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## *Brief Definitive Report*

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### AN EPSTEIN-BARR VIRUS-SPECIFIC CYTOTOXIC T CELL EPITOPE IN EBV NUCLEAR ANTIGEN 3 (EBNA 3)

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All individuals previously infected with Epstein-Barr virus (EBV) harbor life-long a population of virus-infected B cells whose number appears to be controlled by EBV-specific, HLA-restricted (class I and class II) CTLs (CD4 and CD8) that recognize a functionally defined lymphocyte-determined membrane antigen, LYDMA (1, 2). There are two recognized types of EBV (A and B) that show sequence divergence within the reading frames encoding EBV nuclear antigens (EBNAs) 2, 3, 4, and 6 (3, 4) and differences in the molecular weight of these proteins (4, 5).

Our general approach to identifying CTL epitopes has been to isolate EBV-specific T cell clones that recognize autologous A-type transformant but fail to lyse the corresponding B-type transformant (6). The present report defines the first target epitope recognized by EBV-specific CTLs. Our approach to identifying this epitope has been to screen selected peptide sequences from the EBNA proteins using specific CTLs and the autologous B-type transformant.

#### Materials and Methods

**Cell Lines.** EBV-transformed cell lines (LCLs) were established either spontaneously (i.e., by transformation with endogenous virus) or by the addition of exogenous virus (7). A spontaneous LCL from the EBV seropositive donor LC was established using cyclosporin A (8). The type of endogenous virus (A or B) in the spontaneous cell lines was determined using Southern analysis (6) and was used to define donor LC as an A-type donor.

Cell lines were established using exogenous A- or B-type virus by infecting B lymphocytes from an EBV seropositive donor Lc, with virus recovered from two A-type cell lines, IARC-BL74 (8) and IARC-BL36, (4) and two B-type cell lines, Ag876 (3) and L4 (4). Southern and Western analyses were used to confirm the presence of A- or B-type virus in the respective LC transformants (designated LC/BL74, LC/BL36, LC/Ag876, and LC/L4) as previously described (6).

**CTL Activation and <sup>51</sup>Cr-release Assay.** Lymphocytes from donor LC (HLA A1, B8, B18) were cocultivated with the irradiated autologous A-type LCL, LC/BL74 for 3 d and the activated T cells were cloned in agar and expanded in the presence of IL-2 and specific stimulating cells (2). T cell clones were screened in the standard <sup>51</sup>Cr release assay (E/T ratio of 10:1) against a panel of cell lines including autologous and allogeneic A- and B-type LCLs.

**Peptide Selection.** To identify the CTL epitope expressed on the A-type transformants but not on the autologous B-type transformants, a series of peptides (20–25 amino acids) deduced from the known sequences of the A-type EBNA 2, 3, 4, 5, and 6 proteins were synthesized (9) and screened on the autologous B-type transformant LC/Ag876. Peptides selected were

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primarily those in which the A- and B-type sequences had diverged and/or corresponded to the predicted algorithms of De Lisi and Berzofsky (10) and Rothbard and Taylor (11).

**Screening Peptides as Potential CTL Epitopes.** EBNA peptides synthesized were distributed in duplicate into U-well microtiter wells in RPMI 1640 (200  $\mu\text{g}/\text{ml}$ , 20  $\mu\text{l}/\text{well}$ ) and frozen at  $-70^\circ\text{C}$  until required.  $^{51}\text{Cr}$ -labeled PHA blasts or LCLs from autologous and allogeneic donors were added to each well ( $2 \times 10^5/\text{ml}$ , 50  $\mu\text{l}/\text{well}$ ) and incubated at  $37^\circ\text{C}$ . After 1 h, 130  $\mu\text{l}$  of cells from the CTL cloned lines from donor LC were added to the reaction mixture (final E/T ratio of 10 to 1) and the assay was conducted as described above.

### Results and Discussion

Eight EBV-specific T cell clones were isolated (all  $\text{CD8}^+$ , class I restricted) with a pattern of specificity similar to that previously described, i.e., EBV-specific T cell clones that recognized the autologous A-type transformants but failed to recognize the autologous B-type transformants (referred to as A-type-specific T cell clones). Clone 13 was used as a typical representative of these clones and the data presented refer to results obtained with this clone (Fig. 1).

Peptides were screened as possible T cell epitopes by assaying their ability to restore recognition of the B-type transformant by specific CTLs. Of the 76 peptides screened only 1 (peptide 68) restored the level of lysis of the B-type transformant to that of the A-type transformant (Fig. 2). The sequence of this peptide derived from the EBNA 3 protein (BERF1 and BLRF3 reading frames) is TETAQAWNA-GFLRGRAYGIDLLRTE (residues 329–353). This result has been confirmed in 12 consecutive experiments using three separate syntheses of peptide 68. Titration of the activity of this peptide using the B-type transformants from LC revealed that it was 50% active at 2  $\mu\text{g}/\text{ml}$ . It should be noted that we have excluded toxicity of peptide 68 as the reason for the lysis demonstrated. The level of toxicity of this peptide towards the B-type transformant was  $<5\%$  when used at five times that in Fig. 2.

The restricting MHC determinant recognized by clone 13 was defined using LCLs and PHA blasts (grown for 3–5 wk in IL-2) from autologous and allogeneic donors in the presence and absence of peptide 68 (Fig. 3). The results showed that T cell recognition of the peptide was through HLA B8 and that lysis was blocked by mAb to class I determinants. Recent experiments have shown that 8/8 EBV-specific A-type-specific clones established from this donor recognized this amino acid sequence ex-

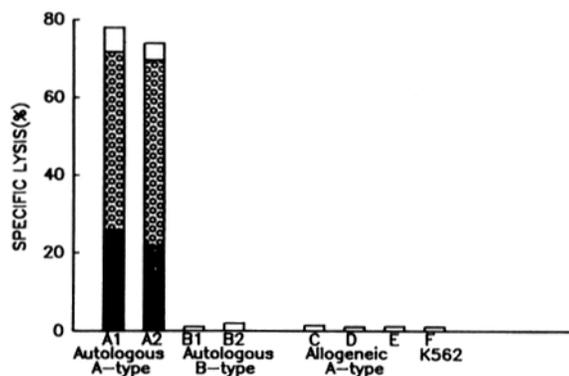


FIGURE 1. Percentage specific lysis by clone 13 of autologous A-type LCLs LC/BL36 and LC/BL 74 (A1 and A2) and B-type LCLs LC/Ag876 and LC/L4 (B1 and B2) and allogeneic LCLs from HLA-unrelated donors (C, D, and E) and K562 (F). The LCLs C and D were transformed with virus from IARC-BL36 and the LCL E was transformed with virus from the B-type transformant Ag876. mAbs specific for the monomorphic determinants on class I (1,620, ■) or class II (1,622, ▨) HLA antigens (provided by I. McKenzie, Melbourne) were tested for their ability to block autologous killing by clone 13.

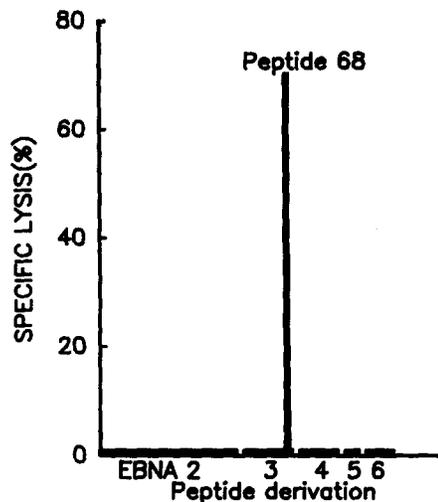


FIGURE 2. Percentage specific lysis of the B-type LCL, LC/Ag876 by clone 13 after preincubation with peptides from EBNA 2 (39 peptides tested), 3 (14 tested), 4 (11 tested), 5 (4 tested), and 6 (8 tested).

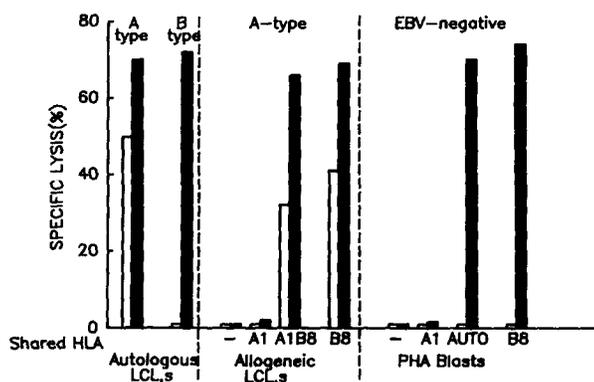


FIGURE 3. Lysis by clone 13 of the autologous A-type target LC/BL74, the autologous B-type target LC/Ag876, autologous (auto) PHA blasts, and HLA-related and -unrelated allogeneic LCLs and PHA blasts in the presence (■) and absence (□) of peptide 68. In this experiment, target cells were incubated with peptide 68 (20  $\mu$ g/ml, 0.5 ml) for 30 min before  $^{51}\text{Cr}$  labeling.

clusively (of these tested), suggesting that this EBV CTL epitope was dominant for this donor.

We have recently shown that similar clones from 2/2 other B8 donors also recognize peptide 68 suggesting that this peptide frequently associates with this allele. In contrast, EBV-specific T cell colonies from 12 non-B8 donors failed to recognize peptide 68 (data not shown).

To further define this epitope, three overlapping 15 mers from peptide 68, 68a (residues 329–343), 68b (residues 334–348), and 68c (residues 339–353) were prepared and tested for their ability to restore the level of lysis of the B-type transformant to that of the A-type transformant. The results demonstrated that all of the activity was included in 68b and 68c (Fig. 4).

The T cell epitope described in this communication is the first report of an EBV-encoded sequence capable of acting as a target for EBV-specific CTL lysis. An earlier report (12) described a sequence from the EBV latent membrane protein that

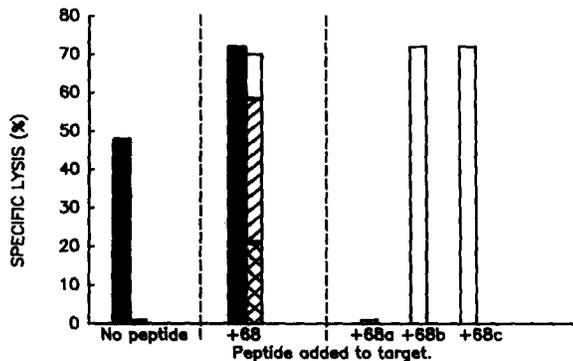


FIGURE 4. Lysis by clone 13 of the A-type LCL, LC/BL74 (■), and the B-type LCL, LC/Ag876 (□) after preincubation with either peptide 68 or the derivatives from peptide 68, 68a, 68b, and 68c (15 mers). mAbs specific for the monomorphic determinants on class I (1,620, ▨) or class II (1,622, ▩) HLA antigens were tested for their ability to block autologous killing by clone 13.

activated a CTL response but was apparently incapable of acting as a target for CTL recognition. The present data are strongly supported by recent results using clone 13 in which it has been demonstrated that LC/Ag876 (B-type transformant) was rendered susceptible to lysis by clone 13 after infection with vaccinia virus containing the recombinant EBNA 3 gene, but was not susceptible to lysis when similar constructs containing EBNA 2, 4, 5, or 6 were used (Murray, R. J., et al., manuscript submitted for publication).

The present study has important implications in the overall biology of EBV, particularly in regard to the ability of EBV-specific T cells to recognize and kill EBV-associated tumors. Burkitt's lymphoma (BL) cells that express only EBNA 1 have already been shown to be refractory to EBV-specific T cell recognition because of downregulation of both class I antigens (13) and adhesion molecules (14). The demonstration that a relevant CTL epitope is included in a protein that is not expressed in BL provides another mechanism of evading T cell recognition. It will be important in the future to determine whether EBNA 1 includes any EBV CTL epitopes.

### Summary

Epstein-Barr virus (EBV)-specific CTL clones were isolated that recognized A-type EBV transformants but not B-type transformants. These A-type-specific CTL clones (HLA B8 restricted) were used to screen peptides derived from the EBV nuclear antigens (EBNAs) 2, 3, 4, and 6 as potential CTL epitopes. Of the 76 peptides screened, one sequence from EBNA 3 (residues 329-353) was recognized by A-type-specific CTL clones after absorption onto target cells (either autologous B-type transformants or PHA blasts). This report is the first description of an EBV target epitope recognized by specific CTL clones.

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