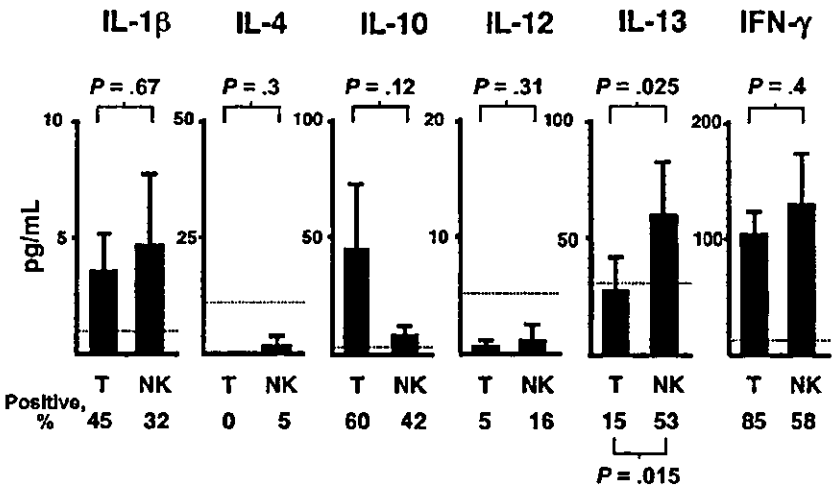

Figure 2. Comparison of plasma cytokine concentrations. Concentrations were estimated by use of an immunoassay and compared between patients with T cell–type ($n = 20$) and NK cell–type ($n = 19$) chronic active Epstein-Barr virus infection. Boxes and error bars indicate means and SEs, respectively; the dotted lines indicate the upper limits of healthy individuals. The Mann-Whitney U test was used to compare plasma cytokine concentrations, and Fisher's exact test was used to compare positivity rates. IFN, interferon; IL, interleukin.

titers of antibodies against the early and late EBV antigens and the existence of cell-free EBV DNA in plasma suggest the possibility of lytic cycle infection [6, 32, 33]. In the present study, a deoxyribonuclease-digestion experiment showed that the presence of EBV DNA in plasma was attributable to free nucleic acids that were likely released from dead or damaged cells. Furthermore, the pattern of EBV gene expression in PBMCs was latency type II, which supports the absence of lytic cycle replication in the PBMCs, at least, of patients with CAEBV infection. Lytic cycle infection may occur in tissue, although our results for tissue samples, while limited, showed no sign of a lytic cycle. It is also possible that we did not detect the occurrence of lytic cycle replication if lytic cycle infection occurred in <1% of EBV-infected cells. There is no definite proof of lytic cycle replication in tissues from patients with CAEBV infection. Some investigators have reported the expression of early or late EBV gene transcripts, such as those for BZLF1 or viral IL-10 [30, 34, 35], whereas other investigators have reported the absence of expression of these transcripts in tissue samples [36].

Our observation here of the absence of lytic cycle infection is particularly important with regard to selection of the treatment strategy for patients with CAEBV infection. Antiviral drugs that suppress viral DNA polymerase and lytic cycle replication may not be suitable for the treatment of CAEBV infection; however, therapies that reduce or eliminate EBV-infected T cells or NK cells may be suitable choices. Chemotherapy or hematopoietic stem-cell transplantation are suitable in this regard—the successful treatment of CAEBV infection by hematopoietic stem-cell transplantation has been reported [37, 38]. Alternatively, EBV-related antigens expressed in T cells or NK cells may be the targets of treatment. Cytotoxic T cells that were generated from LCL and targeted to latency type III antigens have been administered to patients with CAEBV infection [39, 40]. On the basis of the present result that the pattern of EBV gene expression was latency type II, cytotoxic T cells specific for latency type II antigens, such as LMP1 or LMP2A, would be more favorable for the control and eradication EBV-infected cells if they are inducible [41, 42].

In the present study, patients with CAEBV infection had high concentrations of proinflammatory (IL-1β), Th1-type (IFN-γ), and anti-inflammatory (IL-10) cytokines. Transcription of the genes for these cytokines was also high in PBMCs. The upregulation of various cytokine genes has also been reported in patients with CAEBV infection in other studies [43–45]. These cytokines are thought to be produced either by EBV-infected T cells or NK cells or by reacting inflammatory cells. On the one hand, it has been shown that EBV-infected T cells produce proinflammatory (IL-6 and TNF-α), Th1-type (IL-2 and IFN-γ), and anti-inflammatory (transforming growth factor β1) cytokines [14, 46]. Shen et al. reported that, during EBV-infected nasal NK/T cell lymphoma, human IL-10, an anti-inflammatory cytokine that suppresses cytotoxicity against EBV-infected cells, was expressed [47]. On the other hand, reacting inflammatory cells, such as macrophages, can produce most of the cytokines seen in the present study. Unfortunately, because of our study design, it is impossible to determine whether EBV-infected or reacting cells were the main sources of these cytokines. However, the high concentrations of and the elevated

transcription of genes for various cytokines must contribute to the diverse symptoms seen in patients with CAEBV infection.

One of the purposes of the present study was to find virologic differences between the T cell–type and the NK cell–type infection. One main difference was that we found frequent detection and a high concentration of IL-13 in the patients with NK cell–type infection. IL-13 is a Th2-type cytokine that induces the differentiation of B cells, the production of antigen-specific antibody, and a class switch to IgE and that also suppresses the cytotoxic functions of monocytes and macrophages [48]. IL-13 is primarily produced by activated T cells and is not usually detected in plasma from healthy individuals [49]. The cytokine is produced by Reed-Sternberg cells during Hodgkin disease, which is associated with EBV infection [50]. Although the reason why IL-13 was produced in the patients with NK cell–type infection is unclear, the high concentration of IL-13 may explain the high serum IgE levels and the hypersensitivity to mosquito bites, both of which are frequently seen in patients with NK cell–type infection [5].

The other difference between the 2 types of CAEBV infection is that the patients with NK cell–type infection had a higher viral load in PBMCs. This is particularly interesting, because NK cell–type infection is usually milder and progresses slowly [5, 12]. In contrast, the viral load in plasma was similar between the 2 types of CAEBV infection. These results suggest that sources of EBV DNA other than PBMCs exist in patients with T cell–type disease, the more severe, rapid type of CAEBV infection. In patients with T cell–type infection, the cell-free EBV DNA may come from tissue, such as lymph nodes or the spleen, where EBV-infected T cells infiltrate and proliferate. Indeed, patients with T cell–type infection have a higher incidence of hepatomegaly and lymphadenopathy, as was shown in the present study. The higher viral load in plasma could also be explained by the naturally high rate of apoptosis in activated T cells. It is still unclear why T cell–type infection is severe and progresses rapidly. The distribution of infected cells, determined by the differences in homing receptors among cells, may determine the symptoms and prognosis. A recent animal model showed that activated T cells are selectively trapped in the liver, primarily by intracellular adhesion molecule 1, which is constitutively expressed on sinusoidal endothelial cells and Kupffer cells [51]. We previously reported a patient with primary EBV infection who had severe hepatitis and whose liver was infiltrated with EBV-infected $CD8^+$ cells. In patients with T cell–type CAEBV infection, EBV-infected, presumably activated T cells might accumulate in the liver and cause hepatitis. Although further studies are necessary, our findings should help to clarify the pathogenesis of CAEBV infection and facilitate the development of more-effective treatments.

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RESEARCH ARTICLE

Retroviral vector backbone immunogenicity: identification of cytotoxic T-cell epitopes in retroviral vector-packaging sequences

E Kondo[1,3], Y Akatsuka[1], A Nawa[2], K Kuzushima[1], K Tsujimura[1], M Tanimoto[3], Y Kodera[4], Y Morishima[5], K Kuzuya[2] and T Takahashi[1]

[1]*Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan;* [2]*Department of Gynecology, Aichi Cancer Center Hospital, Nagoya, Japan;* [3]*Department of Internal Medicine II, Okayama University Medical School, Okayama, Japan;* [4]*Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan; and* [5]*Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Nagoya, Japan*

*Retroviral vectors are the frequently applied gene delivery vehicles for clinical gene therapy, but specificity of the immunogenicity to the protein encoded by the inserted gene of interest is a problem which needs to be overcome. Here, we describe human cytotoxic T-lymphocyte (CTL) clones recognizing epitopes derived from the protein encoded by the retroviral vector backbone, which were established during the course of our attempts to generate CTLs against cytomegalovirus (CMV) or human papilloma virus (HPV) in vitro. In the case of healthy CMV-seronegative donors, CTL lines specific for retrovirally transduced cells were generated in four out of eight donors by stimulating CD8 T cells with CD40-activated B (CD40-B) cells retrovirally transduced with CMV-pp65. Two CTL clones derived from one of the CTL lines were found to recognize epitopes from gag in the context of HLA-B*4403 and -B*4601, respectively. Similarly, an HLA-B*3501-restricted CTL clone from a cervical cancer patient recognized an epitope located in the junctional regions of the gag and pol sequences. These results show that polypeptides encoded by components of the retroviral vector backbone are in fact immunogenic, generating CTLs in vitro in human cells. Thus, potential CTL responses to retroviral products should also be considered in clinical settings.*

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Keywords: *cytotoxic T lymphocyte; retroviral vector; epitope*

Introduction

Murine leukemia virus (MLV)-based vector is one of the frequently used gene delivery vehicle, being employed in approximately a quarter of the approved clinical protocols worldwide (http://www.wiley.co.uk/genmed/clinical). However, there are still problems with the retroviral vectors used currently in the clinical setting. In particular, the issue of safety has often been raised, mainly because of the possibility of generation of replication-competent retroviruses and unexpected activation of genes adjacent to the viral integral sites. In this regard, it should be noted that development of leukemia manifesting clonal integration of the retrovirus into a proto-oncogene *LMO2* was observed in two SCID-X1 patients in a French trial.[1] Another important problem to overcome is the immunogenicity of the retroviral vectors themselves, that could induce humoral and cellular immune responses in the hosts that eventually diminish effective gene transfer.[2–10] Most retroviral vectors so far reported contain retroviral coding sequences, such as the 5′ end of the *gag* gene to increase their packaging efficiency.[11–14] Because a number of potential start codons, such as ATG or CTG, remain intact in the extended packaging sequences in most of the retroviral vectors,[12] polypeptides translated from them may induce immune responses, in addition to those against transgene products such as α-L-iduronidase[6] or selectable markers (eg, neomycin,[15] hygromycin and/or herpes virus thymidine kinase[16,17]). However, it still needs to be determined whether or not the products encoded by the retroviral vector backbone sequences are immunogenic in humans.

The LZRSpBMN,[18] Molony MLV-derived retroviral vector used in this study, contains full-length long terminal repeats (LTRs) and an extended packaging sequence with a portion of the *pol* gene derived from MFG retroviral vectors,[19] which have been used in a number of clinical trials, including a French trial for SCID-X1.[1] The extended packaging sequence is made by a combination of the original packaging signal and 426 bp of the 5′ end of the *gag* gene, followed by 375 bp of the 3′ end of the *pol* gene which harbors the splicing acceptor sites.[14] In wild-type MLV, Gag and Pol proteins are translated from the unspliced mRNA. The MFG vector still expresses a significant amount of the unspliced messages.[20] There are many potential start

Correspondence: Dr Y Akatsuka, Division of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan
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codons within the extended packaging sequence, such as CTG of gPr80^{gag} and ATG of Pr65^{gag}. Moreover, some (cryptic) polypeptides may be translated from genomic (unspliced) mRNA. Thus, it is possible that these products may give rise to host immune responses which could lead to rapid clearance of retrovirally transduced cells after *in vivo* infusion.

In this study, we demonstrated that human cytotoxic T-lymphocyte (CTL) clones recognizing epitopes derived from the protein encoded by the retroviral vector backbone. Two identified CTL epitopes were located in the *gag* gene, and the other epitope was in the artificial polypeptide joining the *gag* and *pol* sequences.

Results

In a previous study, we achieved efficient generation of cytomegalovirus (CMV)-pp65-specific CTL lines from CMV-seropositive donors using CD40-activated B (CD40-B) cells retrovirally transduced with the CMV-pp65 gene as antigen-presenting cells (APC).[21,22] With all of eight CMV-seronegative donors, however, we failed to generate pp65-specific CTL lines. Thus, to enhance the efficiency of induction of pp65-specific CTLs, we cultured CD8 T cells in the presence of interleukin-12, which is known to be an immunostimulatory cytokine. In fact, CTL lines were then induced in seven out of the eight seronegative donors. Unexpectedly, however, four of them showed cytotoxicity against not only pp65-transduced EBV-transformed lymphoblastic cell lines (LCLs) but also LCLs transduced with an irrelevant antigen, EGFP, whereas untransduced LCLs were not lysed (Figure 1a). These results suggest that the CTL lines were specific for viral-related antigens generated in the retrovirally transduced cells. These interesting findings prompted us to identify the antigens recognized. By limiting dilution, we established two CTL clones, 8C7 and 9C10, from a CMV-seronegative donor and their specificity was evaluated. IFN-γ ELISPOT assays revealed that clones 8C7 and 9C10 recognized 293T cells transfected with the retroviral vector, LZRSpBMN, in combination with HLA-B*4403 and -B*4601, respectively (Figure 1b), indicating that the antigens recognized by the CTL clones were indeed derived from the retroviral vector.

Because, in the retroviral vector we used, 5'LTR is active as an RNA polymerase II promoter, and the start codons of gPr80^{gag} and Pr65^{gag} in the extended packaging signal are not mutated, it is possible that several different polypeptides may be translated from the genomic mRNA.[20] Thus, we subcloned the region spanning the start codon of gPr80^{gag} through a unique *Bam*HI site of the vector into a mammalian expression vector, termed pcDNA3-gPr80, to determine which polypeptides translated from the extended packaging signal are antigenic. Both CTL clones could recognize 293T cells transfected with both pcDNA3-gPr80 and restricting HLA alleles, indicating that the region contains the CTL epitopes.

For their identification, linear expression fragments encoding serial C-terminal truncations of the region were constructed and expressed in 293T cells (Table 1), as previously reported.[22] The HLA-B*4403-restricted CTL

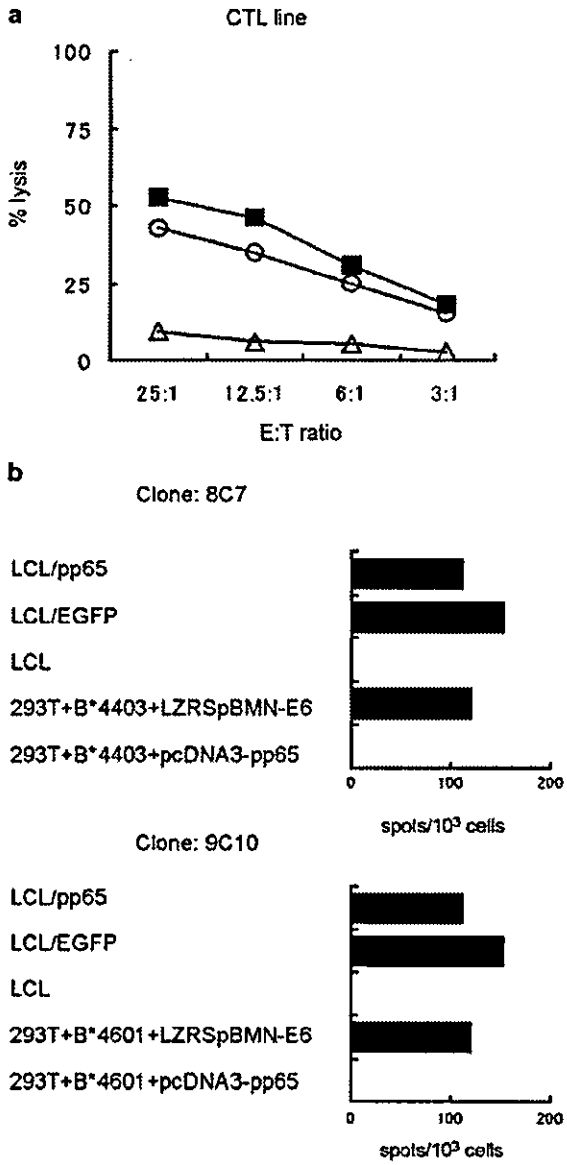

Figure 1 Effector cell activity of the CTL line and clones generated from a CMV-seronegative donor. To generate CTL lines, CD8$^+$ T cells from a CMV-seronegative donor were stimulated three times with autologous CD40-B cells transduced with CMV-pp65 gene retrovirally, and tested. (a) Cytolytic activity of the CTL lines was assessed against autologous LCL retrovirally transduced with the CMV-pp65 (LCL/pp65; closed squares) or EGFP genes (LCL/EGFP; open circles), or untransduced LCL (open triangles) over a range of E/T ratios. Cytolytic activity assessed by ^{51}Cr release is shown as percentage specific lysis. (b) By limiting dilution, two CTL clones (8C7, 9C10) were obtained from the CTL line shown in (a). To determine HLA restriction and specificity of the CTL clones, IFN-γ ELISPOT assays were conducted using autologous LCL/pp65, LCL/EGFP, LCL or 293T cells transfected with the genes indicated. Each bar represents the number of spots per 10³ cells.

clone 8C7 recognized transfectants expressing aa 1–169 or longer, but not 1–138, indicating that the epitope fully or partially lies between aa 139 and aa 169. With the aid of a computer algorithm,[23] peptides presented by HLA-B*4403 within the localized region were predicted (Table 2). Because a fragment encoding a decamer peptide,

AEWPTFNVGW (aa 141–150), was well recognized by the CTL clone 8C7, we defined this decamer as the CTL epitope presented by HLA-B*4403 (Figure 2a).

In the case of CTL clone 9C10, the same region was recognized (Table 1). The binding motif for HLA-B*4601 has been reported as Met at position 2 and Tyr or Phe at the C-terminus,[24] but peptide binding prediction by the computer algorithm for HLA-B*4601 is not currently available. Because there were four Phe, but no Met and Tyr within the region, we generated linear expression fragments encoding four kinds of decamer peptides ending at each Phe. A fragment encoding TFCSAEWPTF (aa 137–146) was well recognized by the CTL clone 9C10. As shown in Figure 2b, C-terminal deletion affected the recognition by the CTL clone, whereas N-terminal deletions did not. By titration assay with synthetic peptides (data not shown), a nonamer peptide, FCSAEWPTF (aa 138–146), was identified as the minimal epitope presented by HLA-B*4601.

Similarly, CTL clones specific for retrovirally transduced cells were obtained by stimulation of CD8 T cells from a cervical cancer patient using HPV16-E6 and E7 transduced CD40-B cells as APC. One of the CTL clones, 3B4, was restricted by HLA-B*3501 and recognized 293T cells transfected with pcDNA3-gPr80 and HLA-B*3501 (Figure 3). With the linear expression fragments of serial C-terminal truncations, a region containing the CTL epitope was localized within aa 237–331 (Table 1). Several HLA-B*3501-restricting peptides within this region were predicted by the computer algorithm (Table 2). Among them, the CTL clone 3B4 well recognized a decamer, APIWPYEILY (aa 240–249, Figure 3b), which interestingly was composed of the artificial junctional region of *gag* and *pol* (Figure 4).[18]

Table 1 Identification of the regions recognized by the CTL clones using deletion mutants in ELISPOT assays

	CTL clone	*8C7*	*9C10*	*3B4*
Length of fragments	*HLA restricted*	*B*4403*	*B*4601*	*B*3501*
1–66		–	–	–
1–138		–	–	–
1–169		+	+	–
1–191		+	+	–
1–248		+	+	–
1–331		+	+	+
1–370		+	+	+

'+' indicates the presence of specific spots by each CTL clone, and '–' indicates the absence of spots.

Discussion

In the present study, we demonstrated that human CTLs specific for HLA class I-bound peptides encoded by retroviral vector backbone sequences can readily be induced *in vitro*. To the best of our knowledge, this is the first identification of epitopes derived from residual retroviral coding sequences rather than inserted cDNAs recognized by human cells. In mice, it has been already shown that Moloney MLV- or other retrovirus-induced tumor cell lines, such as RMA and FBL-3, are lysed by CTL responses, and that the immunodominant epitope of Moloney MLV restricted by H-2D^b is located within the gag leader protein encoded in the extended packaging sequence of the retroviral vector.[25] With regard to humoral immune responses, induction of anti-MLV antibodies has been reported in mice and nonhuman primates[5] as well as in patients treated for brain tumors with murine retroviral vector producer cells.[26] Thus, it is likely that the immune responses against not only inserted gene products but also retroviral coding sequence products can similarly be induced *in vivo*, with vector-based treatment of patients.

The immunogenicity of transferred gene products also remains one obstacle in the development of efficient clinical gene therapy protocols, particularly when introducing artificial or xenogenic sequences. Various attempts have been made to overcome this problem, including asanguineous perfusion after infection of retroviral vectors in liver transplant cases,[4] transplantation of transduced hematopoietic stem cells[7,27,28] or infusion of antigen-specific regulatory T cells,[29] with the aim of establishing immune tolerance to transgene products. In the French clinical trial for the X-linked form of SCID,[1] which is the first recorded case of a therapeutic effect of retroviral gene therapy in humans, the transferred gene is expressed in the patients long term. Immune responses against the transferred common γ-chain gene or MFG-based retroviral vector coding sequences have not been reported so far, which may be due to relative immunodeficiency in these X-SCID patients.

Table 2 Results for HLA-binding motif prediction by computer algorithm

CTL clone	*HLA restricted*	*Range submitted*	*Rank*	*Position*	*Sequence*	*Score*
8C7	B*4403	129–169	1	141–150	AEWPTFNVGW	48
			2	161–169	ITQVKIKVF	2.3
			3	148–156	VGWPRDGTF	2
			4	141–149	AEWPTFNVG	1.6
			5	138–146	FCSAEWPTF	1
3B4	B*3501	237–331	1	240–249	APIWPYEILY	40
			2	240–248	APIWPYEIL	20
			3	305–314	HPYRVGDTVW	10
			4	300–309	RPVVPHPYRV	8
			5	268–276	NSPSLQAHL	5

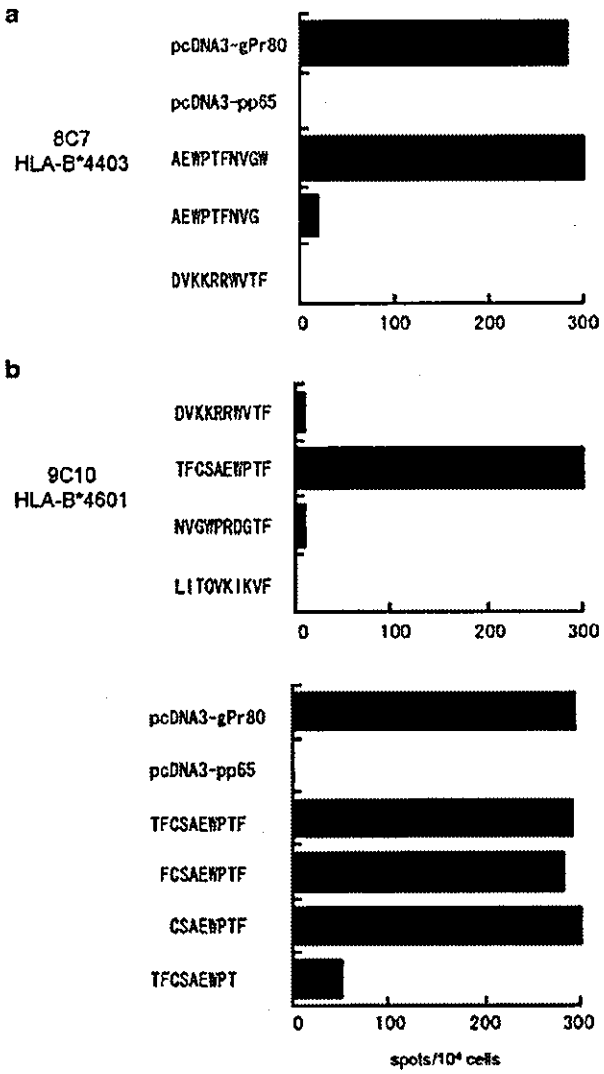

Figure 2 Identification of the CTL epitopes recognized by the CTL clones 8C7 and 9C10. Linear expression fragments encoding various peptides, including the predicted epitopes by computer algorithms, were transfected to 293T cells, together with restricting HLA cDNA. Recognition by the pp65-specific CTL clones was evaluated 48 h later by ELISPOT assay. Each bar represents the number of spots per 10^4 cells. (a) Recognition of the linear expression fragment encoding the predicted peptide, AEWPTFNVGW, or other peptides by the CTL clone 8C7. (b) Recognition of the linear expression fragments encoding the candidate peptides by the CTL clone 9C10 is shown in the upper columns. Effects of N- or C-terminal deletion are shown in the lower panel.

Retention of retroviral sequences may also result in generation of replication-competent retroviruses as a result of homologous recombination.[11] Substitution of the packaging sequence of MLV-based vectors by sequences derived from other viruses has been successfully employed without loss of the transduction efficiency.[30] Indeed, attempts have been made to remove all ATG start codons without losing efficient viral packaging.[12,14] These approaches may be able to provide more effective ways to avoid immunogenicity of retroviral vectors and also generation of replication-competent retroviruses. Finally, the present identification of an

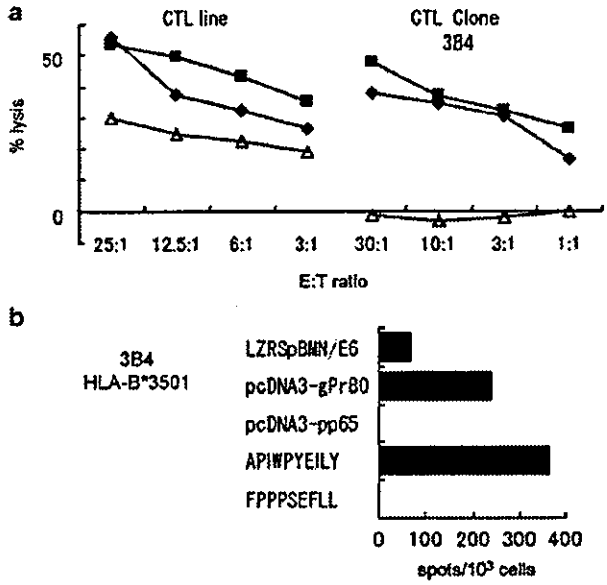

*Figure 3 Effector cell activity of the CTL line and clones generated from a cervical cancer patient. A CTL line was generated by stimulating $CD8^+$ T cells from a cervical cancer patient three times with autologous HPV16-E6 and -E7 transduced CD40-B cells. By limiting dilution, one CTL clone 3B4 was then obtained. (a) Cytolytic activity of the CTL line (left) and the CTL clone (right) was assessed against autologous LCL/E6 (closed squares), LCL/E7 (closed diamonds), or untransduced LCL (open triangles) over a range of E/T ratios. (b) Determination of the epitope for the CTL clone 3B4. ELISPOT assays were conducted using 293T cells transfected with plasmids encoding the indicated gene or with linear expression fragments encoding the indicated peptides, together with HLA-B*3501 cDNA. Each bar represents the number of spots per 10^3 cells.*

immunogenic HLA-B35-restricted peptide derived from the *gag–pol* junctional region may be of clinical significance because vectors encoding various chimeric molecules are currently often used.

In conclusion, peptides encoded by the retroviral vector backbone sequence show potent immunogenicity *in vitro* so as to induce CTL responses. Thus, it is likely that they may also stimulate CTL responses *in vivo*, leading to rapid clearance of retrovirally transduced cells and resulting in adverse effects. We should take into consideration this problem by actively monitoring immune responses in retroviral vector-mediated gene therapies.

Materials and methods

Donors and cells

Peripheral blood samples were obtained from CMV-seronegative healthy donors or cervical cancer patients after we obtained informed consent under a protocol approved by the Institutional Review Board of Aichi Cancer Center according to the Declaration of Helsinki. HLA typing was carried out at The HLA Laboratory (Kyoto, Japan). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by centrifugation on a Ficoll (Amersham Biosciences, Uppsala, Sweden) density gradient, and CD8-positive and -negative fractions were separated using CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cryopreserved until use.

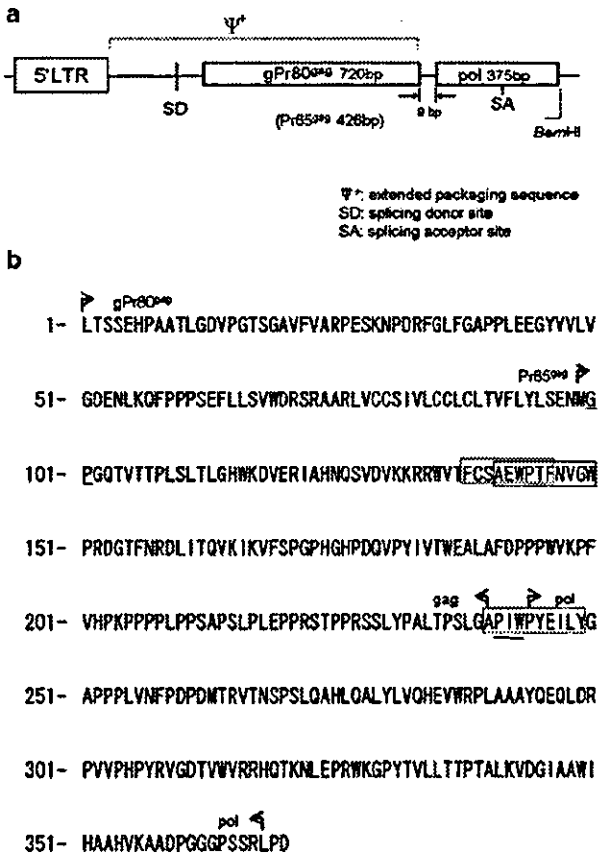

Figure 4 Leader sequence of the retroviral vector, LZRSpBMN. (a) Schematic representation of the leader sequence in LZRSpBMN. This region is identical with that of the MFG vector. (b) The amino-acid sequence translated from the gag–pol fusion region starting at gPr80gag start codon (CTG). The start codon of Pr65gag is Met99 and the junctional region of gag and pol is underlined. The CTL epitopes identified in this study are boxed (see Figures 2 and 3).

Plasmids and synthetic peptides

Plasmids, pcDNA3-pp65, pcDNA3-EGFP, pcDNA3.1. (Invitrogen, Tokyo, Japan) encoding HLA-class I cDNA, LZRSpBMN-pp65 (the backbone plasmid, LZRSpBMN-Z was kindly provided by Dr G Nolan, Stanford University, Stanford, CA, USA), pLBPC-pp65 and pLBPC-EGFP were constructed as previously described.[21,31] All peptides were purchased from Toray Research Center (Tokyo, Japan). HPV16-E6 or -E7 gene (kindly provided by Dr T Kiyono, National Cancer Center Research Institute, Tokyo, Japan) was inserted into the LZRSpBMN vector (LZRSpBMN-E6 or LZRSpBMN-E7, respectively) and the pLBPC vector (pLBPC-E6 or pLBPC-E7, respectively). To generate pcDNA3-gPr80, the region spanning the start codon of gPr80gag through a unique *Bam*HI site of the LZRSpBMN-Z vector (Figure 4a) was inserted into multiple cloning site of the pcDNA3.1 vector.

Generation of CD40-activated B cells and LCLs

CD40-B cells were generated as previously described.[21,32,33] In brief, a thawed CD8-negative fraction of PBMCs was cultured on a γ-irradiated (96 Gy) human CD40L-transfected NIH3T3 cell line[34] (t-CD40L; kindly provided by Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA, USA) in the presence of IL-4 (4 ng/ml; Ono Pharmaceutical, Osaka, Japan) and cyclosporin A (CsA, 0.7 μg/ml; Sandoz, Basel, Switzerland) in 2 ml of Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% pooled human serum. The expanding cells were transferred onto freshly prepared t-CD40L cells and fed with cytokine-replenished medium without CsA every 3–4 days. LCLs were established from the CD40-B cells with supernatant of an EBV producing cell line (B95-8, ATCC, Manassas, VA, USA) in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (FCS; IBL, Takasaki, Japan), referred to as RPMI-10.

Retroviral transduction of CD40-B cells and LCLs

Retroviral transduction was conducted as previously described.[21] In brief, the retroviral construct, for example, LZRSpBMN-pp65, was packaged in the Phoenix GALV cell line[35] (a gift from H-P Kiem, Fred Hutchinson Cancer Research Center, and from G Nolan, Stanford University, Stanford, CA, USA) using FuGENE 6 (Roche Diagnostics, Mannheim, Germany). CD40-B cells and LCLs were infected with the retroviral supernatant in the presence of 10 μg/ml polybrene (Sigma, Chicago, IL, USA), spun at 1000 *g* for 1 h at 32°C, and incubated. Two days after, LCLs transduced with CMV-pp65 (LCL/pp65), HPV16-E6 (LCL/E6), HPV16-E7 (LCL/E7), EGFP (LCL/EGFP) were selected in the presence of puromycin (0.7 μg/ml; Edge Biosystems, Gaithersburg, MD, USA). Transduction efficiency were assessed as previously described.[21]

Generation of antigen-specific CTL lines using retrovirally transduced CD40-B cells

Thawed CD8-positive cells (1×10^6) were cocultured with γ-irradiated (33 Gy) autologous retrovirus-transduced CD40-B (CD40-B/pp65) cells (1×10^6) in 2 ml RPMI 1640 supplemented with 10% pooled human serum, recombinant human IL-7 (50 U/ml; Genzyme, Cambridge, MA, USA) and IL-12 (5 ng/ml; R&D systems, Minneapolis, MN, USA) at 37°C in 5% CO_2. On days 7 and 14, $CD8^+$ cells were restimulated, and 1 day after each stimulation, recombinant human IL-2 (Chiron, Emeryville, CA, USA) was added to the cultures at the final concentration of 20 U/ml. If necessary, rapidly growing cells were split into 2–3 wells and fed with fresh media containing 20 U/ml IL-2.

Epitope selection and construction of linear expression fragments

Linear expression fragments encoding various C-terminus truncated *gag–pol* gene of LZRSpBMN (Table 1) or various peptides (Table 2, Figure 2) were generated using an overlapping PCR method.[22] Targeted region-specific 5′ and 3′ primers incorporating additional sequences (single- and double-underlined, see below) were designed, for example, 5′primer, TCGGATCCACCATGCTGA CGAGTTCGGAAC (30 bp) and 3′primer, GACTCGAGC GCTATAAGATCTCATATGGCC (30 bp) for the fragment encoding aa 1–248, and used for PCR (KOD Plus; Toyobo, Osaka, Japan) with a template retroviral vector, LZRSpBMN. The CMV promoter (P_{CMV}) and BGH

polyadenylation signal (pA) were independently amplified from pcDNA3.1 by PCR using the following primers: 5′ P_{CMV}, CTTAGGGTTAGGCGTTTTGC; 3′ P_{CMV}, NNCATGGTGGATCCGAGCTCGGTA; 5′ pA, NNTAGCGCTCGAGTCTAGAGGG; 3′ pA, GGTTCTTTCCGCCTCAGAAG; 'N' means a mixture of A/C/T/G). The 3′ P_{CMV} and 5′ pA primers contained overlapping sequences (underlined) with the 5′primer and 3′primer, respectively, of the targeted region. The three PCR products, P_{CMV}, the targeted region, and pA, were conjugated by second PCR using primers, 5′ P_{CMV} and 3′ pA. Each linear expression fragment was termed '1-XXX' (C-terminus truncated *gag–pol* gene; 'XXX' indicates the amino-acid positions of the C-terminus) or peptide sequence.

ELISPOT assays

ELISPOT assays were performed as described earlier.[21,22] In brief, a MultiScreen-HA plate (MAHA S4510, Millipore, Bedford, MA, USA) was coated with anti-human IFN-γ mAb (M700A; Endogen, Woburn, MA, USA) and used as an ELISPOT plate. 293T cells were cotransfected with plasmids encoding each of the individual donor's HLA-class I alleles and either antigen encoding plasmids or PCR products of the linear expression fragment by TransIT-293 (Mirus, Madison, WI, USA), and used as stimulator cells after 2 days. The transfected 293T cells, LCLs or retrovirally transduced LCLs were mixed with 10^3 or more effector cells from the CTL lines or clones generated. After cells had been incubated in 200 µl RPMI-10 in a round-bottom 96-well plate (Costar Corning, Cambridge, MA, USA) for 4 h, all the aliquots were transferred into an ELISPOT plate and incubated for an additional 16 h. To visualize spots, a biotin-labeled anti-human IFN-γ antibody (M701B; 1 µg/ml, Endogen), streptavidin–alkaline phosphatase (Biosource International, Camarillo, CA, USA), and substrate were used. Spots were counted after computerized visualization using a scanner (Canon, Tokyo, Japan).

Chromium release assays

LCLs or retrovirally transduced LCLs were labeled in 100 µl RPMI-10 with 3.7 MBq ^{51}Cr for 1 h at 37°C. After 4 h incubation with effector cells, supernatants were counted in a gamma counter. The percentage of specific lysis was calculated as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

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