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Conservation of Epstein-Barr Virus Cytotoxic T-Cell Epitopes in Posttransplant Lymphomas

Implications for Immune Therapy

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Posttransplant lymphoproliferative disease can be treated by the infusion of Epstein-Barr virus-specific cytotoxic T lymphocytes, which were raised against lymphocytes immortalized with a laboratory strain of Epstein-Barr virus (B95.8). Whether the immunodominant epitopes in B95.8 are shared in virus from tumors will affect the general applicability of this therapy. We have characterized the viral strain and the sequence of commonly recognized cytotoxic T-lymphocyte epitopes in 25 posttransplant lymphoproliferative disease specimens from 19 patients. Type A virus was present in 24 of 25 specimens. No variation in two LMP2A epitopes and a few variations mostly outside the targeted epitopes or silent in three EBNA3C epitopes were found, with one variation (Arg to Lys) detected in an EBNA3C epitope in 12 of 24 tumors. However, cytotoxic T lymphocytes to B95-8-derived EBNA3C peptides specifically lysed both B95-8 and the Lys-variant peptide-loaded target cells, although with less efficiency. These results suggest that adoptive immunotherapy using cytotoxic T lymphocytes expanded with B95.8 stimulators or vaccine strategies using B95.8-derived sequence will generally target Epstein-Barr virus strains present in posttransplant lymphoproliferative disease tumors. ***(Am J Pathol 2002, 160:1839–1845)***

Posttransplant lymphoproliferative disorders (PTLDs) are clinically and histologically heterogeneous lymphoid proliferations occurring in organ or bone marrow transplant recipients.[1–3] Generally these are B-cell proliferations and are associated with Epstein-Barr virus (EBV).[4,5] Particular kinds of immune manipulations aimed at preventing or controlling organ rejection or graft-*versus*-host disease such as the use of anti-CD3 antibodies or high doses of cyclosporine are associated with increased risk of PTLDs.[6,7] Withdrawal or reduction of immunosuppressive therapy is sometimes associated with regression of these tumors.[8] In bone marrow transplant recipients, infusion of lymphocytes from the patient's bone marrow donor often leads to disease regression but may be associated with graft-*versus*-host disease.[9] EBV-specific cytotoxic T lymphocytes expanded *in vitro* in response to irradiated EBV-transformed B lymphocytes have also been reported to induce regression of PTLDs in some cases and to prevent the development of PTLDs in the bone marrow transplant setting without graft-*versus*-host disease.[10,11] The virus used to transform stimulator cells in these studies was a laboratory strain of EBV referred to as B95.8.[12] An important question with regard to the general application of this approach is how closely B95.8 corresponds with viral genomes present in PTLD lesions with regard to epitopes recognized by EBV cytotoxic T lymphocytes (CTLs).

Over a wide spectrum of HLA alleles, the immunodominant EBV latency antigens are present in a few proteins: EBNA3A, EBNA3B, and EBNA3C.[13,14] Sequence variations among these immunodominant antigens are well recognized. Two EBV subtypes, A and B, that differ mainly in the coding sequences for EBNA2, EBNA3A, EBNA3B, and EBNA3C have been characterized.[15–17] These differences are such that CTLs have been identified that recognize type A virus but not type B virus and *vice versa*.[18,19] A subdominant response to LMP2 has been consistently detected among individuals who are HLA A0201.[20] Strain differences have also been recognized in LMP2 and these occur independently of subtypes A and B.[21]

To determine whether variations in the sequence of viral proteins are likely to limit the applicability of CTLs raised in response to B95.8-infected lymphocytes as anti-tumor CTLs, we analyzed a series of PTLD lesions and determined the impact of a common variant on CTL killing.

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Table 1. PTLD Cases Cited in Other Reports

This report and Tao et al.[24]	Murray et al.[22]	Tao et al.[23]
2	20	
4	11	
6	10	
7	6	
8	18	
10	7	
12	1	
15	9	
16	19	PTLD2
17	12	
19	5	
20		PTLD1

Materials and Methods

PTLD Tumor Samples

DNA samples from 25 PTLD specimens from 19 organ transplant patients were obtained from the Johns Hopkins Hospital Lymphoma Bank and the Southwest Oncology Group study tumor bank. More than one tumor specimen was available for patients 3, 4, 7, and 9. Some of these cases have been reported previously (Table 1).[22–24] We also studied a tumor specimen from a patient with B-cell lymphoproliferative disease arising in the setting of aplastic anemia treated with immunosuppression (patient 13).[25]

Genotyping for EBV Subtypes

Major EBV subtypes (A and B) were distinguished by polymerase chain reaction (PCR) amplification using primers spanning the EBNA3C region.[17] The B95.8 cell line was used as a positive control for type A, whereas AG876 was used as a positive control for type B virus. The B-cell lymphoma line BJAB was used as a negative control.

Sequence Analysis of EBV CTL Epitopes

For sequence analyses of EBV CTL epitopes, PCR primers were designed to amplify a 195-bp region of EBNA3C that includes three CTL epitopes (HLA A2.1, B44, and B27-restricted)[26,27] and a 148-bp region of LMP2A containing HLA A2.1- and A24.2-restricted epitopes.[28] The primers were: EBNA3C, 5′-TACGCTTCCTTCGTGGTAAA, 5′-CGATTGTCTTGTGAAACCAG; LMP2A, 5′-CTTGCTAT CCTGACCGAATG, 5′-CTGCTGTAAGAATCCAGGCA. The High Fidelity *Taq* enzyme Platinum Pfx DNA polymerase system (Life Technologies, Inc., Gaithersburg, MD) was used to generate the desired PCR products. PCR products were electrophoresed on a 1.8% agarose gel, excised, purified using the Qiaex II Gel Extraction kit (Qiagen, Chatsworth, CA), and sequenced directly with the SequiTherm Excel DNA PCR sequencing kit (Epicentre Technologies, Madison, WI), or the ABI Prism BigDye Terminator Cycle Sequencing kit and the PE ABI 377 sequencer.

For cases with multiple tumors, sequence analyses of EBV CTL epitopes were also performed by cloning sequencing. Briefly, PCR products were electrophoresed and purified by using Spin-X tubes (Costar, Corning, NY), and then cloned into the pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA). Approximately 10 bacterium colonies were analyzed for each DNA sample. Plasmid DNA was extracted and sequenced by using the ABI 377 sequencer.

Peripheral Blood Mononuclear Cells (PBMCs) and Synthetic Peptides

PBMCs were from HLA-B27 EBV-seropositive healthy platelet donors. PBMCs were isolated by density gradient centrifugation using Ficoll-Hypaque 1.077 (Biochrom, Berlin, Germany) and cryopreserved immediately.

Peptides corresponding to viral epitopes recognized by CTLs were synthesized by Macromolecular Resources (Ft. Collins, CO). RRIYDLIEL (referred to as Arg peptide) and its variant RKIYDLIEL (Lys peptide) corresponding to EBNA3C residues 258 to 266.[26] The peptide CLGGLLTMV corresponding to LMP2 residues 426 to 434 and restricted through HLA-A2 was used as a control.[28] Peptides were dissolved in 10% dimethyl sulfoxide (Sigma, St. Louis, MO) to a final concentration of 1 mg/ml, and stored in aliquots at −80°C. This peptide stock solution was further diluted in appropriate assay media for individual experiments.

Establishment of Peptide-Specific CTL Lines

PBMCs were incubated at 10^7/ml in 50 μg/ml of peptide (diluted in RPMI 1640) for 2 hours at 37°C. The peptide-loaded PBMCs were irradiated (2500 rads), washed once, and resuspended in lymphocyte expansion medium, LyEM [45% RPMI 1640, 45% Click's media, 2 mmol/L glutamine, 10 mmol/L HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% v/v fetal bovine serum (FBS) at 0.5×10^6/ml]. One ml was added to each well of a 24-well plate. PBMCs were resuspended in LyEM at 2×10^6/ml and 1 ml added to each well to achieve a responder: stimulator ratio of 4:1. The CTL lines were restimulated on day 7 with irradiated peptide-loaded PBMCs at the same responder:stimulator ratio. Peptide-specific CTL lines were harvested on day 14 and tested in chromium-release assay.

Chromium-Release Assay

Phytohemaglutinin (PHA) blasts for chromium-release assays were generated by culturing PBMCs at a concentration of 2×10^6/ml in the presence of 5 μg/ml of PHA (Sigma) in RPMI-FBS (RPMI 1640, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% v/v FBS) for 3 days. PHA blasts were washed four times and then cultured for at least 4 more days in RPMI-FBS containing 100 U/ml interleukin-2 before used in chromium-release assays. Autologous PHA blasts were incubated with $^{51}CrO_4$ for 90 minutes then

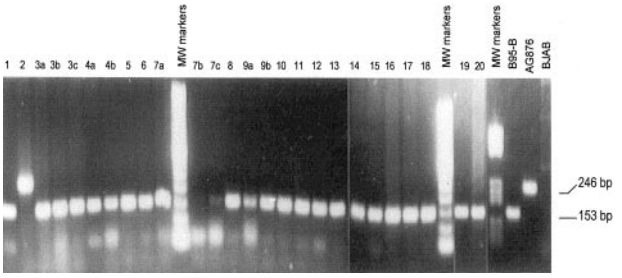

Figure 1. PCR typing for EBV subtypes. A 153-bp band for type A EBV and a 246-bp band for type B virus are shown. Only one PTLD tumor (case 2) is associated with type B EBV infection.

washed four times. Labeled cells (1×10^5/ml, 50 μl/well) were added to 96 V-bottom plates containing 50 μl/well of medium with or without 40 μg/ml of peptide and incubated for 2 hours at 37°C. Peptide-specific CTLs were added to each well (1.5×10^6/ml, 100 μl/well; E:T ratio, 30:1) for the subsequent 5-hour incubation. The concentration of peptides in the final assay volume (200 μl) was 10 μg/ml. Medium for CTL assays was RPMI-FBS. Thirty μl of culture supernatant was harvested and measured for $^{51}CrO_4$ level by using a γ counter (TopCount NXT; Packard Instrument Company, Meriden, CT).

Results

EBV Subtypes in PTLD

Among 25 PTLD tumor specimens from 19 patients, PCR typing of the EBNA3C open reading frame (ORF) showed that 24 specimens from 18 patients harbored type A virus as indicated by the presence of a 153-bp amplification product, whereas only one case (case 2) harbored type B virus as indicated by the presence of a 246-bp product (Figure 1). In the four patients with tumor specimens obtained at different times, the specimens consistently showed the same viral type.

Sequence Conservation of EBV CTL Epitopes

We then performed targeted sequencing to determine whether there was variation in commonly targeted CTL epitopes. Two regions were targeted: part of EBNA3C containing three CTL epitopes (A2.1-, B44-, and B27-restricted) and a part of LMP-2 containing two epitopes (A2.1- and A24.2-restricted). These regions were amplified and directly sequenced in 24 PTLD specimens from 18 patients including one patient with type B EBV. Two hot spots of variation were identified in EBNA3C in patients with type A virus. A missense G to A mutation (RRIYDLIEL to RKIYDLIEL) in the HLA B27-restricted epitope (residues 258 to 266) was detected in 12 specimens (Figures 2 and 3A). A silent C to T mutation in a region of overlapping HLA B44- and A2.1-restricted epitope was present in 14 specimens. A missense T to A mutation (Leu to Met) occurring outside any recognized CTL epitopes was detected in the two type A and the type B specimens. Four other variations that are type B-specific were also recognized, including two A to T changes (Tyr to Phe) with one in the HLA B27-restricted epitope, and two other mutations occurring outside recognized CTL epitopes (A to G, C to G) were also detected (Figures 2 and 3A).

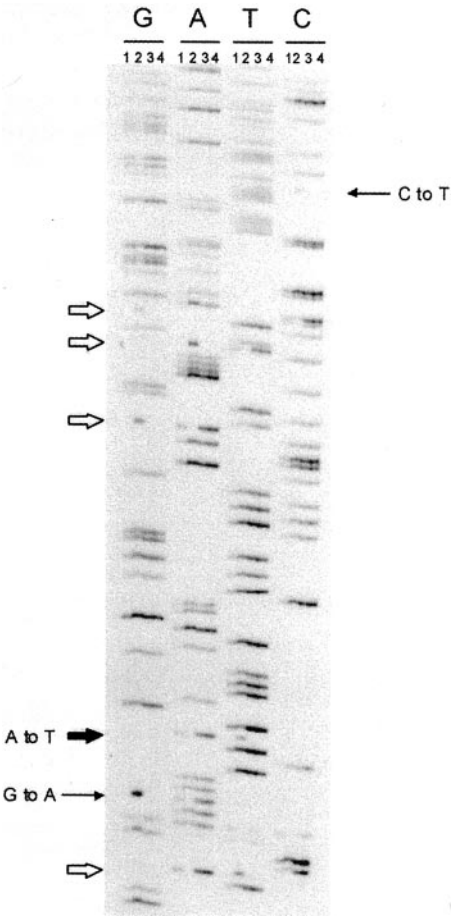

Figure 2. Sequencing for EBNA3C CTL epitopes. **Lane 1**, case 8; **lane 2**, case 2, type B EBV; **lane 3**, case 3a; **lane 4**, case 3b. The variations between type A and B EBV are shown by **thick arrows** including one missense mutation (A to T) within a CTL epitope (**filled thick arrow**). Two mutations (one silent C to T, one missense G to A) among type A EBV isolates are shown by **thin arrows**.

Sequencing of the LMP2A region showed one missense G to C mutation (Cys to Ser) and a silent mutation (T to C) detected in the B-cell lymphoproliferative disease specimen from the patient with aplastic anemia. This silent mutation was also detected in two PTLD tumors from a single patient (case 4). Two missense mutations (both G to A) occurring outside CTL epitopes were also detected in four other PTLD specimens, including three tumors from a single patient (case 7) (Figure 3B).

By direct PCR sequencing, the four patients (cases 3, 4, 7, and 9) with tumor specimens obtained at different times showed no intrapatient sequence variation in either the EBNA3C or LMP2A regions sequenced. Because direct sequencing might miss some small percentage of variants, we also performed sequenced cloned PCR products to check whether there was any evidence of intrapatient variation throughout time. Sequencing of the cloned products confirmed the results of direct sequenc-

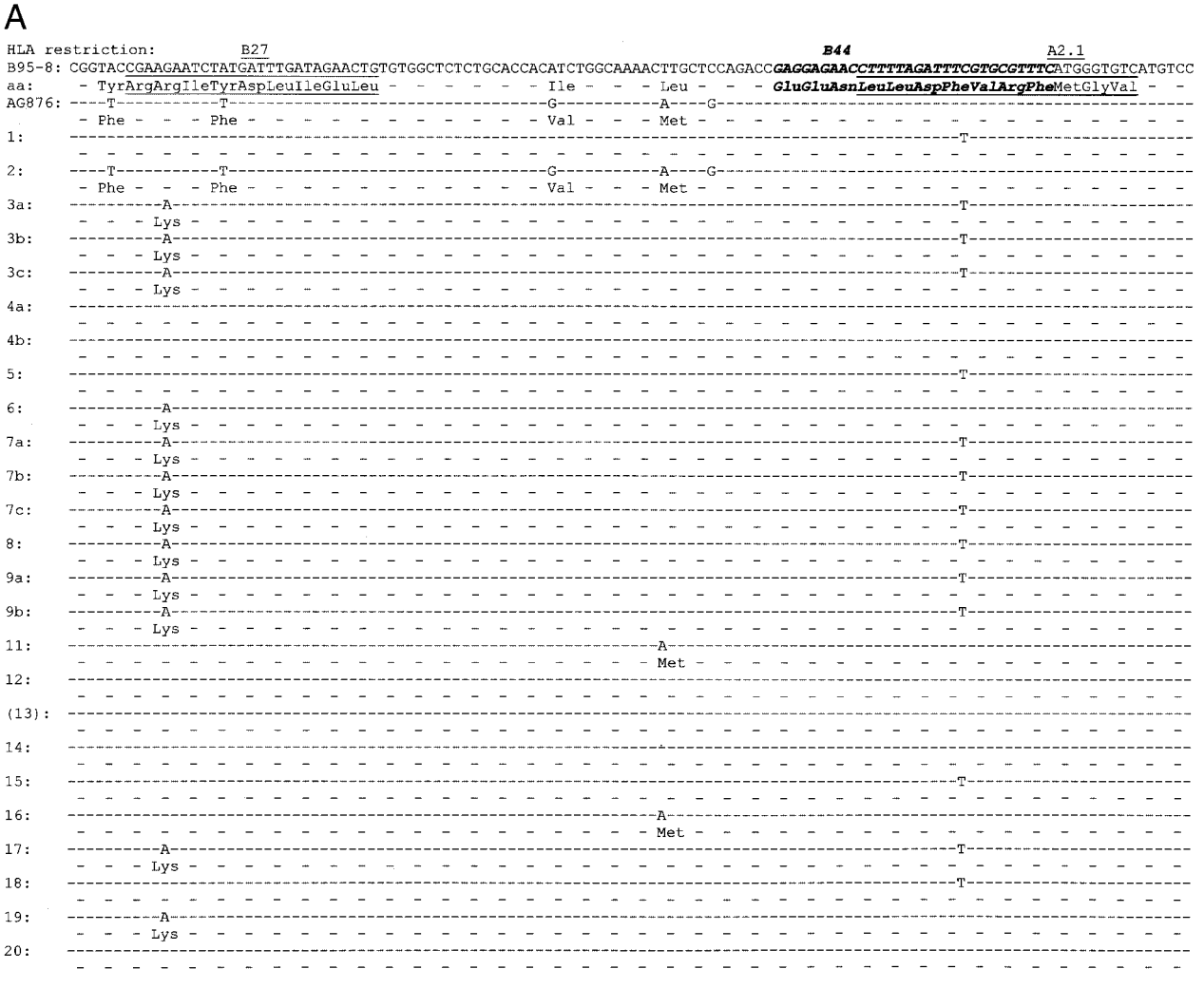

Figure 3. Direct PCR sequencing analyses of EBV CTL epitopes in PTLD. **A:** The HLA A2.1-restricted EBNA3C epitope (**underlined**) is overlapped with the HLA B44 epitope (**italics**). **B:** The HLA A2.1-restricted LMP2A epitope (**underlined**) is overlapped with the HLA A24.2 epitope (**italics**). Tumor samples 3a, 3b, 3c, 4a, 4b, 7a, 7b, 7c, 9a, or 9b are from a single patient at different times.

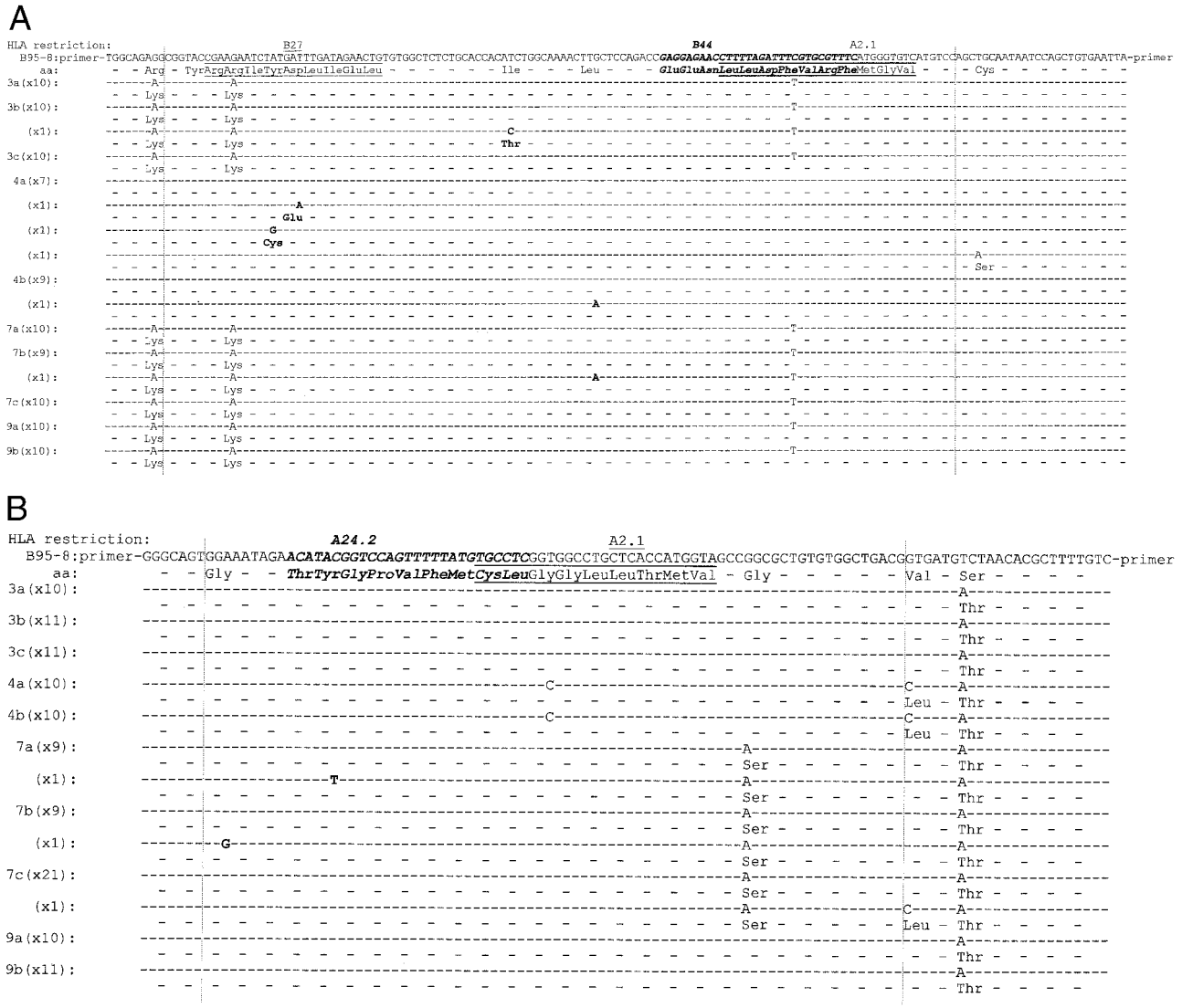

Figure 4. Cloning sequencing for EBV CTL epitopes in PTLD patients (cases 3, 4, 7, and 9) with multiple tumors at different times. **A:** The EBNA3C epitopes. **B:** The LMP2A epitope. The **bolded** variations were only detected by cloning sequencing, but the possibility that these changes represent PCR artifacts cannot be excluded.

ing, but identified additional variations in 1 of 10 or 2 of 10 plasmid clones (Figure 4). These variations are likely to represent artifacts of *Taq* amplification. In any case, only the two variations detected in case 4a would affect the epitope sequence.

CTL Recognition to the EBNA3C Epitope Variant

The missense mutation in EBNA3C resulting in an Arg to Lys substitution has been reported in 40% of virus isolates from healthy Australian Caucasians, but not in any isolates from Africa, Southeast Asia, or Papua New Guinea.[29] To determine whether the presence of this variant would affect CTL-mediated killing, we stimulated PBMCs from a healthy HLA-B27 EBV-seropositive donor with peptide corresponding to the B27 epitope. CTLs were harvested on day 14 and lytic specificity assessed by chromium-release assay. CTLs generated in response to stimulation with the Arg peptide lysed Arg peptide loaded PHA blasts and variant Lys peptide loaded blasts, although somewhat less efficiently (Figure 5). The killing was peptide-specific as neither PHA blasts alone nor PHA blasts loaded with an irrelevant control peptide were killed. In the complementary experiment, CTLs generated by stimulation with the variant Lys peptide were not able to kill any of the targets (Figure 5).

Discussion

The investigations reported here show that type A virus predominates in PTLD lesions and that the general specificity of T cells expanded in response to B95.8 cells is appropriate for the great majority of these tumors. Early studies had suggested that type B virus might be much

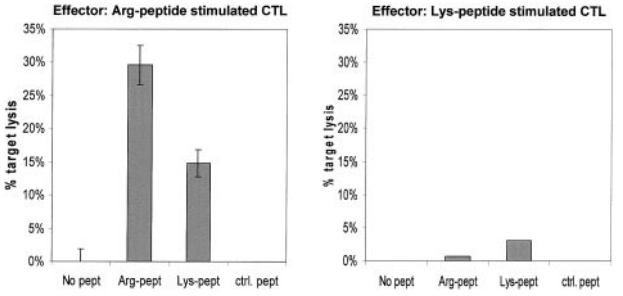

Figure 5. CTL killing of B95.8-derived peptide (Arg peptide) (**left**) and Lys-variant peptide (**right**). CTL clones were raised against these peptide-loaded autologous PBMCs, and tested for CTL recognition to peptide-loaded autologous PHA blasts labeled with $^{51}CrO_4$. Arg peptide, Lys peptide, and ctrl peptide are autologous PHA blasts loaded with different peptides.

more common in immunocompromised patients.[30,31] However, it now seems that the relative increase of type B virus in HIV patients with lymphoma reflects the prevalence of type B virus in the gay male community rather than an effect of immunocompromise.[32] With regard to PTLD lesions, ours is the third series to demonstrate an overwhelming preponderance of type A virus.[33,34] A similar predominance of type A virus has been reported in Hodgkin's disease, nasopharyngeal carcinoma, and healthy Caucasians in England.[35–38]

There remains the possibility that although laboratory strain virus type and PTLD virus type (A *versus* B) generally match, more subtle variations in the genome might limit the effectiveness of T cells expanded *ex vivo* in response to laboratory strain virus. EBV strain variations have been reported from populations in Borneo where HLA A11 predominates such that the A11-restricted epitope of EBNA-3B is absent.[39] Genetic instability in tumors might make the occurrence of such strain variants more likely through mutation. Our sequence analysis of EBV CTL epitopes in EBNA3C and LMP2A showed that with the exception of the Arg to Lys variant in the HLA B27-restricted EBNA3C epitope in 12 of 24 tumors, EBNA3C epitopes and LMP2A epitopes were well conserved in PTLD tumors. Furthermore, in the serial tumor specimens from a single PTLD patient there was no evidence of ongoing mutation of viral CTL epitopes, as analyzed by direct PCR sequencing. The results of cloning sequencing for these serial tumors were inconclusive. In addition, our approach would have missed viral genome deletions of the sort recently been reported.[40]

The Arg to Lys mutation (Lys variant) of the HLA B27 epitope of EBNA3C has been reported in 40% of virus isolates from healthy Australian Caucasians.[29] We detected this variation in 50% of specimens from American patients. The impact of this epitope sequence variation difference on CTL recognition was further tested. Killing of the Lys variant peptide-loaded target cells was observed, although at a lower efficiency than the Arg form. The lack of killing in the Lys peptide-stimulated CTL lines from the same donor may reflect poor immunogenicity of Lys variant virus. Responses to the Arg and Lys variants have recently been analyzed in detail by Brooks and colleagues[41] who found responses to more than one strain in some healthy individuals and inferred infection by co-resident viral strains. They reported that both Arg and Lys variants could be recognized by Arg-specific CTL clones although the Lys variant required a 10-fold higher target peptide concentration to achieve 50% maximum lysis. The only substitution of amino acid found in the HLA A2.1-restricted LMP2A epitope in the B-cell lymphoproliferative disease has previously been reported not to interfere with CTL recognition and killing.[20]

Immunotherapeutic approaches to the prevention or treatment of neoplasia are promising alternatives to conventional cytotoxic therapy with drugs or radiation. Graft-*versus*-leukemia effects have proven to be very potent in the treatment of some leukemias.[42–44] However, in general the cell populations that are associated with antitumor effects have been difficult to differentiate from those associated with graft-*versus*-host disease. The use of EBV-specific CTLs expanded *ex vivo* is a striking exception to this generalization. EBV CTLs have been proven effective at prophylaxis in the allogeneic marrow transplant setting and have not been associated with graft-*versus*-host disease.[11] These dramatic successes have kindled interest in the more general application of EBV CTL therapy to PTLD occurring in the recipients of solid organ transplants. Although we fully expect that exceptions will be noted with time, this first detailed investigation of epitopes in PTLD lesions suggests that CTL lines expanded in response to B95.8-transformed B-cell lines will generally have appropriate specificities for treatment of solid organ transplant patients in the United States.

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