected with EBV previously. The frequency of positivity for anti-EBNA antibody in patients with chronic hepatitis C and non-viral liver diseases was lower than in healthy adults, although the differences were not significant statistically. This might be associated with reactivation of EBV in patients with chronic hepatitis C due to dysfunction of immunoglobulin secretion from B cells.

Clinical Characteristics of Patients Infected With HCV Who Were Positive for BZLF1 mRNA

The clinical characteristics of the 12 patients infected with HCV, who were positive for BZLF1 mRNA, are presented in Table II. A comparison of viral and host factors between patients who were BZLF1 mRNA-positive or -negative showed that positivity for BZLF1 mRNA was associated with higher age than negativity for BZLF1 mRNA (Table III). Abnormalities in markers for lymphoproliferative disorders were observed in

TABLE II. Clinical Characteristics of HCV-Infected Patients Whose PBMCs Were BZLF1 mRNA-Positive

	Age	Gender	Clinical stage	HCV group	HCV-RNA (log IU/ml)	ALT (IU/L)	Platelets ($\times 10^4/\mu l$)	Cg	C4 (mg/dl)	RF (IU/ml)	CH50 (mg/ml)	IgH clonality
1	72	F	CH	1	6.6	51	28.0	(−)	20.6	12.4	<12	(−)
2	61	M	CH	1	5.5	39	14.3	(−)	17.7	54.6	28.8	(−)
3	75	M	LC	1	6.3	26	6.3	(−)	13.1	<7	32.1	(−)
4	77	F	CH, HCC	1	3.7	15	10.6	(−)	20.5	<7	52.2	(+)
5	61	M	CH	1	5.9	17	15.0	(−)	22.6	<7	34.1	(−)
6	76	F	CH	1	6.6	56	14.8	(−)	21.4	19.6	28.8	(−)
7	87	M	LC, HCC	ND	6.7	31	13.7	(−)	15.6	23.2	37.1	(−)
8	80	F	CH	2	4.2	42	11.5	(−)	19.9	<7	21.0	(−)
9	58	M	CH	1	4.2	28	22.8	(−)	30.0	<7	47.0	(−)
10	79	F	LC, HCC	1	6.2	33	12.5	(+)	9.8	15.4	<12	(−)
11	57	F	LC	1	6.5	46	4.9	(−)	12.7	<7	<12	(−)
12	71	F	LC, HCC	1	5.0	43	6.0	(−)	17.9	17.5	44.5	(−)

PBMC, peripheral blood mononuclear cell; BZLF1, *Bam*HI Z leftward reading frame 1; ALT, alanine aminotranferase; Cg, cryoglobulinemia; C4, complement 4; RF, rheumatoid factor; CH50, 50% hemolytic complement activity; IgH, immunoglobulin heavy chain; CH, chronic hepatitis; LC, cirrhosis; HCC, hepatocelluar carcinoma; ND, not determined.

TABLE III. Comparison of Viral and Host Factors Between Patients Positive and Negative for BZLF1 mRNA

	BZLF1(+), N = 12	BZLF1(−), N = 40	*P*-value
Age	73.7 ± 9.9	63.9 ± 12.8	0.023
Gender (male)	5/12 (41%)	17/40 (43%)	NS
Clinical stage			
CH	7/12 (59%)	34/40 (85%)	0.047
LC	5/12 (41%)	6/40 (15%)	0.047
HCC	4/12 (33%)	3/40 (8%)	0.042
ALT (IU/L)	35.6 ± 12.9	44.7 ± 29.2	NS
Platelets ($\times 10^4/\mu l$)	13.4 ± 6.7	15.9 ± 6.8	NS
Cryoglobulinemia	1/12 (8%)	11/40 (28%)	NS
RF (>10 IU/ml)	6/12 (50%)	24/40 (60%)	NS
C4 (<10 mg/dl)	1/12 (8%)	6/40 (15%)	NS
CH50 (<20 mg/ml)	7/12 (58%)	22/40 (55%)	NS
Clonal IgH rearrangement	1/12 (8.3%)	5/39 (12.8%)	NS
Serum HCV-RNA (log/ml)	5.7 ± 1.1	5.5 ± 1.4	NS
Group			
1	10/11 (84%)	27/40 (68%)	NS
2	1/11 (8%)	8/40 (20%)	
LMP1 (+) (%)	5/12 (42%)	4/40 (10%)	0.011
EBNA antibody (+) (%)	8/12 (67%)	33/38 (87%)	NS

BZLF1, *Bam*HI Z leftward reading frame 1; NS, not significant; CH, chronic hepatitis; LC, cirrhosis; HCC, hepatocelluar carcinoma; ALT, alanine aminotranferase; RF, rheumatoid factor; C4, complement 4; CH50, 50% hemolytic complement activity; IgH, immunoglobulin heavy chain; LMP1, latent membrane protein 1; EBNA, Epstein–Barr virus nuclear antigen.
Data are no (%) or the mean ± SD.

patients infected frequently with HCV, but the frequency of abnormalities was not significantly different between patients who were BZLF1 mRNA-positive or -negative. The monoclonal IgH rearrangement was detected in PBMCs in only one patient, and results in Table III show that reactivation of EBV was not associated with the clonal expansion of B cells. The frequency of positivity for expression of LMP1 mRNA in patients positive for expression of BZLF1 mRNA was higher than that in patients negative for expression of BZLF1 mRNA, indicating that expression between the two genes is related. Three of 12 patients with reactivation of EBV were negative for anti-EBNA antibody, but the frequency was not higher significantly compared with the other group (data not shown). With regard to clinical stage, results showed that cirrhosis and HCC were associated with reactivation of EBV in only 11 and 7 patients examined, respectively.

Reactivation of EBV Induced in B Cells of Patients With Chronic Hepatitis C

EBV shows B cell tropism for replication, but several reports have shown that EBV is able to replicate in T, NK, and epithelial cells [Kawa, 2003]. To clarify which lymphoid subsets support reactivation of EBV, expression of BZLF1 mRNA was analyzed in four distinct fractions ($CD8^+$, $CD4^+$ T cells, $CD19^+$ B cells, and others) isolated from 30 patients using affinity beads. Furthermore, B and non-B cells isolated using the system for a touch-free cell isolation were examined for expression of BZLF1 mRNA in 10 patients. Expression of BZLF1 mRNA in B cells of 42 patients was screened first, and the results showed that reactivation of EBV occurred in 10 patients (23.8%). The frequency of positivity for expression of BZLF1 mRNA was almost the same as that in PBMCs from patients with chronic hepatitis C, as shown in Table I (23%). Subsequently, the same study was performed using fractions of other cell subsets from 10 patients. As shown in Figure 2a, BZLF1 mRNA was detected in only B cells from six patients, although it was detected in all fractions examined in one case. All samples from the seven cases showed the same levels as expression of GAPDH mRNA (data not shown). In the three patients studied, analysis of B cells isolated using the system of a touch-free cell isolation showed that reactivation of EBV occurred in only B cells (Fig. 2b). These results suggest that reactivation of EBV was induced in B cells of patients with chronic hepatitis C specifically.

Reactivation of EBV Was Terminated After Eradication of HCV by Interferon Therapy

In order to analyze the effects of eradication of HCV on reactivation of EBV, expression of BZLF1 mRNA in PBMCs was monitored in three patients that attained a sustained viral response (cases 1–3) through combination therapy with pegylated interferon and ribavirin. Figure 3 shows that expression of BZLF1 mRNA disappeared soon after of the start of therapy in these patients. BZLF1 mRNA remained negative even at 3 months after the end of treatment. EBER1 mRNA was expressed throughout the therapy, indicating that the cycle of EBV infection changed from lytic infection into persistent infection in some B cells in the patients. GAPDH mRNA was expressed in all cells investigated equally.

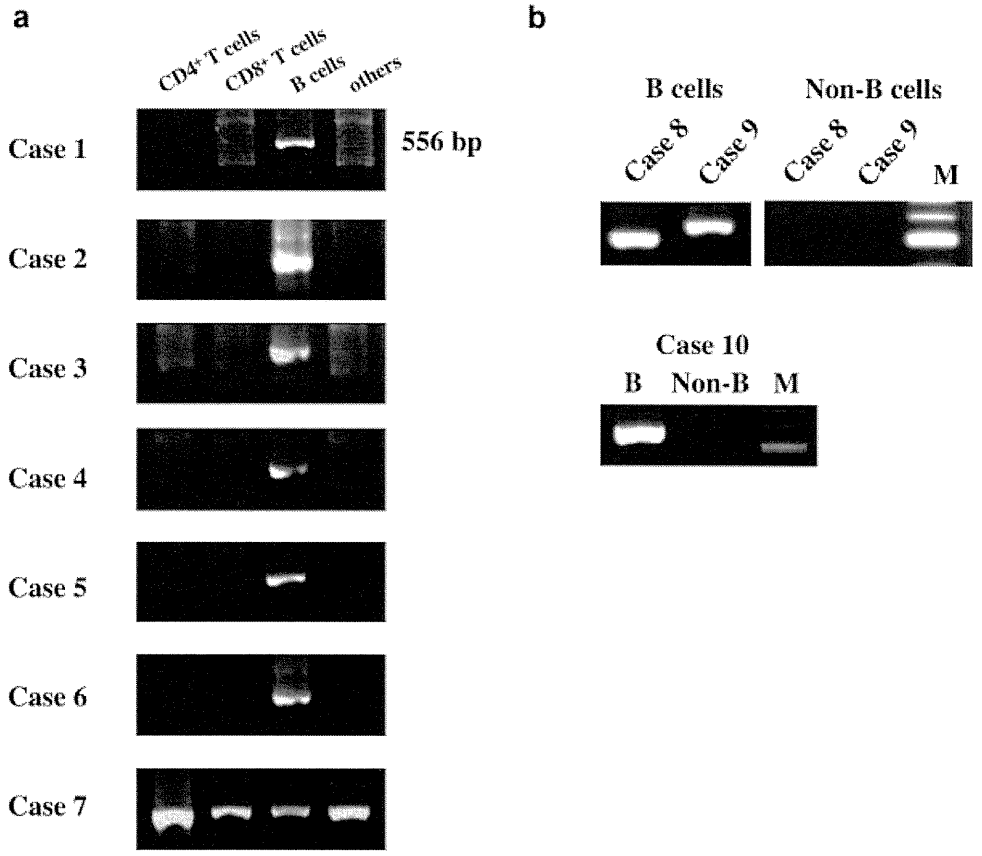

Fig. 2. **a**: Expression of BZLF1 mRNA in four fractions ($CD4^+$, $CD8^+$ T cells, $CD19^+$ B cells, and others) isolated from seven patients with chronic hepatitis C using affinity beads. **b**: Expression of BZLF1 mRNA (556 bp) in B and non-B cells isolated from three patients with chronic hepatitis C using the isolation system of a touch-free cell. Molecular markers are indicated in lane M.

DISCUSSION

HCV infection induces a number of extrahepatic manifestations [Cacoub et al., 1999; Zignego and Brechot, 1999], among which lymphoproliferative disorder is related to HCV infection most closely [Zignego et al., 2007], although the mechanisms remain unknown. Accordingly, there have been many reports speculating on the mechanisms responsible for lymphoproliferative disorders associated with HCV. It has been demonstrated that chronic infection with HCV can lead to the clonal expansion of B cells and that sustained proliferation of B cells promotes the occurrence of genetic mutations. Zignego et al. [2000] have observed translocation of t(14;18) and overexpression of bcl-2 in lymphoid cells from patients with lymphoproliferative disorders frequently in association with HCV infection, although the relationship between clonal expansion of B cells and t(14;18) has not been confirmed yet. Direct association between HCV and B cells is thought to accelerate the clonality of B cells; for example, binding of HCV-E2 to CD81 and infection of B cells with HCV [Machida et al., 2005]. In the previous study, the influence of HCV infection and/or association of B cells on the development of lymphoproliferative disorders were evaluated in patients with persistent infection of HCV [Inokuchi et al., 2009]. The results showed that HCV infection was more prevalent, and HCV RNA levels were higher in B cells than other lymphoid cell subsets. On multivariate analysis, HCV RNA in B cells was an independent factor associated with the presence of at least one marker for lymphoproliferation [Inokuchi et al., 2009]. Based on these results, correlations were

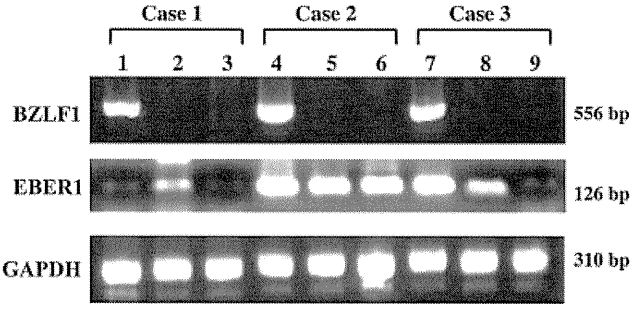

Fig. 3. Disappearance of BZLF1 mRNA in B cells after interferon therapy. BZLF1 mRNA in PBMCs was monitored in three patients (case 1: lanes 1–3; case 2: lanes 4–6; and case 3: lanes 7–9) whose B cells were positive before the start of therapy (lanes 1, 4, and 7). Putative sizes of molecular length for each PCR product are indicated on the right. All patients showed sustained virological responses after treatment with pegylated interferon and ribavirin. This therapy resulted in negativity for BZLF1 mRNA within 1 month (lane 2: 1 month; lane 5: day 1; and lane 8: day 3) and BZLF1 mRNA remained negative at 3 months after the end of therapy (lanes 3, 6, and 9).

demonstrated between infection and/or association of B cells with HCV and occurrence of lymphoproliferative disorders, although the detailed mechanisms remain unknown.

In the present study, BZLF1 mRNA was detected in B cells isolated from PBMCs of patients with chronic hepatitis C, thus suggesting the reactivation of EBV. EBV is a lymphotropic virus that induces lymphoproliferation and is associated with a wide range of tumors; for example, Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal and gastric carcinoma [Niller et al., 2009]. Furthermore, this virus is also associated with autoimmune diseases; for example, rheumatoid arthritis and Sjögren's syndrome [Niller et al., 2008]. Thus, the pathobiology of EBV infection is similar to that of HCV infection. It is possible that HCV infection triggers reactivation of EBV, and that the two viruses induce host lymphoproliferation cooperatively, as well as prolongation of survival and transformation. In fact, EBV-encoded nuclear antigen 1 (EBNA1) enhances replication of HCV in vitro, and EBV DNA has been detected in the tissues of HCC in patients infected with HCV [Sugawara et al., 1999a,b, 2000], although the latter issue remains controversial [Chu et al., 2001; zur Hausen et al., 2003]. LMP1 mRNA, which is a latent viral protein related to the transformation of host cells, has also been detected in B cells of two patients with chronic hepatitis C. The expression of BZLF1 and LMP1 mRNAs confirms the switch from the persistent to the lytic cycle of EBV. This expression was detected by nested RT-PCR only, indicating that the number of reactivated B cells is extremely low.

Anti-VCA antibody was positive in all subjects except for one healthy subject. The subject without anti-VCA antibody had anti-EBNA antibody in the serum, indicating that all subjects studied were in a state of past infection for EBV. The frequency of positivity for anti-EBNA antibody, which is thought to be a neutralizing antibody, is almost the same (healthy adults, 82.8%; and patients infected with HCV, 82%). Furthermore, 8 of 12 (66.7%) patients infected with HCV, who were positive for BZLF1 mRNA, had anti-EBNA antibodies in their sera. This suggests that low levels of anti-EBNA antibody are not the main cause for reactivation of EBV in patients infected with HCV. The possibility that infection of B cells and/or association with HCV induce dysfunction of immunoglobulin itself could not be ruled out. In persistent infection of HCV, an increase in serum is commonly observed. This increase is polyclonal and is determined primarily by an increase of IgG levels. These immunoglobulins include both HCV-specific and non-specific antibodies. Nonetheless, memory B cells do not expand but rapidly differentiate to secrete immunoglobulin and undergo apoptosis [Racanelli et al., 2006]. HCV also induced hypermutation of immunoglobulin to reduce affinity and neutralizing activities against the envelope protein of HCV [Machida et al., 2008]. Based on these observations, HCV infection may impair the function of anti-EBNA antibodies to inhibit reactivation of EBV.

Analysis of EBV reactivation in lymphoid cell subsets showed that reactivation occurred in B cells specifically. EBV infects B cells mainly, but can establish latent infection in other cell types, including T lymphocytes and natural killer (NK) cells [Watry et al., 1991; Kanegane et al., 1996]. The results showed that EBV was reactivated in B cells mainly. In one patient with chronic hepatitis C, however, EBV reactivation was observed in all subsets of lymphoid cells. This patient did not have any lymphoproliferative disorders. HCV RNA in B cells was detected in only 5 of 10 patients studied (data not shown). These results suggest that reactivation of EBV may not be induced by HCV infection of B cells directly. Therefore, further studies are necessary to clarify the association between HCV infection and reactivation of EBV in B cells.

No correlation was observed between reactivation of EBV and the occurrence of lymphoproliferative disorders. There were no differences in the markers for lymphoproliferative disorders between patients with PBMCs that were positive or negative for BZLF1 mRNA. The clonal expansion of B cells is reported to occur in 26% of Italian patients with chronic hepatitis C [Pozzato et al., 1999], while the clonality of B cells was detected in 11% of Japanese patients infected previously with HCV [Inokuchi et al., 2009]. The present study shows that induction of clonality was observed in one case only among patients infected with HCV with PBMCs positive for BZLF1. Several studies have focused on the important role of antigenic stimulation that is sustained, similar to lymphomagenesis due to infection with *H. pylori*, for its possible role in extra-nodal marginal-zone B cell lymphoma arising in lymphoid tissues on mucosae (MALT lymphoma) [Ivanovski et al., 1998; De Re et al., 2000; Sansonno et al., 2004].

Cirrhosis and hepatocelluar carcinoma may be associated with reactivation of EBV. Five of 12 (41%) patients with cirrhosis and 4 of 12 (33%) patients with hepatocelluar carcinoma had reactivation of EBV. These frequencies were statistically higher than those of patients without BZLF1 mRNA, indicating a possible association with reactivation of EBV and advance of clinical stages. But a large number of both patients with cirrhosis and with hepatocelluar carcinoma are needed to conclude this issue. EBV is also associated with the development of malignancies from both B cells and epithelial cells: Burkitt's lymphoma [Epstein et al., 1964], nasopharyngeal carcinoma [Andersson-Anvret et al., 1979], and Hodgkin's disease [Weiss et al., 1989]. Furthermore, about 10% of gastric carcinoma is associated with EBV [Fukayama et al., 1994]. As mentioned above, EBV DNA was detected in HCC tissues in patients infected with HCV [Sugawara et al., 1999a,b, 2000]. It is possible that reactivation of EBV is associated with neoplasm. Again, further large-scale studies are necessary to confirm these issues.

Reactivation of EBV is also associated with other medical problems such as lupus, malaria, and multiple sclerosis [Huggins et al., 2005; Christensen, 2006; Chene et al., 2007]. This may simply indicate that there

are several factors to induce reactivation of EBV in patients suffering from several medical problems. In order to confirm a correlation between HCV infection and reactivation of EBV, BZLF1 mRNA expression was followed in patients who succeeded in eradication of HCV after IFN therapy. After eradication of HCV, expression of BZLF1 mRNA disappeared in three patients with a sustained viral response. Expression of EBER1 RNA as a marker of EBV persistence was detected in PBMCs from all of these patients, although levels of expression were decreased in two of three patients. These results suggest that the interferon therapy eradicated HCV but not EBV in these patients. On the other hand, reactivation of EBV was not observed at 3 months after the end of treatment. Conceivably, the presence of HCV is required for reactivation of EBV in patients. It is possible that interferon therapy affects replication and/or reactivation of EBV directly. However, there have been few reports showing the effects of interferon on chronic infection of EBV directly. Further studies are necessary to clarify the molecular mechanisms responsible for the generation of lymphoproliferative disorders associated with HCV and its relationship with malignant lymphoma.

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J Gastroenterol (2010) 45:451–458
DOI 10.1007/s00535-009-0155-2

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Strong CD8$^+$ T-cell responses against tumor-associated antigens prolong the recurrence-free interval after tumor treatment in patients with hepatocellular carcinoma

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Received: 10 August 2009 / Accepted: 19 October 2009 / Published online: 20 November 2009

Abstract
Aim We investigated whether tumor-specific CD8$^+$ T-cell responses affect tumor-free survival as well as the relationship between CD8$^+$ T-cell responses against tumor-associated antigens (TAAs) and the clinical course after tumor treatment in patients with hepatocellular carcinoma (HCC).
Methods Twenty patients with HCC that were treated by radiofrequency ablation or trans-catheter chemo-embolization (TACE) and in whom HCC was undetectable by ultrasonography, CT, and/or MRI 1 month after treatment were enrolled in the study. Before and after treatment for HCC, analyses of TAA (glypican-3, NY-ESO-1, and MAGE-1)-specific CD8$^+$ T-cell responses were evaluated with an interferon-γ enzyme-linked immunospot (ELISpot) assay using peripheral CD8$^+$ T-cells, monocytes, and 104 types of 20-mer synthetic peptide overlapping by 10 residues and spanning the entirety of the 3 TAAs.
Results Sixteen out of 20 patients (80%) showed a positive response ($\geq$10 TAA-specific cells/10^5 CD8$^+$ T-cells) before or after treatment. When we performed univariate analysis of prognostic factors for the tumor-free period in the 20 patients, platelet count, prothrombin time, and the number of TAA-specific CD8$^+$ T-cells after treatment were significant factors ($P = 0.027$, 0.030, and 0.004, respectively). In multivariate analysis, the magnitude of the TAA-specific CD8$^+$ T-cell response ($\geq$40 TAA-specific cells/10^5 CD8$^+$ T-cells) was the only significant prognostic factor for a prolonged tumor-free interval (hazard ratio 0.342, $P = 0.022$).
Conclusions Our results suggest that strong TAA-specific CD8$^+$ T-cell responses suppress the recurrence of HCC. Immunotherapy to induce TAA-specific cytotoxic T lymphocytes by means such as the use of peptide vaccines should be considered for clinical application in patients with HCC after local therapy.

Keywords Hepatocellular carcinoma · CD8$^+$ T-cell response · Cytotoxic T lymphocyte · ELISpot assay · Immunotherapy

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Introduction

There are about 500,000 new patients with hepatocellular carcinoma (HCC) per year worldwide. Although vaccination against hepatitis B virus (HBV) and interferon (IFN)-based therapy against hepatitis C virus (HCV) will presumably reduce the number of HCC patients in the future, the incidence of HCC is still increasing in Asia and Africa because of the previous prevalence of infection with the virus. Progress in treatments for HCC has improved the prognosis of patients with HCC. However, HCC is usually associated with cirrhosis and often recurs even after complete treatment of the tumors in the remaining part of the cirrhotic liver. Thus, there is a strong need for the development of a new intervention therapy that suppresses the occurrence or recurrence of HCC effectively and that has fewer side effects. Immunotherapy may be such a treatment and may be applicable to the clinical treatment of HCC. In fact, some clinical trials have been performed [1–3].

Cytotoxic T lymphocytes (CTLs) are thought to be potent effector cells against cancers. CTLs recognize specific antigens, and the induction of CTLs specific for tumor-associated antigen (TAA) is an attractive procedure

for tumor therapy. The MAGE-1 gene was first identified as encoding a tumor-specific antigen on MZ-2-MEL cells, a melanoma cell line, in 1991 [4]. MAGE-1 gene and protein can be detected in many cancer tissues, and three articles reported the expression of MAGE-1 in HCC as 30, 68, or 78%, respectively, in a Japanese population [5–7]. In gastrointestinal tumors, immunotherapy using both dendritic cells and MAGE peptides has been performed for patients with primary malignant melanoma of the esophagus, and this therapy was able to induce peptide-specific immune responses [8].

NY-ESO-1 antigen, a member of the cancer-testis antigen family, was initially identified by a serological analysis of recombinant cDNA expression cloning in an esophageal cancer patient [9]. NY-ESO-1 mRNA was detected in 24–37% of HCCs by reverse transcription-polymerase chain reaction [10, 11].

Glypican-3 (GPC3) consists of 580 amino acids and is a heparan sulfate proteoglycan with a potential role in the control of cell division. GPC3 mRNA was detected in 74.8% of HCC tissues, but only in 3.2% of normal liver tissues [12], and GPC3 protein was detected in 72% of HCCs, but not in normal tissue using GPC-specific antibody [13]. The GPC3 protein can also be detected in sera of 40–53% of patients with HCC [14, 15].

These three antigens are thought to be attractive targets for cancer immunotherapy because they are expressed only in tumor tissues and testis, but not in normal tissues other than testis. On the basis of previous reports, it is assumed that most HCCs would express at least one of the three TAAs. Therefore, monitoring immune responses against these TAAs might help in the development of HCC immunotherapy, such as TAA-based vaccination. In this study, we investigated how the magnitude of $CD8^+$ T-cell responses against these TAAs determined by an IFN-γ enzyme-linked immunospot (ELISpot) assay is related to other clinical data and the tumor-free interval in patients with HCC, in order to explore the clinical application of such a TAA-based immunotherapy.

Methods

Patients

Twenty patients who were diagnosed with HCC at Showa University Hospital between 2006 and 2008 were enrolled in the study. They met the following study criteria: (1) pathologically confirmed as having HCC or a lesion with characteristic imaging features of HCC based on ultrasonography, CT, and/or MRI; (2) liver function classed as Child-Pugh A or B; (3) no extrahepatic metastasis or vascular invasion; (4) no previous or simultaneous cancers other than HCC; and (5) an indication for treatment such as radiofrequency ablation (RFA) or trans-catheter chemo-embolization (TACE). RFA was performed by well-trained hepatologists using usual methods according to previous reports [16]. A 16-gauge cooled-tip ablation electrode (Covidien, Boulder, CO) was used in the procedure. TACE was performed by well-experienced hepatologists and radiologists. A microcatheter was inserted from the femoral artery to the artery feeding the HCC superselectively after conventional hepatic angiography, and then a segmental or subsegmental TACE procedure was performed using gelatin, lipiodol, and either epirubicin hydrochloride or cisplatin. All patients were followed every 1–3 months by ultrasonography, CT, and/or MRI to examine the appearance of new lesions in the liver or other organs. The recurrence-free interval was defined as the period from the month of HCC treatment to the month when a recurrent and/or metastatic HCC was first detected after treatment. Clinical data (platelet count, prothrombin time, serum AST, ALT, albumin, total bilirubin level, and AFP level) were collected 1–7 days before HCC treatment. Chronic hepatitis C was diagnosed on the basis of detectable HCV RNA in serum using the Amplicor assay (Roche Diagnostics, Tokyo, Japan). Informed consent was obtained from each patient included in this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethical Committee of Showa University.

Synthetic peptides of TAA

Twenty-mer peptides overlapping by 10 residues and spanning the entire MAGE-1, NY-ESO-1, and GPC3 proteins were synthesized based on the amino acid sequences reported previously as PepSets™ and purchased from Mimotopes (Clayton South, Victoria, Australia). These peptides were >80% pure. A total of 30 MAGE-1, 17 NY-ESO-1, and 57 GPC3 peptides were synthesized, as shown in Table 1. A total of 10–11 TTA peptides were pooled in a mixture (total 10 mixtures) at a concentration of 10 μg/ml each.

Preparation of $CD8^+$ T cells and monocytes from patients with HCC

PBMCs were isolated from heparinized peripheral blood by gradient centrifugation using Ficoll-Paque (Pharmacia-LKB Biotechnology, Uppsala, Sweden). As reported previously, peripheral $CD8^+$ T-cells and monocytes were separated from PBMCs using CD8 microbeads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and a Monocyte Isolation Kit II (Miltenyi Biotec), respectively [17]. These cells were isolated using an autoMACS™ Pro Separator (Miltenyi Biotec). The purity of the cells was >95% on flow cytometry (data not shown).

Table 1 Synthetic peptides and peptide mixtures used in this study

Tumor-associated antigen	Peptide	Amino acid sequence
Glypican-3	GL1	1–20
	GL2	11–30
	GL3	21–40
	⋮	⋮
	GL57	561–580
MAGE-1	MG-1	1–20
	⋮	⋮
	MG-30	290–309
NY-ESO-1	NY-1	1–20
	⋮	⋮
	NY-17	161–180

Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	Mix 9	Mix 10
GL1	GL2	GL3	GL4	GL5	GL6	GL7	GL8	GL9	GL10
GL11	GL12	GL13	GL14	GL15	GL16	GL17	GL18	GL19	GL20
GL21	GL22	GL23	GL24	GL25	GL26	GL27	GL28	GL29	GL30
GL31	GL32	GL33	GL34	GL35	GL36	GL37	GL38	GL39	GL40
GL41	GL42	GL43	GL44	GL45	GL46	GL47	GL48	GL49	GL50
GL51	GL52	GL53	GL54	GL55	GL56	GL57	MG-1	MG-2	MG-3
MG-4	MG-5	MG-6	MG-7	MG-8	MG-9	MG-10	MG-11	MG-12	MG-13
MG-14	MG-15	MG-16	MG-17	MG-18	MG-19	MG-20	MG-21	MG-22	MG-23
MG-24	MG-25	MG-26	MG-27	MG-28	MG-29	MG-30	NY-1	NY-2	NY-3
NY-4	NY-5	NY-6	NY-7	NY-8	NY-9	NY-10	NY-11	NY-12	NY-13
NY-14	NY-15	NY-16	NY-17	–	–	–	–	–	–

IFN-γ ELISpot assay

The ELISpot assay was performed using an IFN-γ ELISpot assay kit (Mabtech AB, Stockholm, Sweden) as previously described [17]. Briefly, a 96-well microtiter plate with a nitrocellulose membrane bottom (Millititer; Millipore, Bedford, MA) was coated with 100 µl anti-IFN-γ monoclonal antibody at a concentration of 15 µg/ml in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by washing 6 times in Hanks' balanced saline solution. After blocking with AIM-V medium (Invitrogen Japan, Tokyo, Japan) containing 10% fetal bovine serum, 1×10^5 $CD8^+$ T-cells, 1×10^4 autologous monocytes, and a TAA peptide mixture at 10 µg/ml of each peptide were placed and incubated in duplicate in 100 µl AIM-V medium at 37°C in a humid atmosphere with 5% CO_2. After incubation for 18 h, the cells were removed by washing the plate 8 times with PBS. Next, 100 µl of biotin-conjugated monoclonal antibody was added to each well, and the plates were incubated further for 2 h at room temperature. Wells were washed 5 times with PBS and incubated with 100 µl streptavidin–alkaline phosphatase for 2 h. Unbound antibodies were removed by washing 6 times with PBS. Then, 100 µl of alkaline phosphatase substrate (Bio-Rad Laboratories, Richmond, CA) was added to each well and incubated until dark spots emerged. Color development was stopped by washing 3 times with water, and the plates were allowed to dry. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted. Numbers of TAA-specific SFCs for each peptide mixture were calculated by subtracting the mean number of SFCs of 2 control wells (without stimulus) from the mean number of SFCs of 2 wells stimulated by TAA antigens. An SFC number was calculated for each patient as the sum of SFCs in each peptide mixture. ELISpot assays were performed before and 3–7 days after treatment. When TAA-specific $CD8^+$ T-cell responses were analyzed in 10 normal subjects, we were unable to detect any responses against TAA peptides in the ELISpot assay (data not shown).

Statistical analyses

The relationship between the number of TAA-specific $CD8^+$ T-cells and the recurrence-free period was analyzed