Localization of Epstein-Barr Virus Cytotoxic T Cell Epitopes Using Recombinant Vaccinia: Implications for Vaccine Development

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Summary

There is considerable interest in designing an effective vaccine to the ubiquitous Epstein-Barr virus (EBV). An important role for EBV-specific cytotoxic T lymphocytes (CTLs) in eliminating virus-infected cells is well established. Limited studies using a small number of immune donors have defined target epitopes within the latent antigens of EBV. The present study provides an extensive analysis of the distribution of class I–restricted CTL epitopes within EBV-encoded proteins. Using recombinant vaccinia encoding individual EBV latent antigens (Epstein-Barr nuclear antigen [EBNA] 1, 2, 3A, 3B, 3C, LP, and LMP 1), we have successfully localized target epitopes recognized by CTL clones from a panel of 14 EBV-immune donors. Of the 20 CTL epitopes localized, five were defined at the peptide level. Although CTL clones specific for nine epitopes recognized both type 1 and type 2 transformants, a significant number of epitopes (7/16 epitopes for which EBV type specificity was determined) were detected only on type 1 EBV transformants. Vaccinia recombinants encoding EBNA 3A and EBNA 3C were recognized more frequently than any other vaccinia recombinants used in this study, while no CTL epitopes were localized in EBNA 1. Surprisingly, epitope specificity for a large number of EBV-specific CTL clones could not be localized, although vaccinia recombinants used in this study encoded most of the latent antigens of EBV. These results suggest that any EBV vaccine based on CTL epitopes designed to provide widespread protection will need to include not only latent antigen sequences but also other regions of the genome. The apparent inability of human CTLs to recognize EBNA 1 as a target antigen, often the only latent antigen expressed in Burkitt's lymphoma and nasopharyngeal carcinoma, suggests that EBV-specific CTL control of these tumors will not be feasible unless the down-regulation of latent antigens can be reversed.

Protective immunity to viral infection requires the development of memory T cells that recognize viral antigens in association with class I MHC. Earlier studies on influenza virus first highlighted the important role of $CD8^+$ CTLs, which recognize virus-encoded proteins in the form of short peptides (1). Since it is well established that immunization with whole viral proteins is unable to elicit an efficient CTL response, interest has been directed towards peptide vaccines based on defined epitope sequences. This is particularly the case with oncogenic viruses, since individual viral genes introduced in recombinant vectors have the potential to initiate tumorigenic processes. Thus, it is important to determine the distribution of these epitopes within viral proteins and the frequency with which infected cells from a significant cohort of immune donors present these epitopes in association with MHC class I alleles. Because of the potential importance of CTL epitopes in the future development of a vaccine to EBV, a herpes virus with known oncogenic potential, there is considerable interest in defining these EBV-encoded molecules recognized by CTLs.

EBV is the etiological agent of infectious mononucleosis (IM)[1] and is associated with Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC) (2), lymphomas in immunocompromised individuals (3), and more recent evidence suggests an association with Hodgkin's lymphoma (4). Two

[1] *Abbreviations used in this paper:* BL, Burkitt's lymphoma; EBNA, Epstein-Barr nuclear antigen; IM, infectious mononucleosis; LCL, lymphoblastoid cell line; LMP, latent membrane protein; LP, leader protein; NPC, nasopharyngeal carcinoma; TP, terminal protein; UM, unfractionated mononuclear.

types of EBV (1 and 2, also referred to as A and B) are recognized that show DNA sequence divergence within the BamHI WYH and E regions of the genome (5–7). In vitro, the virus transforms human B cells into lymphoblastoid cell lines (LCLs), which express a limited number of viral gene products, including a family of Epstein-Barr nuclear antigens (EBNA): 1, 2, 3A, 3B, and 3C, leader protein (EBNA LP), latent membrane proteins (LMP 1 and 2), and terminal proteins (TP 1 and 2) (5). An alternative nomenclature also in current use designates the EBNA family as EBNA 1, 2, 3, 4, and 6, and EBNA-5/LP (4, 5). In contrast, latent antigen expression in BL and NPC is restricted to EBNA 1 (in some instances LMP is also expressed in NPC) (8).

In all previously infected individuals, the virus persists for life as a latent infection in B cells and is apparently restrained by a population of EBV-specific CTLs (9, 10). This CTL response is a classic virus-specific response (CD8, class I restricted) (10), though CD4 class II–restricted cells have also been described (9). The key observation in defining the first peptide epitope recognized by EBV-specific CTLs was that in certain donors it was possible to exploit the allelic polymorphism in the EBNA proteins between type 1 and type 2 EBV by isolating type 1–specific CTL clones (11). These clones provided an opportunity to screen selected EBNA peptides for reactivity on type 2 transformants. This led to the definition of an EBV-specific CTL epitope that was present on type 1 but not type 2 transformants (12). A second epitope, derived from EBNA 3C and present on both type 1 and type 2 transformants, has also been described (13). While this approach was very successful in defining a limited number of CTL epitopes, their overall distribution within EBV latent antigens was largely undetermined.

Two recent technical advances from our laboratories have facilitated this study. First, the construction of recombinant vaccinia capable of expressing individual EBV latent antigens, and second, the establishment of an EBV-negative host cell (anti-μ B cell blasts) for these recombinant vaccinia (14, 15). In the present report, we have localized EBV CTL epitopes recognized by multiple CTL clones from a panel of immune donors to generate the first comprehensive analysis of the distribution of CTL epitopes within the EBV latent antigens. This approach was combined with peptide epitope mapping, which permitted the identification of a number of new CTL epitopes. Moreover, since EBV infection is associated with BL and NPC, another important objective of this study was to determine whether any CTL epitopes are localized within EBNA 1 and/or LMP.

Materials and Methods

Establishment and Maintenance of EBV-transformed Cell Lines. LCLs were established from a panel of healthy EBV-seropositive donors listed in Table 1 by exogenous virus transformation of peripheral B cells using type 1 (B95.8 and IARC-BL74) or type 2 (Ag876) EBV isolates (11), and were routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin plus 10% FCS (growth medium). LCLs were designated with the donors initial followed by the transforming virus source (e.g., LC/B95.8 designates B lymphocytes from donor LC transformed with virus from the B95.8 cell line).

Generation of Anti-μ B Cell Blasts. Unfractionated mononuclear (UM) cells were separated on Ficoll/Paque (Pharmacia, Uppsala, Sweden) and depleted of T cells using E-rosetting (16). The enriched B lymphocytes were cultured in growth medium containing anti-IgM (μ chain specific) coupled to acrylamide beads (Bio-Rad Laboratories, Richmond, CA), human rIL-4 (50 U/ml; Genzyme, Boston, MA), and highly purified human rIL-2 from *Escherichia coli* (20–40 U/ml) (17, 18). After 48–72 h, B cell blasts were suspended in growth medium supplemented with rIL-2 (20–40 U/ml). The B cells continue to divide two to three times/wk for 3 wk in the presence of rIL-2. These cells are referred to as anti-μ B cell blasts.

Vaccinia Virus Recombinants. Recombinant vaccinia constructs for different EBNA genes have been previously described (14, 15, 19). All EBV sequences were derived from the B95.8 strain of virus. The EBNA 3A sequence was derived from the cDNA Clone T216 (7), which consists of the 5′ portion of the EBNA 3A coding sequence, crossing the splice site and extending to the EcoRI site located at position 93166. This plasmid was digested with PstI and collapsed onto itself to remove 5′ noncoding sequences. The resulting plasmid, T216P, was opened at the EcoRI site and ligated to the genomic EcoK fragment derived from plasmid PMH36, recreating the intact full-length EBNA 3A coding sequence. The EBNA 3A coding region was excised with PstI and EcoRV and ligated to Psc11 at the SmaI site. The EBNA 3B was derived from the cDNA clone PMLPT7. The portion of the open reading frame crossing the splice junction was excised with SpeI and XbaI and inserted into SpeI-digested pBluescript. The resulting plasmid was digested with SpeI and EcoRI and ligated to a 440-bp genomic fragment from EcoRI (95243) to SpeI (95683). The resulting plasmid (pBS:E3B) was digested with EcoRI and XbaI to release full-length coding region of EBNA 3B and ligated to the SmaI site of PSC11.

All constructs had the potential to encode the relevant full-length EBV protein except for EBNA 2 deletion mutants. The diagrammatic representation of EBNA 2 deletion mutants is shown in Fig. 1. All constructs utilize the authentic start and stop codons. All constructs are under the control of vaccinia virus P7.5 promotor, except EBNA 1, which has been described elsewhere (19). A vaccinia virus construct made from insertion of the pSC11 vector alone and negative for thymidine kinase (VaccTK$^-$) was used as control.

Source of Generation of EBV-specific CTL Clones. UM cells (10^6/ml) from each donor were cultivated with irradiated (8,000 rad) autologous type 1 (B95.8) LCLs (responder to stimulator ratio of 200:1) in 2-ml culture wells (Linbro Chemical Co., Hamden, CT) for 3 d in growth medium. In the case of donors LC and IM, CTL clones were also established after stimulation with BL74-transformed autologous LCLs. CTL clones generated by seeding in 0.35% agarose were established from these donors and maintained as described earlier (9, 11). Colonies were harvested after 3 d and amplified in culture with biweekly restimulation with rIL-2 and autologous LCL.

Cytotoxicity Assay on LCLs. CTL clones from each donor were screened in a standard 5-h ^{51}Cr release assay (at an E/T ratio of 5:1 or 10:1) for specific reactivity against autologous types 1 and 2 and allogeneic type 1 LCLs as previously described (11). Clones were designated as being EBV-specific on the basis of recognition of the autologous type 1 LCL and lack of recognition of MHC-unrelated LCLs and autologous anti-μ B cell blasts and/or PHA blasts.

Cytotoxicity Assay on Recombinant Vaccinia Virus–infected Targets. Anti-μ B cell blasts or type 2 LCLs were infected with recombinant vaccinia viruses at a multiplicity of infection (M.O.I.) of 10:1 for 1 h at 37°C as described earlier (14). After 14–16 h, cells were washed with RPMI 1640 and incubated with ^{51}Cr for 90 min, washed three times, and used as targets in a standard 5-h ^{51}Cr-release assay as described above. The effector cells were added to the assay at E/T ratios between 5:1 and 10:1. To confirm the expression of EBV antigens in anti-μ B cell blasts and/or LCLs after recombinant vaccinia infection, the infected cells were also processed for immunoblotting and immunofluorescence (14).

Screening of CTL Clones for Peptide Epitope Specificity. To identify the CTL epitopes recognized by EBV-specific CTL clones from each donor, a series of peptides from EBNA or LMP 1 were synthesized (10–15 amino acids) (20) based on the known sequence of the B95.8 strain of EBV. Peptides selected were primarily based on the results of recombinant vaccinia CTL assays and those that corresponded to predicted algorithms (21, 22). Peptides were dissolved in RPMI 1640 and distributed into U-bottomed microdilution plates (200 μg/ml, 20 μl/well) and frozen at −70°C until required. ^{51}Cr-labeled anti-μ B cell blasts were added to each well (2×10^5/ml, 50 μl/ml) and incubated at 37°C. After 1 h, 130 μl of cloned autologous CTLs were added to the reaction mixture (final E/T ratio as indicated), and the assay was conducted as described above.

Table 1. *HLA Antigen (Class I) Type of the EBV-immune Donors Included in this Study*

Donor	HLA typing
LC	A1, B8, B18
IM	A1, A11, B8, B51
DM	A24, A29, B44, B47
CM	A11, A24, B7, B44
PM	A11, A29, B7, B44
AS	A2, A24, B51, B62
NB	A2, A24, B7, B35
DD	A1, A3, B8, B40
LX	A24, B15, B38
LL	A2, B7, B44
CS	A2, A23, B35, B44
SJ	A2, A3, B7, B44
JA	A2, A11, B7, B15
JS	A1, A2, B8, B51

Results

Localization of EBV CTL Epitopes in EBV-immune Donors. A total of 362 clones were isolated from a panel of 14 healthy EBV-immune donors after stimulation with irradiated autologous B95.8- or BL74-transformed LCLs. Of these, 212 were EBV-specific CTLs. The proportion of these specific CTL clones from each donor varied from 100% (17/17 for donor DM) to as low as 16% (8/51 for donor AS) (Table 2).

To define the antigen specificity of the 212 EBV-specific CTL clones, autologous anti-μ B cell blasts or type 2 LCLs were infected with recombinant vaccinia expressing individual EBV latent antigens (and Vacc.TK$^-$) and used as targets in a ^{51}Cr release assay. The reactivity of five EBV-specific clones from one of the 14 donors (DD) is illustrated in Fig. 2, and demonstrates that two of these clones recognize Vacc.EBNA 3A (CTL5 and CTL13), two recognize Vacc.LPM1 (CTL8 and CTL10), while the antigen specificity of one clone (CTL9) was not defined by the panel of vaccinia constructs. Table 2 presents a summary of the EBNA/LMP 1 vaccinia constructs recognized by CTL clones from each of the 14 donors. All vaccinia constructs except Vacc.EBNA 1 and Vacc.EBNA 3B were recognized by EBV-specific CTL clones. However, it should be emphasized that recognition of Vacc.EBNA 3B–infected anti-μ B cell blasts by EBV-specific CTL clones was assessed in only five donors (Table 2). A dominant response through a single vaccinia construct was observed with some donors (DM, CM, and CS recognized primarily Vacc.EBNA 3C) (Table 2). An important feature of these results was that the majority (145/212) of EBV specific clones failed to recognize any of the latent antigens encoded by vaccinia constructs (Table 2). This was particularly evident in the case of donors PM and JA, where none of the clones recognized cells infected with any of the vaccinia constructs.

MHC Class I Restriction of Vaccinia-localized CTL Epitopes. Of the 212 EBV-specific CTL clones investigated in the present study, 20 distinct epitopes were localized using vaccinia constructs. CTL clones specific for seven of these epitopes were type 1 specific, while clones specific for nine

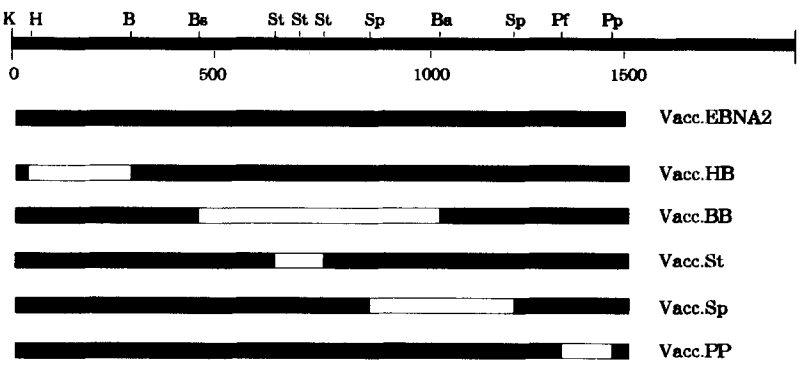

Figure 1. Diagrammatic representation of B95.8 EBNA 2 sequence and of vaccinia constructs encoding EBNA 2 and its deletion mutants. The construct encoding the full-length EBNA 2 is designated (■), while the regions deleted from EBNA 2 are designated (□). Details for the preparation of these mutants has been published earlier (15). Each recombinant vaccinia had the capacity to encode truncated EBNA 2 proteins with the following amino acid residues deleted: Vacc.HB has amino acid residues 19–118 deleted; Vacc.BB has amino acid residues 151–327 deleted; Vacc.St has amino acid residues 203–237 deleted; Vacc.Sp has amino acid residues 251–384 deleted; and Vacc.PP has amino acid residues 405–480 deleted.

Table 2. *Recognition of Vaccinia Recombinants Encoding EBV Latent Antigens by EBV-specific CTL Clones from Immune Donors*

Donor	No. of clones	EBV specific clones‡	Vacc. EBNA 1	Vacc. EBNA 2§	Vacc. EBNA 3A	Vacc. EBNA 3B	Vacc. EBNA 3C	Vacc. EBNA LP	Vacc. LMP 1
LC	47	44	–‖	2	7	–	–	–	1
IM	26	15	–	–	4	–	–	–	–
DM	17	17	–	–	–	NT¶	12	–	–
CM	29	19	–	–	1	NT	6	–	–
PM	25	17	–	–	–	NT	–	–	–
AS	51	8	–	–	1	NT	–	–	3
NB	42	8	–	5	1	NT	–	1	1
DD	21	17	–	–	3	–	–	–	3
LX	14	5	–	–	3	–	–	–	–
LL	19	16	–		1	NT	2	2	1
CS	15	12	–	–	–	NT	4	–	–
SJ	24	19	–	–	1	NT	–	–	–
JA	12	4	–	–	–	NT	–	–	–
JS	20	11	–	2	–	–	–	–	–
Total	362	212	0	9	22	0	24	3	9

* Total number of clones tested for cytolytic activity.
‡ This column summarizes the number of EBV-specific CTL clones isolated from each individual donor.
§ This column refers to the number of clones recognizing autologous anti-μ B cell blasts infected with recombinant vaccinia encoding EBNA 2.
‖ No clones reactive to vaccinia construct.
¶ Not tested.

other epitopes recognized both type 1 and type 2 transformants. The type specificity of four epitopes was undefined (Table 3). The HLA restriction of the specific epitopes was determined by comparing the lysis of autologous LCLs and allogeneic LCLs sharing one or more alleles (Table 3). CTL clones restricted through eight different alleles were observed while the restricting alleles for five EBV CTL epitopes were undetermined (Table 3). An important feature of these results was that different clones restricted through HLA A2, B7, B40, B8, and B51 each recognized epitopes included in two different latent antigens (Table 3). This observation implies that a single allele can present two distinct EBV CTL epitopes.

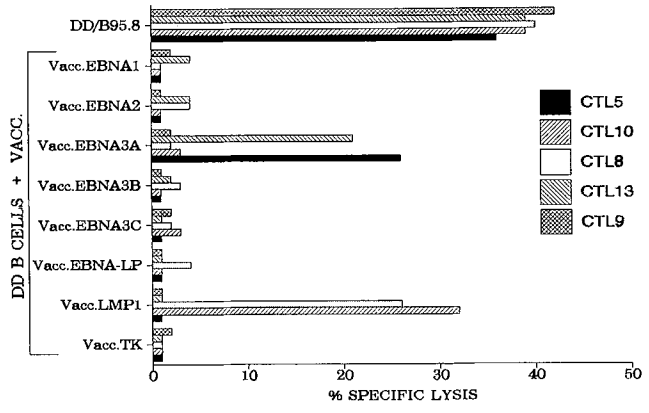

Figure 2. Specific lysis by EBV-specific CTL clones (CTL5, 8, 9, 10, and 13) from donor DD of autologous LCLs (DD/B95.8) and Vacc. EBNA 1, 2, 3A, 3B, 3C, LP, LMP 1, and TK$^-$-infected DD anti-μ B cell blasts. Anti-μ B cell blasts were infected for 12–14 h (M.O.I., 10:1) with vaccinia constructs and processed for standard ^{51}Cr release assay. Vacc.TK$^-$ was used as a control recombinant vaccinia. Results are expressed as percent specific lysis observed in a standard 5-h chromium-release assay. An E/T ratio of 5:1 was used throughout the assay.

More precise localization of CTL epitopes within EBNA 2 was facilitated by the availability of deletion mutants encoding truncated EBNA 2 proteins. In all, nine EBNA 2–specific CTL clones restricted through three different alleles (HLA A2, B18, and B7) were isolated (Table 3). The A2-restricted clones from donors JS and NB recognized four deletion mutants (Vacc.BB, Vacc.St, Vacc.PP and Vacc.Sp) with a level of lysis comparable with Vacc.EBNA 2. In contrast, the Vacc.HB mutant, which had a deletion affecting the NH_2 terminus of EBNA 2 protein (Fig. 1), was not recognized by these clones. Data from one such A2-restricted CTL clone from donor NB is shown in Fig. 3 *a*. In contrast, the HLA B18–restricted EBNA 2–specific CTL clones (from donor LC), failed to recognize Vacc.Sp and Vacc.BB deletion mutants, which had overlapping deletions for amino acids 251–327 of EBNA 2 protein (Figs. 1 and 3 *b*).

Based on the results obtained from the vaccinia experiments, EBV peptides from respective EBNA/LMP 1 regions were screened for their ability to sensitize autologous anti-μ B cell blasts for EBV-specific CTL lysis. In some instances, this involved the selection of 15–20-mer peptides from individual

Table 3. *Summary of the Distribution of MHC Class I–restricted EBV CTL Peptide Epitopes within EBV Latent Antigens Localized by Recombinant Vaccinia*

EBV antigen recognized	HLA restriction	Peptide epitope	EBV type specificity	Donor(s)
EBNA 2	HLA A2	DTPLIPLTIF*	Type 1	JS and NB
EBNA 2	HLA B18	PRSPTVFYNIPPMPL‡	Type 1	LC
EBNA 2	HLA B7	Undefined	Type 1 and 2	NB
EBNA 3A	HLA B8	FLRGRAYGL§	Type 1	LC and IM
EBNA 3A	HLA B7	Undefined	Type 1 and 2	NB and SJ
EBNA 3A	HLA A11 or A24	Undefined	Type 1	CM
EBNA 3A	HLA B51	Undefined	Type 1	AS
EBNA 3A	Undefined	Undefined	ND	LX
EBNA 3A	HLA 40	Undefined	ND	DD
EBNA 3A	Undefined	Undefined	Type 1 and 2	LL
EBNA 3C	HLA A24 or B44	RGIKEHVIQNAFRKA‡	Type 1	CM and DM
EBNA 3C	HLA B44	EENLLDFVRF‖	Type 1 and 2	DM, CM, CS, and LL
EBNA 3C	Undefined	Undefined	Type 1 and 2	CS
EBNA LP	Undefined	Undefined	Type 1 and 2	NB and LL
LMP 1	HLA A2	Undefined	Type 1 and 2	LL and NB
LMP 1	HLA B51	Undefined	Type 1 and 2	AS
LMP 1	HLA B40	Undefined	ND	DD
LMP 1	HLA A24	Undefined	Type 1 and 2	AS
LMP 1	Undefined	Undefined	Type 1	LC
LMP 1	HLA B8	Undefined	ND	DD

* Reference 23.
‡ This study.
§ Reference 12.
‖ Reference 13.

latent antigens that corresponded to predicted algorithms (20–22). However, to define epitopes from EBNA 2, peptides were selected that corresponded to the relevant vaccinia deletion mutant. Of the 20 distinct CTL epitopes localized in this study, five were defined at the peptide level while the other 15 remained undefined (Table 3). Of these five epitopes, three have been previously published (12, 13, 23), while new epitopes from EBNA 2 and EBNA 3C are presented in Table 3. The results of experiments that define these new epitopes are included in Fig. 4, *a* and *b*. The HLA B18–restricted EBNA 2–specific clone recognized autologous anti-μ B cell blasts sensitized with peptide PRSPTVFYNIPPMPL (residue number 276–290), while the EBNA 3C–specific clone, restricted through either HLA A24 or B44, recognized peptide RGIKEHVIQNAFRKA (residue number 332–346) (Fig. 4, *a* and *b*).

Discussion

There is convincing evidence that EBV-specific memory T cells are responsible for controlling the level of EBV-positive B lymphocytes, which all healthy seropositive individuals carry for life after primary infection with the virus. Experimental support for the existence of this protective memory T cell population came from the observation that in virus-infected cultures of mononuclear lymphocytes from seropositive (but not seronegative) donors, the initial proliferation of EBNA-positive B cells was followed by a complete T cell–dependent regression of growth such that LCLs could not be established from subcultures (24, 25). This observation suggested that the latent antigens expressed by these LCLs were a source of target antigens. Indeed, we have recently demonstrated the existence of CTL epitopes within three of the EBNA proteins by screening individual clones against a panel of peptides derived from a range of latent antigens. Although these studies identified several CTL epitopes (12, 13, 23), there has been no previous attempt to determine the relative distribution of CTL epitopes within EBV proteins, recognized by healthy immune donors expressing an array of MHC class I alleles. Such an evaluation is a mandatory prerequisite for any future CTL-based vaccine to EBV. The present study provides an extensive analysis of the distribution of CTL epi-

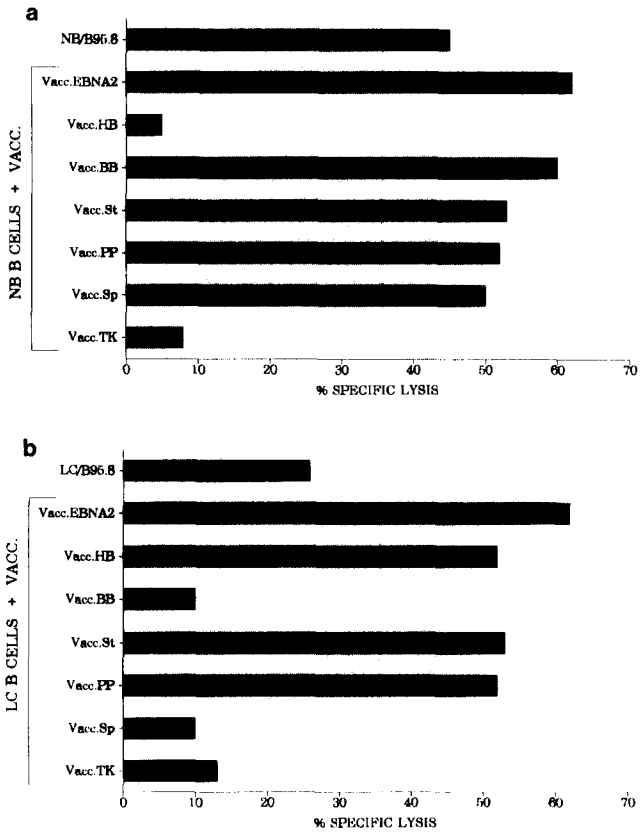

Figure 3. Functional analysis of EBNA 2-specific CTL clones from donors NB (*a*) and LC (*b*) generated from an in vitro stimulation with autologous B95.8 LCLs and tested on autologous type 1 LCL (NB/B95.8 or LC/B95.8) and anti-μ B cell blasts infected with recombinant vaccinia carrying EBNA 2 gene deletions (Vacc.HB, BB, St, PP, and Sp), Vacc.EBNA 2, or Vacc.TK$^-$. Results are expressed as in Fig. 2. For details on the amino acids deleted from each recombinant vaccinia, see legend to Fig. 1.

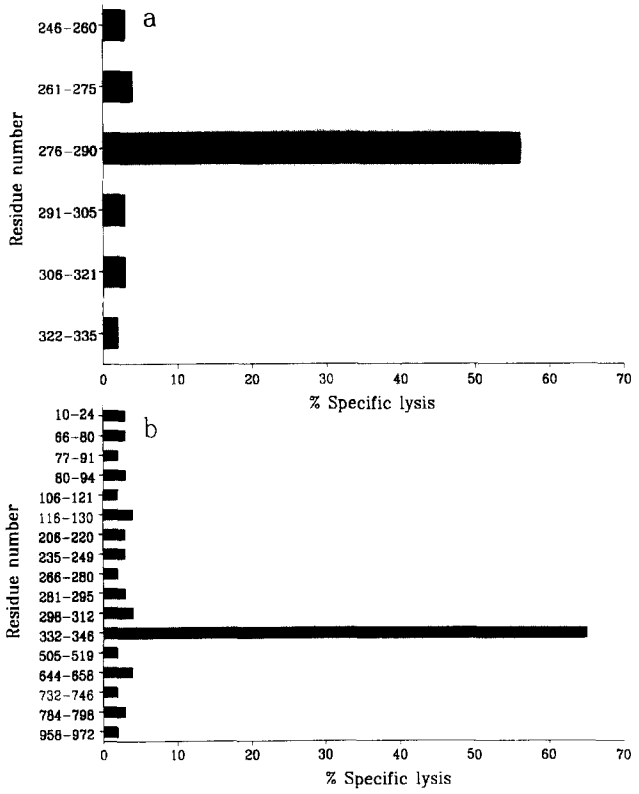

Figure 4. Recognition of peptide-sensitized autologous anti-μ B cell blasts by EBV-specific CTL clones from donors LC (*a*) and CM (*b*). ^{51}Cr-labeled anti-μ B cell blasts were presensitized with each peptide for 1 h and subsequently screened in a CTL assay using the relevant CTL clone. An E/T ratio of 5:1 was used. The results are expressed as in Fig. 2.

topes within viral-encoded proteins. Using recombinant vaccinia encoding individual EBV latent antigens, we have successfully localized target epitopes recognized by CTL clones from a panel of EBV-immune donors.

The location of CTL epitopes within the seven latent antigens, for which vaccinia constructs were available, was unevenly distributed. In particular, EBNA 3A and EBNA 3C were common sites for CTL recognition (11/14 donors had CTL reactivity to either of these antigens), while no epitopes were localized in EBNA 1 and EBNA 3B. Since, in the present study, Vacc.EBNA 3B was not available to test the reactivity of CTL clones from nine donors, it is not possible to draw any conclusions about the occurrence of epitopes in this protein. The immunodominance of the EBNA 3 family of proteins as a source of EBV CTL epitopes seen in this study using EBV-specific CTL clones has been confirmed in a similar study using polyclonal EBV-specific T cells (19). In all, 20 distinct CTL specificities restricted through eight different class I alleles have been defined. Interestingly, HLA A2, B7, B8, B40, and B51 alleles were each shown to present two distinct CTL epitopes derived from different latent antigens. The ability of single MHC alleles to present multiple epitopes has also been reported with HIV (26).

Surprisingly, the specificity of a large number of EBV-specific CTL clones (68%) using the available panel of vaccinia constructs could not be defined. A possible explanation for this result is that the undefined epitopes are located within EBV latent antigens other than those encoded by the vaccinia constructs available for this study; for example, terminal proteins (TP 1 and TP 2), LMP 2, or as yet unidentified latent antigens. Alternatively, antigens associated with the EBV replicative cycle could also include CTL epitopes. These antigens are detected in only a small proportion of latently infected cells by conventional techniques. However, the exquisite sensitivity by which CTLs recognize peptide fragments (27) from viral antigen raises the possibility that low levels of replicative antigens could be processed and presented as target epitopes on LCLs. It is relevant to point out that in another related study, EBNA 3B and LMP 2 were identified as targets for EBV-specific recognition (19). These observations suggest that all latent antigens except EBNA 1 are targets for EBV-specific CTL recognition, although a disproportionate number of CTL epitopes are located within the EBNA 3 proteins.

There are two important implications for the overall biology of EBV if subsequent studies confirm that EBNA 1 does not include CTL epitopes. First, the ability of BL cells (which express only EBNA 1) to proliferate in vivo, in spite of a typically normal EBV-specific CTL response (28), is consistent with the observation that there are no CTL epitopes within EBNA 1. In vitro studies have indicated that BL cells are much less susceptible to EBV-specific CTL lysis than LCLs from the same donor (29). Down-regulation of HLA class I alleles (30) and adhesion molecules (e.g., LFA-1 and -3, and ICAM-1) (31) have been implicated as possible mechanisms for this resistance of BL cells to lysis. However, recent observations from this laboratory using peptide epitopes from EBNA 2, 3A, and 3C have demonstrated that downregulation of latent antigen expression is the most critical factor in the nonrecognition of BL cells (Khanna et al., manuscript in preparation).

The lack of detectable CTL epitopes within EBNA 1 has a second important implication in regard to the persistence of EBV in peripheral B cells. A model for the persistence of EBV in B cells has recently been proposed. A feature of this model is the existence of a long-lived, non-replicating, EBNA 1–expressing B cell (32). The observations from the present study provide a mechanism by which these cells can maintain a nonimmunogenic phenotype by not expressing the critical latent proteins needed for CTL recognition.

Of the 20 CTL epitopes localized by recombinant vaccinia, five were defined at the peptide level. In addition to three previously published epitopes (12, 13, 23), in the present study we have defined two new epitopes, one in EBNA 2 and one in EBNA 3C. Although CTL clones specific for nine epitopes recognized both type 1 and type 2 transformants, a significant number of epitopes (7/16 epitopes for which EBV type was determined) were specific for type 1 EBV. Since we have recently shown that the majority of single amino acid substitutions within CTL epitopes result in loss of recognition (33), the common isolation of type 1–specific CTL clones is not unexpected when the degree of latent antigen sequence variation between the two types is compared (7).

The present study has important implications for any future EBV vaccine designed to control IM or EBV-associated tumors. First, CTL epitopes from a spectrum of individuals are distributed throughout most of the latent proteins. Second, >60% of the CTL epitopes are located in regions outside the EBNA/LMP 1 proteins. Both of these considerations suggest that any EBV vaccine based on CTL epitopes designed to provide widespread protection will need to include not only EBNA and LMP sequences but also other regions of the genome expressed during latent infection. However, since CTLs from the majority of donors recognized EBNA 3A and EBNA 3C as target antigens, incorporation of epitopes derived from these proteins into a vaccine may protect the majority of susceptible individuals from IM. The inability of human CTLs to recognize EBNA 1 as a target antigen, often the only latent antigen expressed in BL and NPC, suggests that CTL control of these tumors will not be feasible unless the downregulation of latent antigens can be reversed. The localization of CTL epitopes within LMP, however, raises the possibility of controlling EBV-associated tumors with normal LMP expression (Hodgkin's lymphoma and some NPC) by boosting the CTL response to this antigen.

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