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Multiple HLA A11-Restricted Cytotoxic T-Lymphocyte Epitopes of Different Immunogenicities in the Epstein-Barr Virus-Encoded Nuclear Antigen 4

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Epstein-Barr virus (EBV), a ubiquitous herpesvirus, induces potent HLA class I-restricted cytotoxic T-lymphocyte (CTL) responses. Analyses of target antigen choice have shown that the very strong CTL responses which are often observed through the HLA A11 allele map are due almost entirely to a single transformation-associated EBV protein, the nuclear antigen EBNA4. Here, we sought to determine the number and relative immunogenicities of HLA A11-restricted epitopes within this 938-amino-acid protein. An initial screening with a series of recombinant vaccinia virus vectors encoding progressively truncated forms of EBNA4 was followed by peptide sensitization experiments using overlapping 14- or 15-mers from the entire sequence. These two approaches allowed the identification of five epitope regions located between residues 101 and 115, 416 and 429, 396 and 410, 481 and 495, and 551 and 564 of the EBNA4 molecule. CTL preparations from all seven HLA A11-positive donors tested had demonstrable reactivities against the 416-to-429 peptide, whereas reactivities against the other epitopes either tended to be lost on serial passage or, for some of the donors, were never detected. The immunodominance of the 416-to-429 epitope was further supported by peptide dilution assays using polyclonal effectors and by CTL cloning experiments. Analysis of the 416-to-429 region identified the nanomer 416–424 (IVTDFSVIK) as the cognate peptide. This peptide was able to sensitize targets to lysis by A11-restricted CTL clones at concentrations as low as 5×10^{-14} M.

Epstein-Barr virus (EBV) is a widespread lymphotropic herpesvirus which causes infectious mononucleosis and is strongly linked to at least three lymphoid malignancies: endemic Burkitt's lymphoma, immunoblastic B-cell lymphomas of immunosuppressed patients, and a subset of Hodgkin's lymphomas (7–9). Primary EBV infection of immunocompetent hosts is usually asymptomatic and leads to the establishment of a life-long carrier state, whereby the virus persists within the B-cell compartment of healthy carriers (5). These infected B lymphocytes can proliferate in vitro, giving rise to lymphoblastoid cell lines (LCLs) which express at least eight latency-associated viral antigens: the nuclear antigens EBNA1 to -6 and the membrane proteins LMP1 and -2 (reviewed in reference 13). The recent demonstration that the immunoblastic lymphomas occurring in immunosuppressed individuals represent the in vivo outgrowth of EBV-positive LCL-like cells (6) emphasizes the role of immune surveillance in controlling this potentially lymphomagenic virus.

EBV induces long-lasting cytotoxic T-lymphocyte (CTL) memory in the infected host. Thus, EBV-specific CTL precursors can be reactivated in relatively large numbers from the T-cell pool of EBV-seropositive donors by challenging in vitro with autologous virus-infected B cells (22, 27). Recent studies have shown that in each individual such CTL responses are composites of several virus-induced reactivities (3, 11, 18). Each reactivity is directed against one or another of the transformation-associated viral proteins presented in the context of particular HLA class I restricting determinants. An individual's HLA class I genotype can, therefore, strongly influence EBV-specific CTL responses both qualitatively, in terms of target antigen choice, and quantitatively, in that strong CTL responses are frequently associated with restriction through certain HLA class I alleles. The HLA A11 allele provides a particularly clear example of this phenomenon, since CTLs restricted through this determinant are often dominant in polyclonal CTL cultures reactivated in vitro from HLA A11-positive individuals (17, 26). Using recombinant vaccinia virus vectors, this A11-restricted response was shown to be directed largely to the transformation-associated viral antigen EBNA4 (3, 18). Here, we show that the EBNA4 protein contains multiple HLA A11-restricted epitopes with different immunogenicities.

MATERIALS AND METHODS

Abbreviations used in this article. EBV, Epstein-Barr virus; EBNA, EBV-encoded nuclear antigen; LCL, lymphoblastoid cell line (transformed with EBV); CTL, cytotoxic T lymphocyte; TK, thymidine kinase; PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline. HPLC, high-pressure liquid chromatography.

Cells. LCLs were obtained by transformation of purified B lymphocytes from HLA-typed donors with the B95-8-derived strain of EBV (16). Fibroblast lines were established

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from skin biopsies obtained from HLA-typed donors and were used for vaccinia virus infection and cytotoxic assays within the first 20 in vitro passages. The cell lines were maintained in RPMI 1640 or modified Eagle medium (GIBCO/BRL) supplemented with 2 mM glutamine, antibiotics, and 10% heat-inactivated fetal calf serum (complete medium). PHA-activated T blasts were obtained from HLA-typed donors after activation of lymphocytes with 1 μg of PHA (Wellcome) per ml for 3 days and subsequent expansion in interleukin 2-containing medium.

Construction of EBNA4 deletion mutants and generation of vaccinia virus recombinants. The Vacc-EBNA4 recombinant carrying the entire EBNA4-coding sequence was described previously (11). Mutations in the coding region of EBNA4 were constructed by using plasmid pBS:E3b (12), which encodes a full-length cDNA from the B95-8 strain of EBV. All truncated mutants (Fig. 1A) were produced by insertion of the stop codon linker 5′-d(CTAGTCTAGACTAG)-3′ (New England Biolabs, Beverly, Mass.) at the specified site at the linker-to-plasmid ligation ratio of 100:1. pBS:E3b-Sp was constructed by opening pBS:E3b at the unique *Sph*I site (genomic position 96073), repairing the ends with T4 DNA polymerase, and ligating the plasmid to the stop codon linker. pBS:E3b-Sm and pBS:E3b-M were constructed by opening pBS:E3b at the unique *Sma*I (position 97027) and *Msc*I (position 97302) sites and ligating the plasmid to the stop codon linker. pBS:E3b-A was constructed by opening pBS:E3b at the unique *Afl*II site (position 97793), repairing the ends, and ligating the plasmid to the stop codon linker. pBS:E3b-SS was constructed by digesting pBS:E3b with *Spe*I (position 95683) and *Sma*I, repairing the *Spe*I-cut end with T4 DNA polymerase, and ligating the plasmid to itself. All six mutants were digested with *Eco*RI and *Xba*I to release the fragments and were subsequently, after repair of the ends with T4 DNA polymerase, ligated to pSC11, which had been opened at the unique *Sma*I site. Appropriate orientations of inserts relative to the p7.5 promoter of pSC11 were selected for subsequent transfection. Vaccinia virus recombinants were produced by standard techniques (19). Briefly, CV-1 cells were infected with vaccinia virus strain WR and, subsequently, transfected with the above DNA constructs. After 48 h, the virus was harvested and plated onto TK^-143 cells in the presence of 25 μg of bromodeoxyuridine. After three plaque purifications, the viral isolates were amplified twice in the presence of bromodeoxyuridine and finally grown in CV-1 cells without selection. Viruses were titrated in CV-1 cells. Fibroblasts from HLA A11-positive donors were infected as previously described (3) for 12 h at a multiplicity of infection of 5 before being used as targets in the cytotoxicity tests. Under these conditions, the viability was higher than 90%, as a rule.

Detection of EBNA4 in vaccinia virus-infected cells. Protein samples were prepared from 3×10^6 infected cells, separated in 7.5% polyacrylamide-SDS gels (14), blotted onto nitrocellulose filters (25), and probed with the human serum A1 containing high titers of antibody to EBNA1 to -6. The serum was used at a 1:15 dilution. EBNA staining was performed as previously described (3) by indirect immunofluorescence using fluorescein-conjugated rabbit anti-human immunoglobulin G (Dakopatts).

Synthetic peptides. Peptides, synthesized by the Merrifield solid-phase method (10), were purchased from Alta Bioscience, University of Birmingham School of Biochemistry, Birmingham, United Kingdom. The whole EBNA4 protein deduced from the prototype B95-8 DNA sequence was covered by a total of 185 14- or 15-amino-acid synthetic peptides overlapping by 10 amino acids. In addition, all the possible 9-mers corresponding to the 416-to-429 14-mer were also synthesized. All peptides were dissolved in dimethyl sulfoxide at the concentration of 10^{-2} M and further diluted in PBS to obtain the indicated concentrations before the cytotoxicity assays. The protein concentrations of the dimethyl sulfoxide stock solutions were determined by a biuret assay (2). Where indicated, the peptides were HPLC purified by running 200 μg of peptide resuspended in 500 μl of H_2O

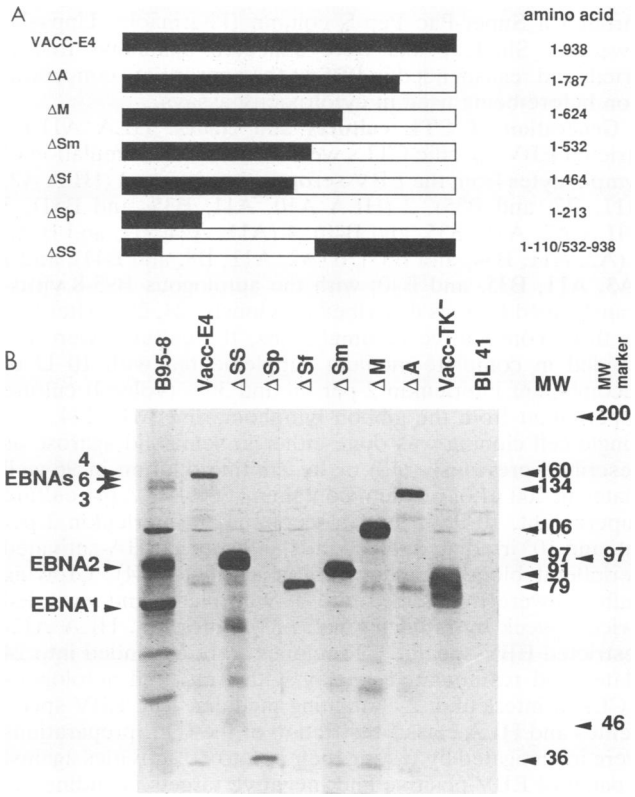

FIG. 1. (A) Schematic diagram illustrating the EBNA4 (B95-8 strain) deletion mutants encoded by recombinant vaccinia viruses. The full-length (938 amino acids) EBNA4 protein encoded by Vacc-E4 (Vacc-EBNA4) is represented by a filled bar. Each deletion (Δ) vaccinia virus recombinant has the capacity to encode a truncated EBNA4 protein indicated by the filled area of the bar and containing the amino acid sequence shown. Note that the ΔSS construct contains an internal deletion which removes residues 111 to 531 of the EBNA4 sequence. (B) Immunoblot demonstrating the expression of truncated EBNA4 proteins by the recombinant vaccinia viruses. Protein extracts of fibroblasts infected for 12 h with the various EBNA4 recombinant vaccinia viruses at a multiplicity of infection of 5 were separated by SDS-polyacrylamide gel electrophoresis, and immunoblots were probed with human serum A1 with high titers of antibodies to EBNA1 to -4 and -6. The expression of those EBNAs in B95-8-transformed cells is apparent from the B95-8 reference track; the EBV-negative cell line BL41 served as a negative control. The positions and molecular weights (MW) (in thousands) of the full-length and the truncated forms of EBNA4 and of MW markers are indicated on the right. Note that levels of vaccinia virus-encoded EBNA4 proteins are considerably higher than the level of the full-length virus-encoded EBNA4 in B95-8 cells. The control track of fibroblasts infected with the Vacc-TK^- recombinant shows a broad band of proteins detected by the serum A1 in the 70- to 90-kDa region of the gel; these proteins are likely to be vaccinia virus-related products.

through a Super-Pac Pep-S column (Pharmacia, Uppsala, Sweden). Single peaks were collected manually, freeze-dried, and resuspended in PBS at the appropriate concentration before being used in cytotoxicity assays.

Generation of CTL cultures and clones. HLA A11-restricted EBV-specific CTLs were obtained by stimulation of lymphocytes from the EBV-seropositive donors 1 (HLA A2, A11, B7, and B35), 2 (HLA A10, A11, B35, and B51), 3 (HLA A2, A11, B35, and B40), 4 (A11, A24, B7, and B35), 5 (A2, A11, B44, and B55), 6 (A2, A11, B8, and B44), and 7 (A3, A11, B35, and B40) with the autologous B95-8 virus-transformed LCL as described previously (24, 28). After two or three consecutive restimulations, the cultures were expanded in complete medium supplemented with 10 U of recombinant interleukin 2 per ml and 30% (vol/vol) culture supernatant from the gibbon lymphoma line MLA144 (21). Single-cell cloning was done either in semisolid agarose as described previously (17) or by limiting dilution in 96-well plates in 200 μl of medium containing 30% MLA144 culture supernatant, 10 U of human recombinant interleukin 2 per ml, and 10^5 irradiated (3,000 rads) allogeneic PHA-activated peripheral blood lymphocytes as a feeder (24). Growing cultures were transferred into 48-well plates and were fed twice a week by replacing half of the medium. HLA A11-restricted EBV-specific CTL clones were expanded into 24 plates and restimulated weekly with irradiated autologous LCLs in interleukin 2-containing medium. The EBV specificities and HLA class I restriction of the CTL preparations were investigated by testing their cytotoxic activities against a panel of EBV-positive and -negative targets including the autologous LCLs, allogeneic LCLs and PHA-activated blasts matched through single HLA class I alleles, HLA-mismatched LCLs, and the prototype NK-sensitive target, K562.

Cytotoxicity assay. The cytotoxic activity was assayed in standard 5-h ^{51}Cr release assays (24). The targets were labelled with $Na^{51}CrO_4$ (0.1 μCi/10^6 cells) for 1 h at 37°C. The cytotoxicity tests were routinely run at effector/target ratios of 10:1, 3:1, and 1:1 in triplicate. Under the conditions of vaccinia virus infection described above, the spontaneous release of uninfected and infected cells did not exceed 15% of the total incorporation, as a rule. In order to test the abilities of synthetic EBNA4 peptides to sensitize PHA blasts to CTL lysis, 20 μl of each peptide was added to 4×10^3 ^{51}Cr-labelled cells (in 20 μl of complete medium) in triplicate V-shaped wells of 96-well plates. The plates were incubated for 1 h at 37°C before addition of CTLs (4×10^4 per well in 100 μl). Peptide toxicities were checked for each assay and were always ≤3%.

RESULTS

Expression of truncated EBNA4 proteins in recombinant vaccinia virus-infected fibroblasts. Recombinant vaccinia viruses carrying different parts of the EBNA4-coding sequence (Fig. 1A) were screened for expression of the relevant EBNA4 fragment after infection of human fibroblasts. Figure 1B shows the results obtained when protein extracts from fibroblasts infected for 12 h at a multiplicity of infection of 5 were immunoblotted and probed with the polyclonal human serum A1. This serum contains high titers of antibodies to all the individual EBNAs expressed in B95-8 cells including EBNA4 and readily detects the full-length EBNA4 and the truncated species which are expressed at much higher levels from the recombinant vaccinia virus vectors. Specific bands of 134, 106, 91, 79, 36, and 97 kDa were detected in immunoblots of fibroblasts infected with recombinant viruses carrying the ΔA, ΔM, ΔSm, ΔSf, ΔSp, and ΔSS deletions, respectively. These apparent molecular sizes are broadly in line with the expected sizes of the mutant EBNA4 gene products. Expression of EBNA4 and its truncated variants was also examined at the single-cell level by immunofluorescence. This confirmed that, in each case, more than 90% of the infected fibroblasts expressed the relevant EBNA4 protein (data not shown).

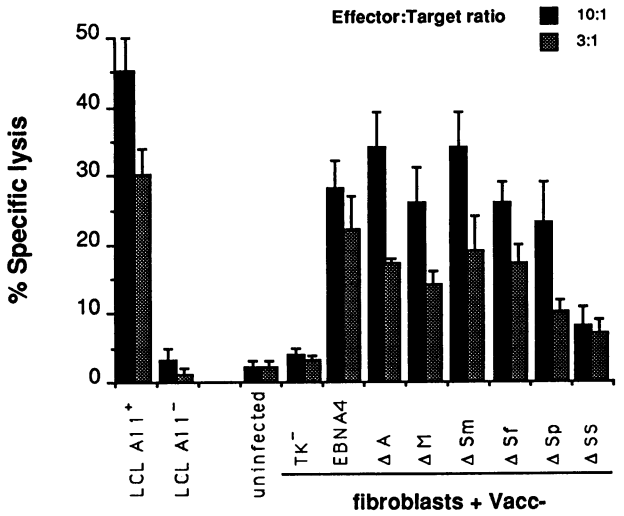

FIG. 2. Sensitivity of recombinant vaccinia virus-infected fibroblasts to polyclonal HLA A11-restricted EBNA4-specific CTL cultures. Uninfected and recombinant vaccinia virus-infected fibroblasts from the HLA A11-positive donor 8 (HLA A11, B13, and B51) were tested for sensitivity to lysis by EBV-specific A11-restricted CTLs obtained by stimulation of lymphocytes from the EBV-seropositive donor 1 (HLA A2, A11, B7, B62) with the autologous B95-8 virus-transformed LCL. The means ± standard errors of percent specific lysis recorded at effector/target ratios of 10:1 and 3:1 in four separate experiments are shown. The specificity of the effector population was in each case confirmed by the capacity to lyse allogeneic HLA A11-positive but not HLA A11-negative LCLs. In addition, HLA A11-positive T blasts and the prototype NK-sensitive target, K562, were not lysed (data not shown).

Mapping of HLA A11-restricted EBNA4 epitopes. (i) Mapping with recombinant vaccinia viruses. Experiments in which HLA A11-restricted EBNA4 epitopes were mapped by using recombinant vaccinia viruses used EBV-specific polyclonal CTL preparations from seven different HLA A11-positive donors, each containing a strong A11-restricted component. The EBV specificity and HLA A11 restriction of the CTL cultures were first assessed with a standard panel of targets including autologous PHA blasts and autologous HLA A11-matched and -mismatched B95-8 virus-transformed LCLs. HLA A11-restricted EBV-specific CTL cultures were then assayed for their abilities to lyse fibroblasts which were matched with the CTLs only through HLA A11 and which had been infected with the panel of EBNA4-carrying vaccinia recombinants. Figure 2 shows the mean results of four successive experiments performed with effector populations from one of the donors, donor 1. As expected, fibroblasts expressing the full-length EBNA4 protein were lysed by these effectors. While expression of EBNA4 fragments containing the first 787 and 624 residues (as encoded by the Vacc-ΔA and -ΔM recombinants, respec-

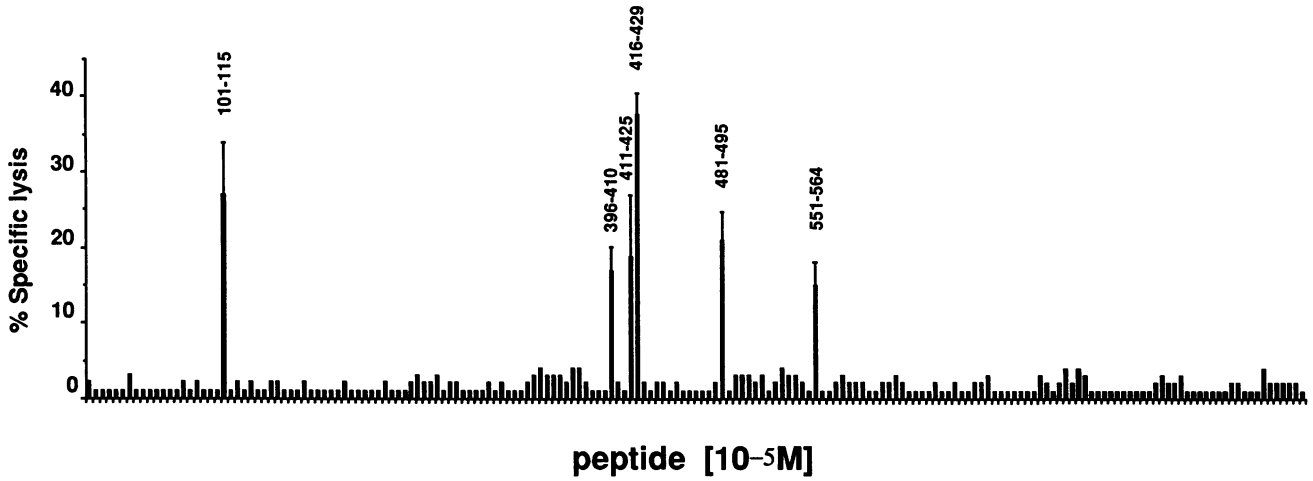

FIG. 3. Recognition of EBNA4 peptides by HLA A11-restricted polyclonal cultures. HLA A11-positive PHA blasts were pretreated at 37°C for 1 h with a 10^{-5} M concentration of synthetic peptides before the cytotoxicity assay. Synthetic peptides covering the entire EBNA4 sequence were synthesized as a series of 14- or 15-mers overlapping by 10 amino acids; results for all 185 peptides tested are shown from left (N-terminal region) to right (C-terminal region) along the horizontal axis. The effector populations were polyclonal CTL cultures derived from seven donors, each matched with the targets through HLA A11 only. The data are means ± standard errors of percent specific lysis recorded at a 10:1 effector/target ratio in 5 to 20 experiments with each peptide. Peptides mediating significant levels of lysis are identified above the corresponding bars.

tively) consistently gave similar levels of lysis, we observed a slightly reduced level of lysis for fibroblasts infected with the Vacc-ΔSm, -ΔSf, and -ΔSp recombinants, expressing the first 532, 484, and 213 residues, respectively. In contrast, fibroblasts infected with the Vacc-ΔSS recombinant, which carries an internal deletion between amino acids 110 and 532, were recognized much less well, although still at a level significantly above the background level of fibroblasts infected with the control Vacc-TK$^-$ recombinant. The above pattern of reactivity was reproduced by polyclonal CTLs from six of the seven HLA A11-positive donors tested. Results from the remaining donor, donor 3, were similar except that in this case fibroblasts infected with Vacc-ΔSp (1 to 213) and Vacc-ΔSS (internal deletion 110 to 532) were not recognized.

(ii) Mapping with synthetic peptides. The fine specificity of A11-restricted EBNA4-specific effectors was further investigated by using as targets HLA A11-positive PHA blasts preincubated with a 10^{-5} M concentration of synthetic peptides (14- or 15-mers overlapping by 10 amino acids) covering the predicted amino acid sequence of the B95-8 strain EBNA4. Initial experiments, using polyclonal effector populations from three donors (donors 1, 3, and 6) to screen across the complete range of 185 synthetic peptides, identified six peptides which were able to sensitize the PHA blasts to lysis; these were peptides 101–115, 396–410, 411–425, 416–429, 481–495, and 551–564. Subsequently, polyclonal CTLs from the seven A11-positive donors were tested in repeated experiments with these peptides and, as a control, with peptides from adjacent parts of the molecule. Figure 3 is a compilation of the results obtained in this series of experiments, showing the mean level of lysis observed for each peptide. Note that two of the peptides (411–425 and 416–429) identified in this work were overlapping and, as shown below, contain the same epitope; since the 416–429 peptide was consistently more active, we will refer to this as the 416–429 epitope region. In summary, the above peptide screening identified five different epitope regions of EBNA4.

The recognition of these epitopes differed among the seven CTL donors. Table 1 summarizes the individual patterns of results obtained. Peptide 416–429 was strongly recognized in all experiments with effector populations derived from all seven donors; thus, the level of lysis of blasts sensitized with this peptide was consistently as high as that observed with the same assays for an HLA A11-positive LCL. By comparison, peptides from the other four epitope

TABLE 1. Recognition of HLA A11-restricted EBNA4 epitopes by CTLs from different HLA A11-positive donors[a]

Peptide	Reactivity of CTLs from donor:						
	1	2	3	6	4	5	7
101–115	++	+	−	+	+	NT	+
396–410	+	+	++	++	+	++	+
416–429	+++	+++	+++	+++	+++	+++	+++
481–495	+	+	−	+	+	+	+
551–564	+	+	−	−	NT	NT	NT

[a] HLA A11-restricted EBNA4-specific polyclonal CTL cultures from seven donors were tested for their capacities to lyse HLA A11-matched PHA blasts which had been preincubated with a 10^{-5} M concentration of each of the indicated 15-mer. At least two independent CTL cultures were tested for each donor in 2 to 10 different experiments. Reactivities relative to the reactivity of a reference HLA A11-positive LCL were scored as follows: +++, comparable level of lysis observed in all experiments; ++, up to 50% lower levels of lysis observed in the majority or all experiments; +, up to 50% lower levels of lysis recorded in at least two separate experiments; −, no lysis recorded in at least two separate experiments. NT, not tested.

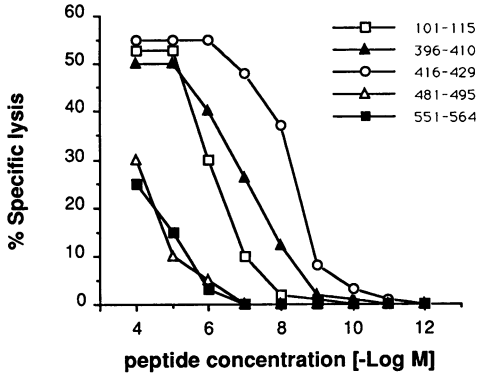

FIG. 4. Titration of EBNA4-derived synthetic peptides. HLA A11-positive PHA blasts (donor 8) were preincubated for 1 h with 10-fold serial dilutions of peptides 101–115, 396–410, 416–429, 481–495, and 551–564 and then used as targets for polyclonal A11-restricted CTL cultures from donor 1. The data are percent specific lysis recorded at a 10:1 effector/target ratio in one representative experiment of four.

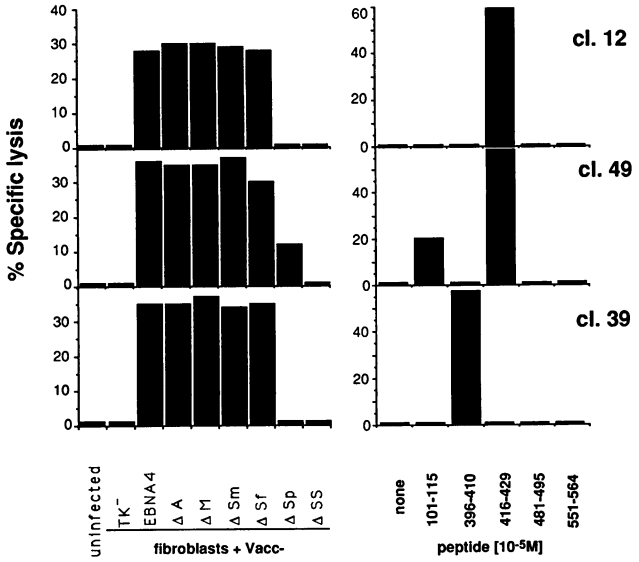

FIG. 5. Recognition of EBNA4 epitopes by HLA A11-restricted CTL clones. HLA A11-restricted CTL clones derived from donor 1 were tested for cytotoxicity against HLA A11-matched fibroblasts infected with the indicated panel of vaccinia virus recombinants encoding truncated forms of EBNA4 (as in Fig. 2) and against HLA A11-positive PHA blasts pretreated with the indicated synthetic peptides (as in Fig. 3). Different patterns of cytotoxic response are represented by one clone for each category. Of 26 clones investigated, 22 showed the pattern of reactivity of cl.12 and 3 showed the pattern of cl.49; cl.39 was unique. The data are percent specific lysis recorded at a 10:1 effector/target ratio in one experiment of three with each clone-target combination.

regions generally produced lower levels of lysis and/or were not recognized by CTLs from some of the donors. Thus, reactivity against peptide 396–410 was detected, albeit at different levels, for all seven donors, while reactivities against peptides 101–115 and 481–495 were each detected for all but one donor; recognition of peptide 551–564 was observed for only two of the four donors. Furthermore, we noted that the dominant activity against peptide 416–429 was retained upon prolonged in vitro growth of the polyclonal CTL cultures whereas the activities against the other peptides tended to decline.

The efficiencies with which the individual peptides sensitized PHA blasts to lysis were assessed by using a polyclonal effector population from donor 1, which showed reactivities to all five epitope regions. Figure 4 shows representative results from such an experiment, in which sensitization was titrated across a 10^{-4} to 10^{-13} M range of peptide concentrations. Peptide 416–429 was by far the most efficient, giving a titration curve with a half-maximal lysis point of 5×10^{-9} M. Higher concentrations were required to achieve half-maximal lysis with the other four peptides, with peptides 396–410 and 101–115 being 10^2-fold less efficient and peptides 481–495 and 551–564 being at least 10^4-fold less efficient than peptide 416–429. Note that similar titration curves were obtained whether we used HPLC-purified 15-mers (Fig. 4) or the original peptide preparations used in the first screening assay; thus, the different efficiencies of sensitization are likely to reflect properties of the individual 15-mer peptides rather than the presence of other contaminants.

Recognition of EBNA4 epitopes by HLA A11-restricted clones. We next attempted to dissect these reactivities by cloning the HLA A11-restricted CTL responses from three of the donors, donors 1, 6, and 7. A total of 26 A11-restricted clones isolated from polyclonal cultures of donor 1 were tested for recognition of the five epitope regions within EBNA4 by using both the panel of recombinant vaccinia virus-infected fibroblast targets described earlier and, in parallel assays, PHA blasts preincubated with 10^{-5} M concentrations of peptides 101–115, 396–410, 416–429, 481–495, and 551–564. Three different patterns of clonal reactivity were observed, and these are illustrated in Fig. 5. The majority of clones (22 of 26) followed the pattern shown by cl.12; such clones lysed fibroblasts infected with Vacc-EBNA4, -ΔA, -ΔM, -ΔSm, and -ΔSf and recognized only blasts preincubated with the 416–429 peptide. Another 3 of the 26 clones followed the pattern shown by cl.49 and lysed fibroblasts infected with Vacc-EBNA4, -ΔA, -ΔM, -ΔSm, -ΔSf, and, to a lesser extent, -ΔSp. Interestingly, these cells recognized blasts preincubated with peptide 416–429 and, to a lesser extent, those preincubated with peptide 101–115. This pattern of reactivity was maintained upon recloning in limiting dilution from a seeding of 0.1 cell per well. Finally, one clone, cl.39, showed the same pattern of reactivity on vaccinia virus-infected fibroblasts as cl.12 but selectively recognized only blasts preincubated with peptide 396–410.

Analysis of a smaller number of A11-restricted CTL clones obtained from donors 6 and 7 confirmed the dominance of peptide 416–429 reactivity. For both donors, the majority of clones (5 of 6 from donor 7 and 3 of 4 from donor 6) displayed a pattern of killing similar to that of cl.12 (donor 1). In each case, the remaining clones showed a dual peptide recognition like that illustrated for cl.49 (donor 1) (data not shown).

Identification of 9-mer peptide 416–424 as the cognate HLA A11-restricted EBNA4 epitope. As the results shown in Fig. 4 and 5 indicate, 14-mer peptide 416–429 (IVTDFSVI KAIEEE) appears to contain the dominant A11-restricted EBNA4 epitope. Since earlier experiments with other HLA class I molecules indicate a preference for peptides of 8 or 9 residues in length, all possible 9-mers contained in the above 14-mer were synthesized and titrated, alongside the 14-mer, for their abilities to sensitize PHA blasts to A11-restricted EBNA4-specific lysis. As shown in Fig. 6, the 9-mer 416–424 (IVTDFSVIK) was active even at very low concentrations,

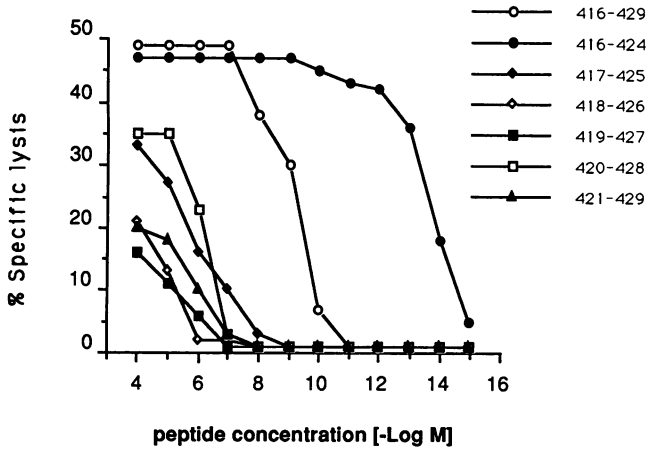

FIG. 6. EBNA4 peptide 416–424 is the cognate epitope. HLA A11-positive PHA blasts (donor 8) were preincubated for 1 h with 10-fold serial dilutions of peptide 416–429 and its derived 9-mers and then used as a target for A11-restricted CTLs (cl.12 [donor 1]) previously shown to be reactive against peptide 416–429. The data are percent specific lysis recorded at a 10:1 effector/target ratio in one representative experiment of four, with each experiment involving a different clone from donor 1.

reaching a half-maximal lysis point at 5×10^{-14} M, and was thus 10^4 times more efficient than the original 14-mer 416–429. The other 9-mers were significantly less active, with half-maximal lysis points at concentrations between 10^{-5} and 10^{-6} M; these peptides were, therefore, virtually inactive at a concentration of 10^{-7} M, whereas the 416–424 and 416–429 peptides still induced maximal lysis. The data in Fig. 6, obtained by using cl.12 from donor 1, were reproduced in subsequent peptide titration assays with three other donor 1 CTL clones.

DISCUSSION

Detailed analysis of the human CTL response to viral infections has been confined mainly to three systems, influenza virus, human immunodeficiency virus, and EBV. The early work with influenza virus clearly showed the influence of the responder's HLA class I genotype upon target epitope choice and, in accordance with the results with animal models, suggested that responses restricted through a particular HLA class I antigen were directed largely towards a single immunodominant epitope (4, 15). Somewhat in contrast, more recent work with human immunodeficiency virus-infected individuals has indicated a greater variety of target choice and revealed examples in which multiple peptide epitopes, sometimes all derived from the same viral antigen, were recognized in the context of the same restricting determinant (for a review, see reference 20). However, these studies did not seek to determine the relative importance of these multiple epitopes within the context of the overall response. Work on the EBV system conducted to date has, likewise, identified certain alleles (for example, HLA B8) which can present epitopes from several different EBV target proteins, while other alleles show a strict preference for one particular protein (for example, the preference of HLA A11 for presentation of EBNA4) (3, 11, 18). The latter combination is particularly interesting because A11-restricted EBNA4-specific responses tend to be the major component in polyclonal CTL cultures reactivated from HLA A11-positive individuals (17, 26). Here, we show that while this strong response is a composite of multiple reactivities against different peptide epitopes within the EBNA4 protein one of the epitopes is, in fact, immunodominant for all the donors tested.

It was already clear from the initial series of experiments using vaccinia virus recombinants expressing progressively truncated EBNA4 species that most, but not all, CTL reactivities mapped to sequences within the 101–532 fragment deleted from the Vacc-ΔSS recombinant (Fig. 2). Furthermore, the fact that the CTLs of at least one of the donors (donor 3) failed to recognize the Vacc-ΔSp recombinant (1–213) suggested that the 110-to-532 region contains multiple CTL epitopes. Such indications were confirmed with the peptide sensitization assays, which clearly identified five epitope regions within the EBNA4 primary sequence (Fig. 3). CTL preparations from all seven donors consistently showed recognition of the 416–429 peptide, whereas recognition of the other epitopes either tended to be lost on serial passage or, for some donors, was never detected (Table 1). Two further observations support the view that the individual epitopes are differentially immunogenic. Firstly, when the 15-mer peptides were titrated in sensitization assays using polyclonal CTL effectors with reactivities to all five epitopes, the 416–429 peptide was active at 10^2- to 10^4-fold lower concentrations compared with the other peptides (Fig. 4). Secondly, cloning experiments with CTLs from three different donors yielded an overwhelming frequency of 416–429-specific clones. These experiments raised the possibility that recognition of the 101–115 epitope by polyclonal populations may be due to the presence of a small subset of CTLs which are cross-reactive with both 416–429 and 101–115. It is clear, however, that the 396–410-specific CTLs represent a separate population. The failure to isolate clones reactive to the 481–495 and 551–564 epitopes is in line with the peptide titration assays, which indicate that these are the least abundant reactivities within the overall response.

Given the strong immunodominance of the 416–429 peptide reactivity in HLA A11-positive donors, we sought to identify the optimal peptide epitope. The analysis of 9-mer peptides from this region clearly identified the 416–424 peptide IVTDFSVIK as by far the most active (Fig. 6). Indeed, sensitization by this peptide was effective even at extremely low concentrations (half-maximal lysis at 5×10^{-14} M). To our knowledge, this is the lowest effective concentration yet reported for a synthetic peptide epitope in CTL sensitization assays and corresponds to a peptide loading of, at most, 150 molecules per target cell for half-maximal lysis. We, therefore, infer that 416–424 is the cognate target peptide for A11-restricted lysis.

At least three parameters could influence the different relative immunogenicities of the five epitopes identified here. One obvious possibility is that they have different affinities for the peptide-binding groove of HLA A11 molecules, and this possibility is currently being tested. Another possibility is that these epitopes are generated with different efficiencies during the natural processing of EBNA4 molecules in EBV-transformed cells. Either phenomenon could result in differential representation of the individual epitopes within the cell surface pool of HLA A11-peptide complexes. Furthermore, we cannot exclude the possibility that differences in the T-cell repertoires of individual donors can bias the response (see, for example, the lack of reactivity of CTLs from donor 3 against peptide 101–115, which was apparent with both recombinant vaccinia virus-infected targets and in

peptide sensitization assays). In addition, we should stress that there are occasional HLA A11-positive EBV-immune donors (not studied here) who do not generate detectable HLA A11-restricted responses on in vitro reactivation of CTLs using autologous B cells transformed with the B95-8 virus strain. This again may reflect individual differences in the T-cell repertoire, as suggested by the failure to induce HLA B8-restricted EBNA3-specific CTLs for one EBV-immune donor (23). Alternatively, these particular donors may be infected with a viral strain lacking the most immunogenic HLA A11-restricted epitope; we have indeed recently identified rare EBV strains with a nonconservative substitution at position 424 of the EBNA4 sequence, which abrogates CTL recognition (1).

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