Efficient Class II Major Histocompatibility Complex Presentation of Endogenously Synthesized Hepatitis C Virus Core Protein by Epstein-Barr Virus-Transformed B-Lymphoblastoid Cell Lines to CD4\(^+\) T Cells

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The induction of an efficient CD4\(^+\) T-cell response against hepatitis C virus (HCV) is critical for control of the chronicity of HCV infection. The ability of HCV structural protein endogenously expressed in an antigen-presenting cell (APC) to be presented by class II major histocompatibility complex molecules to CD4\(^+\) T cells was investigated by in vitro culture analyses using HCV core-specific T-cell lines and autologous Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCLs) expressing structural HCV antigens. The T- and B-cell lines were generated from peripheral blood mononuclear cells derived from HCV-infected patients. Expression and intracellular localization of core protein in transfected cells were determined by immunoblotting and immunofluorescence. By stimulation with autologous B-LCLs expressing viral antigens, strong T-cell proliferative responses were induced in two of three patients, while no substantial stimulatory effects were produced by B-LCLs expressing a control protein (chloramphenicol acetyltransferase) or by B-LCLs alone. The results showed that transfected B cells presented mainly endogenously synthesized core peptides. Presentation of secreted antigens from adjacent antigen-expressing cells was not enough to stimulate a core-specific T-cell response. Only weak T-cell proliferative responses were generated by stimulation with B-LCLs that had been pulsed beforehand with at least a 10-fold-higher amount of transfected COS cells in the form of cell lysate, suggesting that presentation of antigens released from dead cells in the B-LCL cultures had a minimal role. Titrating numbers of APCs, we showed that as few as 10\(^4\) transfected B-LCL APCs were sufficient to stimulate T cells. This presentation pathway was found to be leupeptin sensitive, and it can be blocked by antibody to HLA class II (DR). In addition, expression of a costimulatory signal by B7/BB1 on B cells was essential for T-cell activation.

Hepatitis C virus (HCV) has been known as a major etiologic agent of posttransfusion and sporadic community-acquired non-A, non-B hepatitis. Like the other members in the family Flaviviridae, HCV contains a single, positive-strand RNA genome with a single long open reading frame (ORF) coding for a polyprotein precursor of about 3,000 amino acids (aa) (12). HCV infection is frequently persistent in the majority of patients and is closely associated with the later development of liver cirrhosis and hepatocellular carcinoma (3, 12, 16, 32). The effective control of HCV infection has been limited by the high frequency of viral genetic heterogeneity (7), the low rate of response to alpha interferon (46), and inadequate production of protective immunity (44, 45). These features strongly suggest that there is a great need to establish a new, highly effective therapy.

CD4\(^+\) T cells are considered to play a central role in the generation of protective immunity against infections, because they can provide help to B cells for antibody production (42) and to cytotoxic precursor T cells for their maturation to effectors (21). Some CD4\(^+\) T cells may also act as cytotoxic effectors (30). It has been recognized that CD4\(^+\) T-cell response to HCV antigens is important for determining the clinical course of HCV infection (17, 37). Generally, T-cell proliferation is more frequent and stronger in patients with a benign course (6, 17, 20, 33, 37) that is accompanied by the normalization of serum alanine aminotransferase and, in some cases, the clearance of viral RNA (17, 37). In contrast, patients who have a poor T-cell response tend to develop persistent infection (17, 37). These findings support the hypothesis that a sufficient CD4\(^+\) T-lymphocyte response is critical for limiting HCV infection.

Activation of T lymphocytes depends on the recognition of processed viral peptides, but not native antigens, in the context of major histocompatibility complex (MHC) molecules that are presented by antigen-presenting cells (APCs) (56). The B cell is an important professional APC, and its role in mediating antigen-specific immune response has been described extensively (11). Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cells are frequently used as APCs in in vitro analyses for antigen processing and presentation to T cells. These cells are characterized by high-level expression of class I and class II MHC molecules, along with accessory molecules such as ICAM-1, B7/BBI, and LFA-3, known to be important costimulatory molecules for T-cell activation (9, 15, 24). Importantly, transfected EBV-immortalized B cells expressing a tumor antigen have been shown to be capable of eliciting both T-helper and cytotoxic-T-lymphocyte (CTL) responses following in vivo
inoculation (40). Nevertheless, dendritic cells have been shown to be critical for initiating responses by naïve T cells (53), and in some situations presentation by B cells has been suggested to be tolerogenic (35). To date, the role of B cells in processing and presenting HCV antigens has not been studied in detail and the mechanisms underlying T-cell–B-cell interaction are still being worked out.

In the present study, EBV-transformed B-lymphoblastoid cell lines (B-LCLs) established from HCV-infected patients were transfected with an expression vector coding for the whole structural region and part of the NS2 region of the HCV genome. The capacity of transfected B-LCL APCs for presenting intracellularly synthesized peptides was assessed by in vitro induction of the HCV-specific lymphoproliferative response of autologous T-cell lines. Our results indicated that core protein was properly expressed and efficiently presented by B-LCL APCs to CD4+ T cells. We demonstrated that the endogenous core peptides were presented through the class II MHC pathway and that they need B7/BB1 for presenting costimulatory signals.

MATERIALS AND METHODS

Subjects. Three patients with chronic hepatitis C were selected from the Kagawa Medical School Medical Center (Kagawa, Japan) based on histological diagnosis of chronic hepatitis, seropositivity for anti-HCV antibodies (detected by second-generation enzyme immunoassay; Abbott Laboratories, North Chicago, Ill.) and for HCV RNA of the 5′ noncoding region (detected by nested PCR [51]), and seronegativity for hepatitis B surface antigen (HBsAg) (detected by enzyme-linked immunosorbent assay [ELISA]; Abbott Laboratories). Informed consent was obtained from all subjects studied.

B-LCLs and cultures. Blood was drawn from each patient before interferon therapy, and the peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). B-LCLs were established from PBMCs as described previously (50), and they were referred to as B-LCL-P1, B-LCL-P2, and B-LCL-P3. The B-LCLs were maintained in RPMI 1640 medium containing 10% inactivated fetal calf serum (FCS), penicillin/ml, and 50 mg of streptomycin/ml.

Transfection. EBV-transformed B-lymphoblastoid cell lines (B-LCLs) established from HCV-infected patients were transfected with an expression vector coding for the whole structural region and part of the NS2 region of the HCV genome. The capacity of transfected B-LCL APCs for presenting intracellularly synthesized peptides was assessed by in vitro induction of the HCV-specific lymphoproliferative response of autologous T-cell lines. Our results indicated that core protein was properly expressed and efficiently presented by B-LCL APCs to CD4+ T cells. We demonstrated that the endogenous core peptides were presented through the class II MHC pathway and that they need B7/BB1 for presenting costimulatory signals.

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Characterization of structural gene products of HCV. The translational products from pC980 have been expressed previously by using an in vitro expression system in the presence of microsomal membranes with [35S]methionine for labeling, showing four major cleavage proteins of 70, 35, 22, and 19 kDa that are relevant to the E2, E1, core, and NS2A regions of the HCV ORF, respectively (29). In this study, the same HCV cDNA sequence was subcloned into the pCMV980 expression vector and expressed intracellularly in COS7 and B-LCL cells. Analyses of cell lysates from transiently (48 h) transfected COS7 and B-LCL cells by SDS-PAGE and immunoblotting revealed results similar to those obtained by in vitro expression (29) (Fig. 1). Three major bands, of 70, 35, and 22 kDa, were identified by using MAbs to E2 (A11), E1 (A4), and core protein (H-29), respectively (Fig. 1a). The supernatants of B-LCL980 cultures (2 × 10^5 cells/ml) over different times were probed with H-29. The signal of staining was generally stronger in COS cells than in B-cell APCs (Fig. 2A and B). Both cells showed a cytoplasmic staining pattern. The core products were further assessed by double immunostaining. The IF pattern of the core protein was well colocalized with that of the ER (Fig. 2C). For each independent experiment, negative cell controls (untransfected cells, or cells transfected with pcDNA3/ CAT) and antibody controls (pools of normal human or mouse sera, or goat polyclonal antibody to CAT; Boehringer Mannheim Biochemicals) were included. Nonspecific binding was not noticed in our assays (data not shown).

Efficient induction of T-cell proliferation by B-LCL APCs. Stable B-LCL980 transfectants were obtained from all three patients and subsequently used to stimulate autologous T cells after MMC treatment. By using B-LCL980 APCs, strong T-cell proliferation was induced in two of three patients (Fig. 3). The level of response to antigens expressed by B-LCL980 was highest in patient P1 (28,819 ± 602 cpm; SI = 57) and moderate in patient P2 (15,477 ± 1,130 cpm; SI = 25) but was within background levels in patient P3 (1,930 ± 201 cpm; SI = 3). No proliferative responses above background levels to CAT or B-LCL APCs alone were observed in any of the patients. On the other hand, T-cell lines from all three patients responded equally well to IL-2 (34,581 ± 671, 27,868 ± 672, and 33,570 ± 1,457 cpm and SI values of 68, 46, and 59 for patients P1, P2, and P3, respectively). These results indicated that the T-cell proliferation was specific to HCV antigens but not to nonlated protein (CAT) or to EBV-derived viral antigens (B-LCL APCs alone). It was not understood why T-cell line P3 failed to respond to autologous APCs. To test whether B-LCL-P3 APCs were functional, they were cultured with T-cell lines from two responders, P1 (HLA-DR compatible) and P2 (HLA-DR incompatible). An additional experiment was performed to stimulate P3 T cells with P1 B APCs (HLA-DR compatible). Figure 4 showed that B-LCL980-P3 did stimulate T cells from HLA-DR-compatible (P1) (6710 ± 321 cpm; SI = 13) patients but not from HLA-DR-incompatible (P2) (791 ± 138 cpm; SI = 1.6) patients, indicating that B-LCL-P3 APCs were functional and that the presentation was HLA restricted. Again, the P1 B APCs (HLA-DR compatible) failed to stimulate T-cell line P3. To further assess the efficacy of antigen presentation by B-LCL980-P1 and B-LCL980-P2, the number of APCs used to stimulate autologous T cells was titrated. The results indicated that as few as 10^5 APCs were sufficient to induce significant T-cell proliferation (7,434 ± 560 and 6,580 ± 401 cpm and SI values of 15 and 11 for P1 and P2, respectively) (Fig. 5). Usually, 10^5 APCs produced maximal proliferation, but further increases in APC numbers resulted in no substantial additional increase in T-cell proliferation (data not shown).

Bystander presentation. Theoretically, some B cells may present exogenous antigens (bystander presentation) by reabsorbing and processing antigens that were secreted into the culture medium by transfected cells or shed by adjacent dead or dying cells during the time of culture. To test this possibility, 5 × 10^5 untransfected B-LCL-P1 APCs were precultured with an equal number of allogeneic transfected APCs (B-LCL980- P2, HLA mismatched) in 1 well of a 24-well culture plate for various periods. Two hundred thousand APCs from the mixed culture were then tested for stimulation of autologous T cells (P1) as described above. As shown in Fig. 6A, the untransfected B-LCL-P1 APCs that had been precultured with allogeneic B-LCL980-P2 APCs were incapable of inducing T-cell
proliferation above background levels. One reason for this result could be that the core protein is retained in the ER and not secreted to the culture medium, at least not at a level detectable by immunoblotting. Nevertheless, the medium should contain some antigens that were released from the dead cells. To further address this question, $10^6$ untransfected B-LCL APCs were cultured for 3 days in the presence of cell lysate from $10^7$ transfected COS cells, which was prepared by repeatedly freeze-thawing COS980 cells in PBS, then passing the cells through a sterile 25-gauge needle. The cell lysate prepared from untransfected COS7 cells was used as a negative control. As shown in Fig. 6B, presentation of exogenous antigens in the form of cell lysate could induce only weak T-cell proliferative responses ($3,338 \pm 272$ cpm; SI = 6.6) and the level was just about 1/10 of that obtained by using B-LCL980 APCs. Taken together, these results suggested that presentation with intracellularly synthesized rather than reabsorbed exogenous antigens contributed most, if not exclusively, to the observed T-cell proliferation stimulated by B-LCL APCs.

**Presentation of endogenous HCV core peptides is leupeptin sensitive.** B-LCL-P1 and B-LCL-P2 APCs were precultured in complete medium containing 33, 100, and $300 \mu g$ of leupeptin/ml for 24 h and then were used for stimulating autologous T cells in the presence of the same concentration of the drug. A clear inhibition of the presentation of core peptides to T cells was seen in both APCs in a dose-dependent manner (Fig. 7). When leupeptin was added at $300 \mu g$/ml, T-cell stimulation was inhibited to a level below background (SI = 2.9) in P2 and to 26% of that in the control (without leupeptin; SI = 32.4) in P1 (SI = 8.5). These data indicated that processing of endogenously synthesized core peptides by B-LCL APCs is leupeptin sensitive.

**Presentation of endogenous core peptides requires class II MHC and costimulatory molecules.** The efficiency of presen-
tation of endogenous peptides is correlated with the surface expression of MHC class II and costimulatory molecules such as B7/BB1 and ICAM-1 on APCs (14, 18, 26, 34). ICAM-1 was consistently expressed on both large (activated) and small (resting) B cells (26), while expression of B7 was found only on activated B cells (26, 34). In this study, we determined the relative roles of HLA class I, HLA class II, and B7 in the activation of in vivo primed T cells. As shown in Fig. 8, addition of the soluble MAbs to class II (L243, DR) (50) to the cultures led to the complete inhibition of T-cell proliferation, whereas a MAb to HLA class I (LW6/32) (50) had no substantial inhibitory effects (15.2 and 18.6% inhibition relative to the control responses for P1 and P2, respectively). These findings were consistent with the phenotype analyses showing that more than 90% of proliferating cells were CD4+ CD8- T cells (data not shown). In addition, a MAb to BB1 also inhibited T-cell pro-

FIG. 3. Lymphoproliferative responses of T-cell lines to autologous B-LCL980 APCs. T cells (3 × 10^5 cells/well) were stimulated for 2 days in the presence of autologous B-LCL APCs (1 × 10^5 cells/well), of pCMV980- or pcDNA3/CAT-transfected cells, or of untransfected cells, or in the absence of APCs with IL-2 alone. T-cell proliferative responses are expressed as SI. The background level (expressed as mean counts per minute of triplicate cultures ± SD) for each T-cell line was 508 ± 98 (P1), 609 ± 61 (P2), and 569 ± 66 (P3) cpm.

FIG. 4. T-lymphoproliferative response to allogeneic HLA-compatible B-LCL APCs. B-LCL980-P3 APCs were used to stimulate T-cell lines from patients P1 (HLA-compatible) and P2 (HLA incompatible). Similarly, the T-cell line from patient P3 was stimulated by allogeneic B-LCL980 APCs (B-LCL980-P1). Untransfected APCs were included in each experiment as B-LCL controls. T-lymphoproliferative responses were expressed as SI.

FIG. 5. Efficient presentation of endogenous HCV antigens to autologous T-cell lines. T cells (3 × 10^5 cells/well) from patients P1 (squares) and P2 (circles) were stimulated with various numbers of autologous B-LCL980 APCs. The response of each T-cell line is expressed as the SI.

FIG. 6. Bystander presentation played a minimal role. (A) Untransfected B-LCL-P1 APCs were precultured with HLA-incompatible transfected APCs (B-LCL980-P2) for 12, 24, 48, and 72 h. Two hundred thousand cells from the coculture were then used to stimulate autologous T cells (3 × 10^5 cells/well) for 2 days. (B) Untransfected B-LCL-P1 APCs were prepulsed for 3 days with a cell lysate of pCMV980-transfected or untransfected COS7 cells. One hundred thousand pulsed APCs were then used to stimulate autologous T-cell lines.
The capacity of B-lymphoblastoid cells for presenting endogenous HCV core peptides to autologous T cells has been investigated by stimulation of patient-derived core-specific T-cell lines. B-LCL APCs expressing the endogenous viral antigens were found to be highly effective restimulators of in vivo-primed T cells. The endogenous presentation of core peptides by B-LCL APCs is leupeptin sensitive and requires class II MHC and B7/BB1 molecules.

CD4+ (Th) and CD8+ (CTL) T cells recognize antigenic peptides in association with MHC class II and class I molecules, respectively, expressed on APCs (57). Classically, presentation of endogenous antigens is mediated by MHC class I molecules, while the exogenous antigens are presented by MHC class II molecules (47, 59). However, accumulating reports have evidenced that MHC class II molecules can also present endogenous viral antigens such as HBsAg (31), influenza A matrix protein (39), and measles virus-derived hemagglutinin (HA) antigen to CD4+ T cells. Extending these findings, we show here that HCV core protein can also be presented through the HLA class II (DR) pathway to CD4+ T cells. The endogenous presentation of core peptides involves in the hydrolytic digestion of endogenous HCV polypeptides and the consequent binding of processed peptides with MHC class II molecules. The core protein contains two hydrophobic domains (55), and the COOH-terminal hydrophobic fragment (aa 171 to 187) is considered responsible for the cytoplasmic retention of the protein (43). Analysis by immunoelectron microscopy has shown that core antigen is distributed along the ER membrane or in the cisternae (38). Supporting these findings, our results demonstrate that the core antigen is well colocalized with an intrinsic ER membrane protein. The fact that core protein usually exhibits strong cytoplasmic retention 

![FIG. 7. Inhibition of antigen presentation by leupeptin. B-LCL980 APCs were pretreated with leupeptin at various concentrations as indicated for 24 h prior to the T-cell proliferative assay. One hundred thousand APCs were added to the T-cell culture in the presence of the same concentration of leupeptin that was used for pretreatment.](image)

**DISCUSSION**

It is well documented that the endosome/lysosome compartments are the site of loading of MHC class II molecules with endogenous peptides (25, 28, 36), and they might be involved in the hydrolytic digestion of endogenous HCV polypeptides and the consequent binding of processed peptides with MHC class II molecules. The core protein contains two hydrophobic domains (55), and the COOH-terminal hydrophobic fragment (aa 171 to 187) is considered responsible for the cytoplasmic retention of the protein (43). Analysis by immunoelectron microscopy has shown that core antigen is distributed along the ER membrane or in the cisternae (38). Supporting these findings, our results demonstrate that the core antigen is well colocalized with an intrinsic ER membrane protein.
toplamic staining in our study and others' (27, 38, 43) studies suggests that there is high expression and/or retention of the protein in transfected cells. It seems likely that the core protein in association with the ER membrane is selectively removed by autophagy during cell remodeling to generate an autophageosome (19). This intermediate vesicle might then fuse with endosome/lysosome compartments to initiate the process for hydrolytic digestion of the "excessive" proteins. Another route for endogenous core protein traveling may also use endocytosis, since the protein can be found on the cell surface when unfixed cells are used (data not shown). The core polypeptide is a basic protein and can bind avidly with other negatively charged surface molecules once the protein is secreted to the surface by an as-yet-unknown mechanism. The core antigen can then be endocytosed by spontaneous internalization of the cell membrane and transported to the site for MHC class II loading. The processing of endogenous core peptides in endosome/lysosome compartments is evidenced by our results in leupeptin inhibition experiments. Leupeptin is a commonly used cysteine-serine protease inhibitor that blocks degradation of the protein in the endocytic pathway. Therefore, it also blocks the degradation of the MHC class II-associated invariant chain. Limited studies have also suggested a nonendosomal processing pathway that involves the proteolytic degradation of endogenous proteins and consequent binding with MHC class II molecules in the ER compartment (39, 49). So far, this pathway has not been well characterized. In fact, the newly synthesized MHC class II heterodimers (αβ) are competitively occupied by the invariant chain (Ii), and the complexes do not disassociate until they reach the acidic environment of the endosomal/lysosomal compartment (23), thus preventing binding with endogenous peptides in the ER. Whether MHC class II loading with HCV endogenous peptides can occur physiologically in a nonendosomal compartment such as the ER in "professional" APCs remains to be established.

A solid base of evidence has confirmed that optimal activation of T cells requires, in addition to the first signal for triggering the CD3–T-cell receptor complex to initiate the activation process, a second signal that is delivered by APC-mediated accessory molecules such as B7/BB1, ICAM-1, VCAM-1, or LFA (all of which belong to the Ig supergene family) (14, 18, 26, 34). B7/BB1 is one of the most important costimulatory ligands and functions by interacting with its receptor, CD28/CTLA-4, expressed on the surfaces of T cells. The triggering of CD28 by B7/BB1 is found to be especially involved in IL-2 production and proliferation (24, 34). Furthermore, the presence of B7/BB1 during the cross-linking of T-cell receptors with antigenic peptide-MHC class II complex is thought to play a critical role in preventing T-cell clonal inactuation or anergy (48). Since B7/BB1 is constitutively expressed on EBV-transformed B-cell lines (58), the molecule may contribute to the efficient presentation of endogenous HCV peptides in our assays. This possibility is supported by the significant or even complete inhibition of T-cell activation by a MAb to BB1. Although other adhesion and costimulatory molecules may possibly provide costimulatory signals, such effects appear to be less efficient or to depend on the synergistic expression of B7/BB1 for costimulation of in vivo-primed T cells. This is consistent with the previous observations that ICAM-1 and VCAM-1 regulate resting CD4+ T-cell preferentially, whereas B7/BB1 costimulates antigen-primed T cells most potently. LFA-3 has a behavior similar to that of B7/BB1, but it is less efficient (14).

Although endogenous antigens are classically presented through the class I MHC pathway to stimulate CD8+ T cells, we demonstrate here that core antigens endogenously synthesized in B cells can also enter the class II MHC pathway for presentation and efficiently induce an autologous CD4+ T-cell reaction. These findings provide useful information for the design of new approaches, such as DNA-mediated vaccination, for augmenting both helper and CTL responses. Importantly, data from a recent in vivo study have shown that subcutaneous immunization of chimpanzees with transfected autologous EBV-immortalized B cells expressing a tumor antigen induced both CD4 and CD8 antitumor T-cell responses (40). Since the protocols for de novo gene delivery into the primary B cells have already described and tested for somatic gene therapy in a murine model (54) and a clinical trial (5), delivery of HCV genes or other viral and tumor immunodominant genes into the B-cell compartment may serve as an alternative approach in this regard. These potential applications await further examination.

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REFERENCES

ICAM-1 (CD54) expression on B lymphocytes is associated with their co-stimulatory function and can be increased by coactivation with IL-1 and IL-7. Cell Immunol. 154:414–423.


37. Steinman, R. M. 1991. The dendritic cell system and its role in immunogene-


