

HLA A2.1-Restricted Cytotoxic T Cells Recognizing a Range of Epstein-Barr Virus Isolates through a Defined Epitope in Latent Membrane Protein LMP2

S. P. LEE,¹ W. A. THOMAS,¹ R. J. MURRAY,¹ F. KHANIM,¹ S. KAUR,¹ L. S. YOUNG,¹ M. ROWE,¹
M. KURILLA,² AND A. B. RICKINSON^{1*}

Cancer Research Campaign Laboratories, Department of Cancer Studies, University of Birmingham, Birmingham B15 2TJ, United Kingdom,¹ and Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908²

Received 21 July 1993/Accepted 13 September 1993

Cytotoxic T-lymphocyte (CTL) responses induced by persistent Epstein-Barr virus (EBV) infection in normal B-lymphoid tissues could potentially be directed against EBV-positive malignancies if expression of the relevant viral target proteins is maintained in tumor cells. For malignancies such as nasopharyngeal carcinoma and Hodgkin's disease, this will require CTL targeting against the nuclear antigen EBNA1 or the latent membrane proteins LMP1 and LMP2. Here we analyze in detail a B95.8 EBV-reactivated CTL response which is specific for LMP2 and restricted through a common HLA allele, A2.1. We found that in vitro-reactivated CTL preparations from several A2.1-positive virus-immune donors contained detectable reactivity against A2.1-bearing target cells expressing either LMP2A or the smaller LMP2B protein from recombinant vaccinia virus vectors. Peptide sensitization experiments then mapped the A2.1-restricted response to a single epitope, the nonamer CLGGLTMV (LMP2A residues 426 to 434), whose sequence accords well with the proposed peptide binding motif for A2.1. Most Caucasian and African virus isolates (whether of type 1 or type 2) were identical in sequence to B95.8 across this LMP2 epitope region, although 2 of 12 such isolates encoded a Leu→Ile change at epitope position 6. In contrast, most Southeast Asian and New Guinean isolates (whether of type 1 or type 2) constituted a different virus group with a Cys→Ser mutation at epitope position 1. CTLs raised against the B95.8-encoded epitope were nevertheless able to recognize these variant epitope sequences in the context of A2.1 whether they were provided exogenously as synthetic peptides or generated endogenously in B cells transformed with the variant viruses. A CTL response of this kind could have therapeutic potential in that it is directed against a protein expressed in many EBV-positive malignancies, is reactive across a range of virus isolates, and is restricted through a relatively common HLA allele.

Epstein-Barr virus (EBV), a gammaherpesvirus widespread in human populations, appears to persist in the B-lymphoid system through one or more forms of latent infection (19, 37, 49). Since at least one such form of latency leads to growth transformation of the infected B cell (24), maintenance of asymptomatic virus carriage in vivo and protection from lymphoproliferative disease almost certainly depend upon efficient host controls. In this context one important effector mechanism appears to be mediated by HLA class I-restricted cytotoxic T lymphocytes (CTLs) (31). Thus, memory CTLs specific for EBV can readily be reactivated from the blood of healthy virus-carrying individuals by in vitro stimulation with the autologous EBV-transformed B-lymphoblastoid cell line (LCL). Furthermore, immunosuppressive regimens specifically directed against T-cell function render patients susceptible to EBV-associated lymphoproliferations (42, 52).

In this as in other viral systems, the CTL response recognizes viral antigens in processed form as peptide fragments presented at the cell surface in association with HLA class I molecules (47). Thus, all of the EBV latent proteins constitutively expressed in LCLs, including the six nuclear antigens EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP and the two latent membrane proteins LMP1 and LMP2 (24), are potential sources of target peptides, and for any one individual, target antigen choice will be heavily

influenced by their HLA class I type. Studies of more than 30 EBV-immune individuals with a range of HLA backgrounds showed that reactivities against one or more of the EBNA3A-EBNA3B-EBNA3C family of proteins were very often major components of the memory CTL response (23, 33), and in several such cases the immunodominant viral peptides have now been identified (5, 8, 18). The original studies (23, 33) also revealed less frequent examples of responses directed against EBNA2, EBNA-LP, LMP1, and LMP2 but significantly, to date, not against EBNA1.

One of the most interesting implications of these findings concerns the efficiency of the EBV-induced CTL response as a control against EBV-positive malignancies (34). On one hand, immunoblastic lymphomas express all the known virus latent proteins (20, 50) and accordingly remain sensitive to a recovery of the virus-specific CTL response (46). In contrast, endemic Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), and the malignant cells of EBV-positive Hodgkin's disease (HD) are much more likely to escape immune control because the frequently immunodominant target antigens such as EBNA3A, EBNA3B, and EBNA3C are not expressed in tumor cells (34). Thus, virus latent protein expression is restricted to EBNA1 in BL (38) and to EBNA1, LMP1, and LMP2 in NPC and HD (6, 9, 12, 15, 45, 51). This highlights the potential importance for immunotherapy of those components of the virus-induced CTL response directed towards epitopes from this restricted subset of latent proteins.

In our earlier studies, the best documented example of such

* Corresponding author.

a response involved LMP2 as the target antigen and HLA A2.1 as the presenting allele (33). Although this particular reactivity was never strong in *in vitro*-reactivated CTL preparations, it was clearly worthy of further investigation since HLA A2.1 is a very common allele in human populations (22). Here we analyze this response in further detail, identify the LMP2 peptide epitope presented by HLA A2.1, and screen a range of geographically distinct EBV isolates for conservation of that epitope.

MATERIALS AND METHODS

Cell lines. LCLs were generated by transforming B cells from donors of known HLA type with the reference type 1 EBV isolate B95.8 (29) and, in the case of donor 7 (HLA A2.1,11; B27,40), with a number of other viral isolates of different geographical origin and including representatives both of type 1 and of type 2 (40). The BL-derived cell line Eli-BL (HLA A2.1,23; B7,22) was isolated from an African patient and has been described previously (36). All the above cell lines were cultured in RPMI 1640 (GIBCO, Paisley, United Kingdom) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU of penicillin per ml, and 100 µg of streptomycin per ml (growth medium). Fibroblast cultures were established from small skin biopsy samples from two normal donors (HLA A2.1,29; B8,40 and HLA A2.1,11; B35,40) and were cultured in DME (GIBCO) supplemented as described above.

Recombinant vaccinia viruses. Recombinant vaccinia virus vectors coding for each of the EBV latent proteins (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, and LMP2A) and a control vector (vTK⁻) have been described previously (33). The LMP2B vaccinia virus recombinant (vLMP2B) was likewise generated with an LMP2B cDNA (39), expression of the relevant protein being confirmed by immunofluorescence staining of infected cells with a NPC patient serum with high anti-LMP2 reactivity. For cytotoxicity assays, fibroblasts were infected for 2 h with recombinant vaccinia virus at a multiplicity of infection of 10:1 and then incubated in culture medium for a further 16 h before being trypsinized and used as targets in a cytotoxicity assay.

Synthetic peptides. Peptides were synthesized by J. Fox (Alta Bioscience, University of Birmingham, Birmingham, United Kingdom) using fluorenylmethoxycarbonyl chemistry and dissolved in dimethyl sulfoxide, and protein concentrations were measured by a modified biuret assay (14).

Cytotoxic T-cell lines and clones. The HLA A and B antigen types of the 15 healthy laboratory donors used in this study were as follows: donor 1, A2.1 and B14,15; donor 2, A2.1 and B18,44; donor 3, A2.1 and B7; donor 4, A2.1,11 and B8,44; donor 5, A2.1,11 and B35,40; donor 6, A2.1,24 and B7,44; donor 7, A2.1,11 and B27,40; donor 8, A2.1 and B27; donor 9, A2.1,24 and B27,35; donor 10, A2.1,3 and B7,39; donor 11, A2.1 and B27,44; donor 12, A1,2.1 and B17,55; donor 13, A2.1 and B31,44; donor 14, A2.1,3 and B15,44; and donor 15, A2.1,11 and B44,55. EBV-specific polyclonal CTLs were generated by stimulation of peripheral blood mononuclear cells from these donors with the autologous B95.8-transformed LCL (γ irradiated) at a responder-to-stimulator ratio of 40:1, followed by short-term expansion in growth medium containing interleukin-2 as described elsewhere (48). EBV-specific CTL clones were obtained by seeding autologous LCL-stimulated peripheral blood mononuclear cells in semisolid agar as described previously (32).

Cytotoxicity assays. Target cells were labelled for 1 h with ⁵¹CrO₄, washed, and incubated with CTLs in a standard 5-h

chromium release assay at known effector/target ratios. When vaccinia virus-infected targets were used, supernatants from the assay were harvested into 1% formaldehyde before counting. To screen CTLs for recognition of synthetic peptides, ⁵¹CrO₄-labelled target cells (80-µl volume) were preincubated with peptide (20 µl) in 96-well V-bottom plates for 1 h or with the appropriate dilution of dimethyl sulfoxide solvent as a control. CTLs (100 µl) were then added to the wells, and the assay was harvested after a further 5-h incubation. Peptide concentrations refer to those present in the final 200-µl volume.

DNA sequencing by polymerase chain reaction (PCR). DNA for PCR was extracted by incubating cells in a proteinase K lysis solution containing 100 µg of proteinase K per ml, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, and 0.005% Tween 80 for 1 to 2 h at 55°C. Crude DNA (10 to 100 ng) was amplified in a 100-µl reaction mix as described previously (40) by using the following oligonucleotide primers which span exon 7 of LMP2: LMP2 S1, 5'-GCAGCACTG AATTTATACCC-3' (coordinates 1176 to 1195), and LMP2 S2, 5'-GCTCCTCACTTTCCAGTGTA-3' (coordinates 1648 to 1629). Coordinates correspond to the prototype B95.8 virus strain published sequence (4). Following amplification, aliquots of the PCR mix were run out on 1.5% low-melting-point agarose (Ultrapure) gels and PCR products were purified by using a Magic PCR Preps DNA Purification System Kit (Promega) according to the manufacturer's instructions. Cycle sequencing was carried out with the Ampliqaq Cycle Sequencing Kit (Perkin-Elmer Cetus) using the oligonucleotide LMP2 S1 and 3' nested primer LMP2 SP (5'-CTAATGACC CCAAAGAGGGC-3' [coordinates 1597 to 1578]) which were ³²P end labelled with T4 polynucleotide kinase (Boehringer Mannheim).

RESULTS

Mapping of the HLA A2.1-restricted CTL response to LMP2B. From a panel of healthy EBV-carrying individuals in the laboratory, we elected to study 15 donors with the HLA A2.1 allele (see Materials and Methods for donors' HLA types). Each donor was tested for EBV-specific CTL responsiveness on at least three occasions by *in vitro* stimulation of peripheral blood mononuclear cells with the autologous B95.8 virus-transformed LCL. Effector populations with clear evidence of EBV specificity were obtained from 12 of these donors, and further testing of such effectors on a panel of appropriately HLA-matched LCLs identified 6 individuals whose response contained an A2.1-restricted component.

Figure 1 presents data from two such effector populations, from donors 1 and 3, tested on HLA A2.1-matched and on mismatched LCLs and on HLA A2.1-matched fibroblasts infected with recombinant vaccinia viruses expressing the individual EBV latent proteins. The A2.1-restricted response clearly recognized fibroblasts expressing the LMP2 protein but not any of the other vaccinia virus-infected targets. This pattern of results was obtained from all six of the relevant donors (donors 1, 2, 3, 6, 7, and 8), although in several of these individuals the A2.1-restricted reactivity was only a minor component of the total EBV-induced CTL response. Interestingly, in the one donor (donor 1) in whom the HLA A2.1 allele appeared to be the dominant restriction element for the polyclonal CTL response, we could occasionally detect a minor A2.1-restricted component recognizing EBNA3C in addition to consistent reactivity against LMP2. This alternative antigenic specificity was not observed with the other five donors.

There are two forms of LMP2 constitutively expressed in

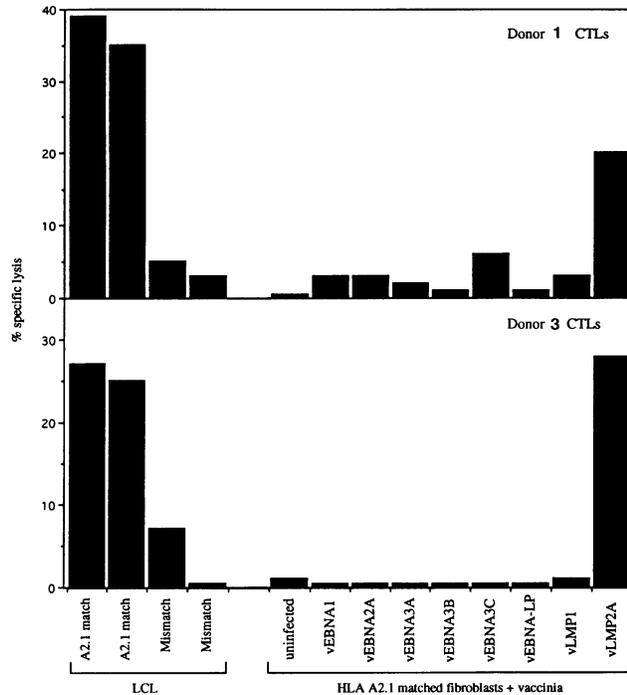


FIG. 1. Viral target antigen specificity of polyclonal CTL preparations from donors 1 (A2.1; B14, B15) and 3 (A2.1, B7). Data from representative cytotoxicity assays on fibroblast targets (HLA matched with these donors only through A2.1) either uninfected or following infection with recombinant vaccinia viruses expressing individual EBV (B95.8 strain) latent proteins. Control targets included B95.8 EBV-transformed LCLs either HLA matched through A2.1 or completely mismatched. Results are shown as percent specific lysis observed in 5-h chromium release assays at an effector/target ratio of 10:1.

LCLs, the full-length LMP2A protein (497 amino acids) and a truncated LMP2B protein which is initiated at an internal methionine representing residue 120 in the LMP2A sequence (25, 39). Using further A2.1-restricted effector populations from donors 1 and 3, we went on to test their ability to recognize fibroblasts expressing these two alternative forms of LMP2 from recombinant vaccinia virus vectors. As illustrated by representative results in Fig. 2, there was clear recognition of both the LMP2A-positive and the LMP2B-positive target cells.

Identification of the LMP2-derived target epitope. Synthetic peptides (14-mers overlapping by 10 amino acids) representing the entire LMP2B primary sequence were then prepared and screened at a standard concentration of 2×10^{-7} M for their ability to sensitize A2.1-matched target cells to LMP2-specific recognition. Note that the source of target cells in these experiments was the A2.1-positive Eli-BL cell line; although it is EBV genome positive, this line does not naturally express LMP2 (37) but was clearly recognized by A2.1-restricted CTLs following vaccinia virus LMP2 infection (data not shown). Figure 3 shows typical results of such a peptide sensitization assay using the complete panel of LMP2B peptides and polyclonal CTLs from donor 1 as the source of effectors. Significant lysis above background was observed with two overlapping 14-mer peptides representing residues 421 to 434 and 425 to 438 in the LMP2 sequence but not with any of the other peptides.

To define the target epitope(s) more precisely, we used the

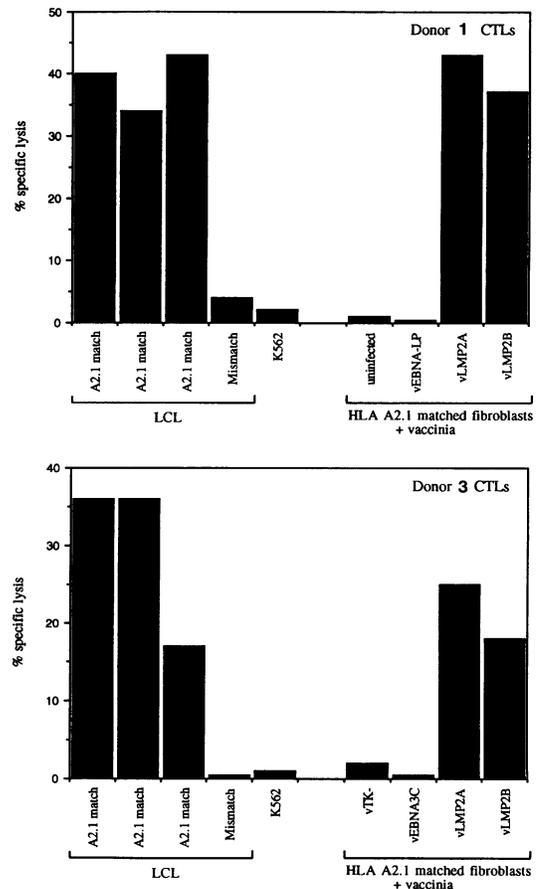


FIG. 2. HLA A2.1-restricted CTLs recognize both LMP2A and LMP2B proteins. Polyclonal CTL preparations from donors 1 and 3 were tested as in Fig. 1 on HLA A2.1-matched fibroblast targets either uninfected or following infection with a control vaccinia virus (vTK⁻) or with recombinant vaccinia viruses expressing EBV latent protein LMP2A, LMP2B, EBNA-LP, or EBNA3C. Control targets included A2.1-matched and mismatched LCLs as in Fig. 1 and the EBV-negative K562 cell line.

same approach to screen a selection of 9- and 10-mer peptides from within the region 421 to 438. LMP2-specific CTL preparations from three different A2.1-positive donors, 1, 2, and 3, were tested in this way, and each gave the same pattern of results. This is illustrated by the data in Table 1 from an experiment in which CTLs from donor 3 were assayed on targets preexposed to peptides at 2×10^{-7} and 2×10^{-8} M concentrations. Significant lysis was observed not just with the original 14-mers of residues 421 to 434 and 425 to 438 but also with the 10-mer peptide of residues 425 to 434 (i.e., the precise region of overlap between these 14-mers) and with the constituent 9-mer of residues 426 to 434 (sequence CLGGLLT MV). In contrast, other peptides tested, including the immediately adjacent nonamers of residues 425 to 433 and 427 to 435, gave only background levels of killing, thus demonstrating the importance both of the Cys residue at 426 and of the Val residue at 434. These initial observations were subsequently confirmed in peptide titration experiments of the kind illustrated in Fig. 4. In this case the A2.1-positive Eli-BL cell line was preexposed to a wide range of peptide concentrations (2×10^{-5} to 2×10^{-13} M) and then tested for lysis by CTLs from donor 3. For both 14-mer peptides of residues 421 to 434 and

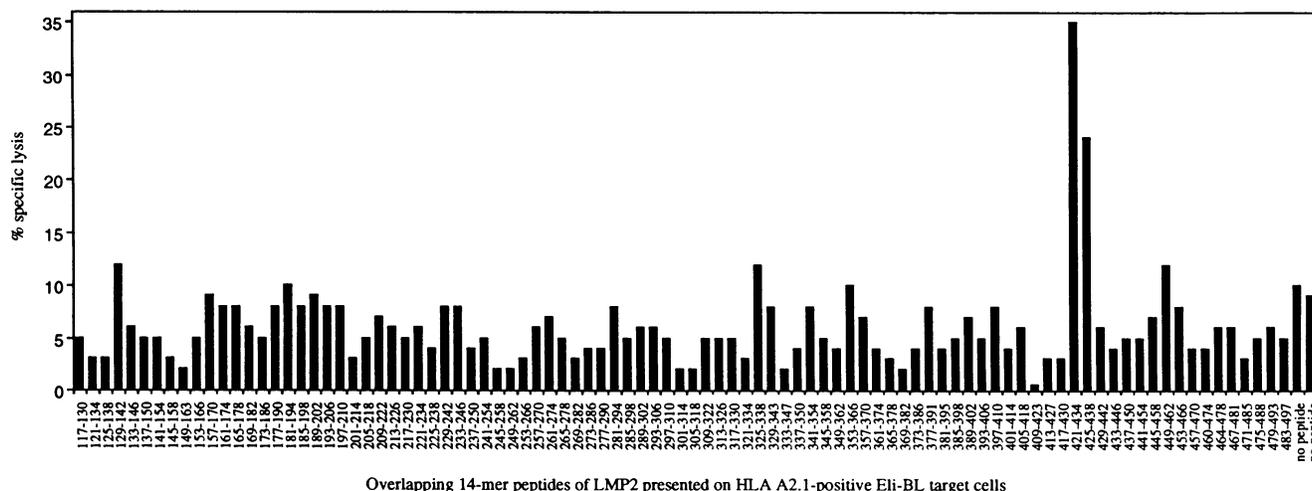


FIG. 3. Peptide screening of A2.1-restricted, LMP2-specific CTLs. Polyclonal CTL preparations from donor 1 were tested on the A2.1-matched Eli-BL cell line preexposed to a 2×10^{-7} M concentration of individual 14-mer peptides from the LMP2A sequence (representing the amino acid residues shown) or to an equivalent dilution of dimethyl sulfoxide solvent (no peptide [controls]). Results are shown as percent specific lysis observed in a 5-h chromium release assay at an effector/target ratio of 10:1.

425 to 438 and for the 10-mer of residues 425 to 434, half-maximal lysis was achieved at concentrations between 10^{-7} and 10^{-8} M, whereas the 9-mer of residues 426 to 434 was significantly more efficient, mediating half-maximal lysis at 5×10^{-10} M. Interestingly, with all four peptides titrated in this way, a reduction in the levels of lysis was observed at a high peptide concentration.

Analysis of the A2.1-restricted epitope in a range of EBV isolates. The work described above was entirely based upon CTLs activated against autologous B cells transformed in vitro by the standard type 1 virus strain, B95.8. We next sought to determine to what extent the LMP2 position 426 to 434 epitope was conserved in other virus strains; this was important because there are examples in the literature of EBV genome polymorphisms which alter target epitope sequences and thereby abrogate CTL recognition (3, 13). Exon 7 of the LMP2 gene, encoding residues 390 to 462 of the LMP2 protein

encompassing the epitope, was therefore amplified by PCR and sequenced from both the B95.8 reference strain and 18 other virus isolates. These isolates were derived from geographically distinct areas and included representatives both of the type 1 and of the type 2 virus families. Table 2 presents sequence data from these isolates for that region of LMP2 exon 7 encoding amino acid residues 421 to 438. From such data, the majority of viral isolates divided into two groupings which correlated closely with geographic origin rather than with virus type. Thus, four of five Caucasian isolates and six of seven African isolates were identical to the B95.8 sequence in the epitope region, whereas only one of four Southeast Asian isolates carried this sequence. In contrast, the three other Southeast Asian and two New Guinean isolates displayed a different sequence, having three nucleotide changes with respect to B95.8; two of these were conservative, while the third encoded a Cys→Ser mutation at position 1 of the nonamer CTL epitope. Two other isolates (one Caucasian and one African) showed a different mutation encoding a Leu→Ile change at position 6 of the epitope.

To determine to what extent such changes affected CTL recognition, nonamer peptides representing the three different epitope sequences were synthesized and tested over a range of concentrations in peptide sensitization assays. Figure 5 presents the results from an experiment in which CTLs generated from donor 3 by stimulation with autologous B95.8 virus-transformed LCL cells were assayed on peptide-treated targets of the A2.1-positive Eli-BL line. Interestingly, the Cys→Ser mutant peptide mediated higher levels of lysis than the B95.8 sequence, though the two titration curves had similar end points, while the Leu→Ile mutant was recognized but only at a 100-fold-higher peptide concentration. Finally, target LCLs were generated by in vitro transformation of B cells from the A2.1-positive donor 7 with representative EBV isolates from the panel described above. These lines were then tested for lysis by B95.8-reactivated CTL populations from donor 1 in which the only detectable EBV reactivity was against the LMP2 epitope of residues 426 to 434. Figure 6 shows the pattern of results reproducibly obtained in such experiments. All donor 7 LCLs tested were equally well recognized and

TABLE 1. Screening of peptides in LMP2 region 421 to 438

LMP2 peptide (residues)	Peptide sequence	% Lysis ^a at peptide concn of (M):	
		2×10^{-7}	2×10^{-8}
417-430	NRTYGPVFMCLGGL	2	0
421-434	GPVFMCLGGLLTMV	30	13
425-438	MCLGGLLTMVAGAV	30	23
429-442	GLLTMVAGAVWLTV	1	0
423-430	VFMCLGGL	2	6
424-432	FMCLGGLT	3	1
425-433	MCLGGLLTM	1	1
425-434	MCLGGLLTMV	28	27
426-434	CLGGLLTMV	21	27
427-435	LGGLLTMVA	6	2
429-438	GLLTMVAGAV	4	2
No peptide		0	1

^a Specific lysis of peptide-coated Eli-BL cells in a 5-h chromium release assay with LMP2-specific A2.1-restricted CTLs from donor 3 at an effector/target ratio of 20:1.

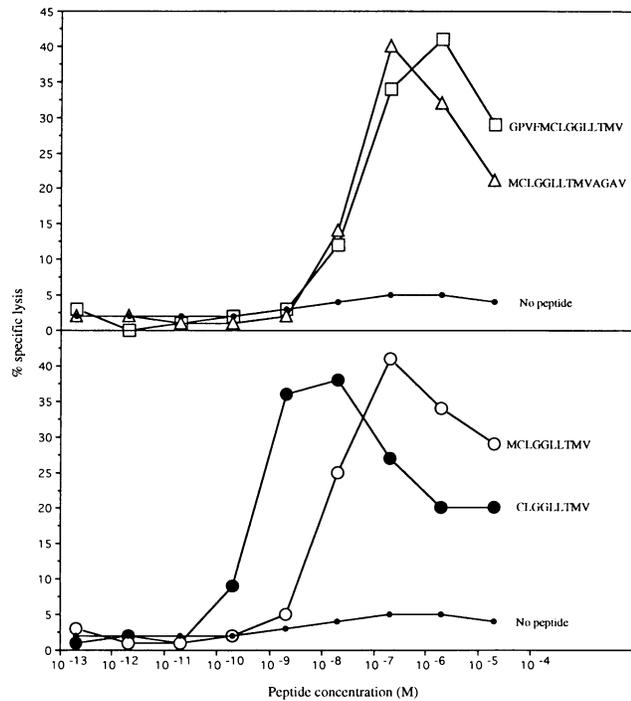


FIG. 4. Titration of different peptides from the LMP2 epitope region. Effector CTLs from donor 3 were tested on the A2.1-matched Eli-BL cell line preexposed to a series of 10-fold dilutions of the peptides LMP2 residues 421 to 434 (□), LMP2 residues 425 to 438 (△), LMP2 residues 425 to 434 (○), and LMP2 residues 426 to 434 (●) or to an equivalent dilution of dimethyl sulfoxide solvent (No peptide [control]). Results are shown as percent specific lysis observed in a 5-h chromium release assay at an effector/target ratio of 20:1; for clarity, the data from one representative experiment are presented on two graphs.

killed by these A2.1-restricted effectors, whether the resident virus encoded an LMP2 with the B95.8 epitope sequence, with the Cys→Ser mutation at position 1, or with the Leu→Ile mutation at position 6.

DISCUSSION

Recent work in several laboratories has begun to identify the major EBV antigens eliciting CTL responses in asymptomatically infected virus carriers (17, 23, 33). A natural progression therefore is to ask whether such responses can be turned to advantage in the control of EBV-positive malignancies, particularly tumors such as NPC and HD in which viral antigen expression appears to be limited to EBNA1, LMP1, and, at least from the evidence of transcriptional analysis, LMP2 (6, 9, 12, 15, 45, 51). While CTL responses to EBNA1 were not detected in studies of as many as 30 virus-immune individuals, some of these donors did show detectable reactivities against LMP1 or LMP2, albeit often as minor components of their total virus-induced response (23, 33). The present work concentrates on the most frequently observed reactivity of this type, namely, on the HLA A2.1-restricted CTL response to LMP2. This response was of interest not only in being directed against a viral protein constitutively expressed in many EBV-positive tumors but also because the HLA A2.1 allele is common in virtually all human populations (22). Lessons learned from this particular example may therefore be applicable in the long run to an unusually high proportion of patients.

The results from the recombinant vaccinia virus experiments (Fig. 1 and 2) clearly showed that the A2.1-restricted response recognizes both the 497-amino-acid LMP2A protein and the shorter LMP2B protein which lacks the 119-amino-acid N-terminal region of LMP2A (26, 39). This was an important observation not just for the subsequent epitope mapping studies but also in the context of the potential effectiveness of this response against tumors, since there appear to be some

TABLE 2. DNA sequences of EBV isolates in A2.1 CTL epitope region of LMP2

Isolate	Type	Origin	Epitope sequence ^a
B95.8	1	Caucasian	
IB	1	Caucasian	
RT	1	Caucasian	
PB	1	Caucasian	421 438
AW	2	Caucasian	GGTCCAGTTTTTATGTGCCTCGGTGGCCTGCTCACCATGGTAGCCGGCGCTGTG
BL72	1	North African	
C17	1	North African	
C18	1	North African	G P V F M C L G G L L T M V A G A V
ODHI	1	Central African	
MW1	1	Central African	
AG876	2	Central African	
YKO	1	Southeast Asian	
WW1	1	New Guinean	421 438
WW2	2	New Guinean	GGTCCCGTTTTTATGTCCCTCGGCGGCTGCTCACCATGGTAGCCGGCGCTGTG
CKL	1	Southeast Asian	
DH	1	Southeast Asian	G P V F M S* L G G L L T M V A G A V
OY	1	Southeast Asian	
BL74	1	Caucasian	421 438
MAK	1	Central African	GGTCCAGTTTTTATGTGCCTCGGTGGCCTGATCACCATGGTAGCCGGCGCTGTG
			G P V F M C L G G L I* T M V A G A V

^a Nucleotide changes with respect to the B95.8 sequence are underlined; amino acid changes are marked with an asterisk. Boxes identify the minimal epitope sequence.

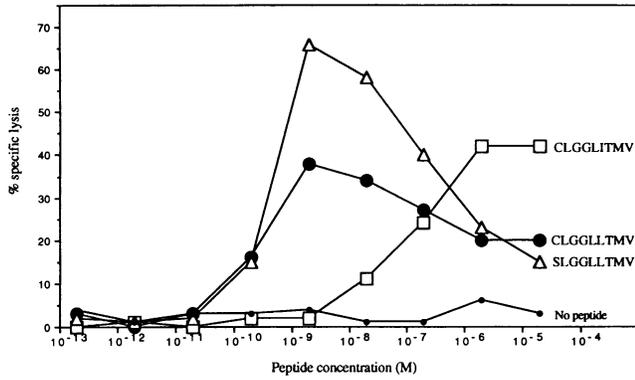


FIG. 5. CTL recognition of the B95.8 LMP2 residue 426 to 434 peptide and of variant nonamer peptide sequences from other EBV isolates. Effector CTLs from donor 3 were tested as in Fig. 4 against A2.1-matched Eli-BL cells preexposed to a series of 10-fold dilutions of the B95.8 epitope sequence (●) and of variant peptides with the Cys→Ser change at position 1 (△) or the Leu→Ile change at position 6 (□).

cases of NPC and HD in which only the LMP2B form of the protein is expressed (12, 45). By using synthetic peptides covering the 378-amino-acid sequence common to both forms of LMP2, clear evidence of A2.1-restricted CTL recognition was confined to one particular region defined by two overlapping 14-mer peptides representing residues 421 to 434 and 425 to 438 of the LMP2A sequence (Fig. 3). Subsequent studies with shorter peptides mapped recognition to a single nonamer epitope within that region (Table 1; Fig. 4). Although the original two 14-mers and the 10-mer sequence by which they overlap (425 to 434) were each capable of sensitizing targets to A2.1-restricted effectors, peptide titration experiments clearly

showed greater efficiency of sensitization by the nonamer of residues 426 to 434. Interestingly, with all four active peptides, their presence in the assay at high concentrations was associated with reduced levels of target cell lysis. We attribute this effect to increased lysis within the A2.1-positive effector cell population itself through mutual peptide presentation leading to T-cell-T-cell killing (30).

The sequence of the cognate epitope in LMP2, CLGGLL TMV, accords well with the consensus motif proposed for A2.1-binding peptides (16, 21) in that the anchor residues at positions 2 and 9 are occupied by leucine and valine, respectively. HLA refolding assays using the T2 mutant cell line (10) indeed confirmed the ability of this peptide to bind nascent A2.1 molecules. In this context, of 35 nonamer peptides from the LMP2 sequence which satisfied the A2.1-binding motifs, 30 gave significant levels of binding in the above assay and 5 bound A2.1 either as strongly as or more strongly than the residue 426 to 434 peptide (25a). Yet the A2.1-restricted CTL response to LMP2 appeared to focus only on the residue 426 to 434 peptide, with no clear evidence of reactivity against any of the other A2.1-binding sequences (Fig. 3 and data not shown).

Since all of the CTL assays had used memory cells reactivated against autologous B-cell lines carrying the B95.8 EBV isolate, it was important to determine whether the CLGGLL TMV epitope was conserved across a range of different isolates. The major subdivision between natural EBV strains is into types 1 and 2, reflecting sequence polymorphisms in the EBNA2, EBNA3A, EBNA3B, and EBNA3C genes (2, 11, 40). Given the concentration of CTL epitopes found to date in these EBNA proteins, it is not surprising that some of these epitopes are type specific in their antigenicity (41) whereas others are conserved between both virus types (7). There are in addition more subtle polymorphisms within the type 1 group of viruses which can alter epitope residues critical for CTL

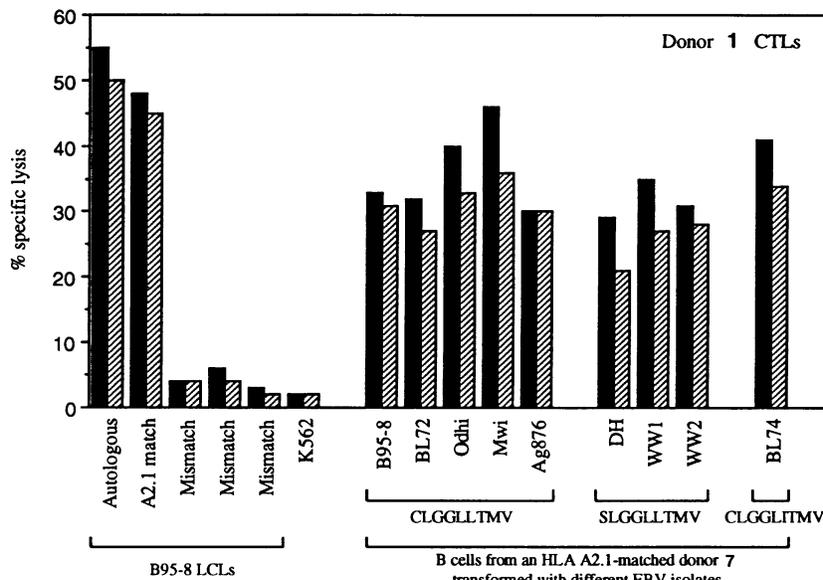


FIG. 6. HLA A2.1-restricted LMP2-specific CTLs recognize A2.1-positive LCL targets transformed with different EBV isolates. Effector CTL preparations from donor 1, in which the only EBV-specific reactivity detectable was against the LMP2 residue 426 to 434 epitope, were tested on a panel of LCL targets from the A2.1-matched donor 7 transformed with different virus isolates from Table 2 (LMP2 residue 426 to 434 target epitope sequences as shown). Control targets included B95.8 EBV-transformed LCLs, either autologous, HLA matched through A2.1, or completely mismatched, and the EBV-negative K562 cell line. Results are shown as percent specific lysis observed in a 5-h chromium release assay at effector/target ratios of 20:1 (■) and 10:1 (▨).

recognition (13), and indeed one such example sets the reference B95.8 isolate apart from all other type 1 strains (3). Sequence analysis across the LMP2 residue 426 to 434 epitope did indeed reveal some strain polymorphisms which, interestingly, correlated more closely with geographic origin of the viruses than with type 1 or type 2 status.

Thus, while most Caucasian and African isolates mirrored the B95.8 sequence, a Cys→Ser mutation at epitope position 1 was prevalent among Southeast Asian and New Guinean isolates. This adds to the evidence that Southeast Asian and New Guinean viruses, whether of type 1 or type 2, are distinguishable from other isolates at a number of polymorphic sites (1, 13, 28). The above Cys→Ser mutation did not, however, reduce the antigenicity of the peptide epitope. Indeed, CTLs generated by B95.8 LCL stimulation from donor 3, whose endogenous EBV is B95.8-like in the LMP2 epitope region (Table 2, isolate IB), produced higher overall lysis of targets precoated with the Cys→Ser mutant peptide than with the CLGGLTMTV peptide itself (Fig. 5). A second epitope mutation, Leu→Ile at position 6, was found in just two isolates, and this peptide was also recognized by B95.8-induced CTLs in peptide sensitization assays, though less efficiently (Fig. 5). Interestingly, these differences between the three target epitopes in peptide titration assays were not obviously reflected in the levels of killing observed for LCL targets transformed with the corresponding viruses; all three groups of LCL targets appeared equally sensitive to epitope-specific T cells (Fig. 6). We therefore conclude that, at least in the context of an LCL cell, the LMP2 residue 426 to 434 epitope is sufficiently well processed and presented on A2.1 molecules to allow CTL recognition to occur whichever of the three alternative epitope sequences is being encoded by the resident viral genome. We do not yet know, however, whether A2.1-positive individuals infected with one of the non-B95.8-like EBV strains mount a CTL response against the variant LMP2 residue 426 to 434 epitope, but in light of the present findings this seems quite possible.

The above response, restricted through a common HLA allele and recognizing a wide range of virus isolates through a defined epitope in LMP2, remains an interesting candidate for immunotherapy of LMP2-positive malignancies such as NPC or HD. However, we should point out that there is as yet no indication that the HLA A2.1 allele is naturally protective against either of these tumors. Indeed, a very early analysis of Chinese populations suggested that A2 (then not discriminated into its subtypes) may even be a weak risk factor for NPC (43, 44), although this may now be explained by linkage disequilibrium with another, as yet unidentified susceptibility gene (27). The lack of clear evidence for A2.1 as a protective allele may reflect the fact that the LMP2-specific response, when present, constitutes only a minor component of EBV-specific CTL memory in most A2.1-positive individuals studied to date. It may nevertheless prove possible to amplify this component by vaccination either with the relevant peptide or with an LMP2-expressing viral vector or to augment this response by passive transfer of *in vitro*-amplified effectors (35). For this reason it becomes important to determine whether the relevant tumors carry EBV isolates in which the epitope sequence is conserved and whether all the tumor cells not only express the LMP2 protein but can also process and present the necessary target epitope.

ACKNOWLEDGMENTS

This work was supported by the Cancer Research Campaign and the Medical Research Council, United Kingdom.

We are grateful to C. K. Sam and U. Prasad, University of Malaysia, for access to the YKO and CKL virus isolates and to Deborah Williams for excellent secretarial help.

REFERENCES

1. Abdul-Hamid, M., J. J. Chen, N. Constantine, M. Massoud, and N. Raab-Traub. 1992. EBV strain variation: geographical distribution and relation to disease state. *Virology* **190**:168–175.
2. Addinger, H. K., H. Delius, U. K. Freese, J. Clarke, and G. W. Bornkamm. 1985. A putative transforming gene of Jijoye virus differs from that of Epstein-Barr virus prototypes. *Virology* **141**:221–234.
3. Apolloni, A., D. Moss, R. Stumm, S. Burrows, A. Suhrbier, I. Misko, C. Schmidt, and T. Sculley. 1992. Sequence variation of cytotoxic T cell epitopes in different isolates of Epstein-Barr virus. *Eur. J. Immunol.* **22**:183–189.
4. Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. G. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Séguin, P. S. Tuffnel, and B. G. Barrell. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature (London)* **310**:207–211.
5. Brooks, J. M., R. J. Murray, W. A. Thomas, M. G. Kurilla, and A. B. Rickinson. 1993. Different HLA-B27 subtypes present the same immunodominant Epstein-Barr virus peptide. *J. Exp. Med.* **178**:879–887.
6. Brooks, L., Q. Y. Yao, A. B. Rickinson, and L. S. Young. 1992. Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. *J. Virol.* **66**:2689–2697.
7. Burrows, S. R., I. S. Misko, T. B. Sculley, C. Schmidt, and D. J. Moss. 1990. An Epstein-Barr virus-specific cytotoxic T-cell epitope present on A- and B-type transformants. *J. Virol.* **64**:3974–3976.
8. Burrows, S. R., S. J. Rodda, A. Suhrbier, H. M. Geysen, and D. J. Moss. 1992. The specificity of recognition of a cytotoxic T lymphocyte epitope. *Eur. J. Immunol.* **22**:191–195.
9. Busson, P., R. McCoy, R. Sadler, K. Gilligan, T. Tursz, and N. Raab-Traub. 1992. Consistent transcription of the Epstein-Barr virus LMP2 gene in nasopharyngeal carcinoma. *J. Virol.* **66**:3257–3262.
10. Cerundolo, V., J. Alexander, C. Lamb, P. Cresswell, A. McMichael, F. Gotch, and A. Townsend. 1990. Presentation of viral antigen controlled by a gene in the major histocompatibility complex. *Nature (London)* **345**:449–452.
11. Dambaugh, T., K. Hennessy, L. Chamnankit, and E. Kieff. 1984. U2 region of Epstein-Barr virus DNA may encode Epstein-Barr nuclear antigen 2. *Proc. Natl. Acad. Sci. USA* **81**:7632–7636.
12. Deacon, E. M., G. Pallesen, G. Nielsen, J. Crocker, L. Brooks, A. B. Rickinson, and L. S. Young. 1993. Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. *J. Exp. Med.* **177**:339–349.
13. de Campos-Lima, P. O., R. Gavioli, Q. J. Zhang, L. E. Wallace, R. Dolcetti, M. Rowe, A. B. Rickinson, and M. G. Masucci. 1993. HLA-A11 epitope loss isolates of Epstein-Barr virus from a highly A11+ population. *Science* **260**:98–100.
14. Dumas, B. T. 1975. Standards for total serum protein assays—a collaborative study. *Clin. Chem.* **21**:1159–1166.
15. Fahraeus, R., H. L. Fu, I. Ernberg, J. Finke, M. Rowe, G. Klein, K. Falk, E. Nilsson, M. Yadav, P. Busson, T. Tursz, and B. Kallin. 1988. Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. *Int. J. Cancer* **42**:329–338.
16. Falk, K., O. Röttschke, S. Stevanovic, G. Jung, and H.-G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature (London)* **351**:290–296.
17. Gavioli, R., P. O. de Campos-Lima, M. G. Kurilla, E. Kieff, G. Klein, and M. G. Masucci. 1992. Recognition of the Epstein-Barr virus-encoded nuclear antigens EBNA4 and EBNA6 by HLA-A11-restricted cytotoxic T lymphocytes: implications for down-regulation of HLA-A11 in Burkitt lymphoma. *Proc. Natl. Acad. Sci. USA* **89**:5862–5866.
18. Gavioli, R., M. G. Kurilla, P. O. de Campos-Lima, L. E. Wallace,

- R. Dolcetti, R. J. Murray, A. B. Rickinson, and M. G. Masucci. 1993. Multiple HLA-A11-restricted cytotoxic T-lymphocyte epitopes of different immunogenicities in the Epstein-Barr virus-encoded nuclear antigen 4. *J. Virol.* **67**:1572-1578.
19. Gratama, J. W., M. A. P. Oosterveer, F. E. Zwaan, J. Lepoutre, G. Klein, and I. Ernberg. 1988. Eradication of Epstein-Barr virus by allogeneic bone marrow transplantation: implications for the site of viral latency. *Proc. Natl. Acad. Sci. USA* **85**:8693-8699.
 20. Gratama, J. W., M. M. Zutter, J. Minarovits, M. A. P. Oosterveer, E. D. Thomas, G. Klein, and I. Ernberg. 1991. Expression of Epstein-Barr virus growth-transformation-associated proteins in lymphoproliferations of bone-marrow transplant recipients. *Int. J. Cancer* **47**:188-192.
 21. Hunt, D. F., R. A. Henderson, J. Shabanowitz, K. Sakaguchi, H. Michel, N. Sevilir, A. L. Cox, E. Appella, and V. H. Engelhard. 1992. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* **255**:1261-1263.
 22. Imanishi, T., T. Akaza, A. Kimura, K. Tokunaga, and T. Gojbori. 1992. W15.1 reference tables, p. 1066-1074. *In* K. Tsuji, M. Aizawa, and T. Sasazuki (ed.), *HLA—1991*. Oxford University Press, Oxford.
 23. Khanna, R., S. R. Burrows, M. G. Kurilla, C. A. Jacob, I. S. Misko, T. B. Sculley, E. Kieff, and D. J. Moss. 1992. Localisation of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. *J. Exp. Med.* **176**:169-178.
 24. Kieff, E., and D. Liebowitz. 1990. Epstein-Barr virus and its replication, p. 1889-1920. *In* B. N. Fields, D. M. Knipe, et al. (ed.), *Virology*. Raven Press, New York.
 25. Laux, G., M. Perricaudet, and P. J. Farrell. 1988. A spliced Epstein-Barr virus gene expressed in immortalized lymphocytes is created by circulation of the linear viral genome. *EMBO J.* **7**:769-774.
 - 25a. Lee, S., and M. Rowe. Unpublished data.
 26. Longnecker, R., and E. Kieff. 1990. A second Epstein-Barr virus membrane protein (LMP2) is expressed in latent infection and colocalizes with LMP1. *J. Virol.* **64**:2319-2326.
 27. Lu, S.-J., N. E. Day, L. Degos, V. Lepage, P.-C. Wang, S.-H. Chan, M. Simons, B. McKnight, D. Easton, Y. Zeng, and G. de Thé. 1990. Linkage of nasopharyngeal carcinoma susceptibility locus to the HLA region. *Nature (London)* **346**:470-471.
 28. Lung, M. L., W. P. Lam, J. Sham, D. Choy, Z. Yong-Sheng, H.-Y. Guo, and M. H. Ng. 1991. Detection and prevalence of the "F" variant of Epstein-Barr virus in Southern China. *Virology* **185**:67-71.
 29. Miller, G., and M. Lipman. 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proc. Natl. Acad. Sci. USA* **70**:190-194.
 30. Moss, D. J., S. R. Burrows, G. D. Baxter, and M. F. Lavin. 1991. T cell-T cell killing is induced by specific epitopes: evidence for an apoptotic mechanism. *J. Exp. Med.* **173**:681-686.
 31. Moss, D. J., S. R. Burrows, R. Khanna, I. S. Misko, and T. B. Sculley. 1992. Immune surveillance against Epstein-Barr virus. *Seminars in Immunology* **4**:97-104.
 32. Moss, D. J., I. S. Misko, S. R. Burrows, K. Burman, R. McCarthy, and T. B. Sculley. 1988. Cytotoxic T cell clones discriminate between A and B type Epstein-Barr virus transformants. *Nature (London)* **331**:719-721.
 33. Murray, R. J., M. G. Kurilla, J. M. Brooks, W. A. Thomas, M. Rowe, E. Kieff, and A. B. Rickinson. 1992. Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus (EBV): implications for the immune control of EBV-positive malignancies. *J. Exp. Med.* **176**:157-168.
 34. Rickinson, A. B., R. J. Murray, J. Brooks, H. Griffin, D. J. Moss, and M. G. Masucci. 1992. T cell recognition of Epstein-Barr virus-associated lymphomas, p. 53-80. *In* L. M. Franks (ed.), *Cancer surveys, a new look at tumour immunology*, vol. 13. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 35. Riddell, S. R., K. S. Watanabe, J. M. Goodrick, C. R. Li, M. E. Agha, and P. D. Greenberg. 1992. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science* **257**:238-241.
 36. Rooney, C. M., C. D. Gregory, M. Rowe, S. Finerty, C. Edwards, H. Rupani, and A. B. Rickinson. 1986. Endemic Burkitt's lymphoma: phenotypic analysis of tumour biopsy cells and of derived tumour cell lines. *JNCI* **77**:681-687.
 37. Rowe, M., A. Lear, D. Croom-Carter, A. H. Davies, and A. B. Rickinson. 1992. Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. *J. Virol.* **66**:122-131.
 38. Rowe, M., D. T. Rowe, C. D. Gregory, L. S. Young, P. J. Farrell, H. Rupani, and A. B. Rickinson. 1987. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. *EMBO J.* **6**:2743-2751.
 39. Sample, J., D. Liebowitz, and E. Kieff. 1989. Two related Epstein-Barr virus membrane proteins are encoded by separate genes. *J. Virol.* **63**:933-937.
 40. Sample, J., L. Young, B. Martin, T. Chatman, E. Kieff, A. Rickinson, and E. Kieff. 1990. Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. *J. Virol.* **64**:4084-4092.
 41. Schmidt, C., S. R. Burrows, T. B. Sculley, D. J. Moss, and I. S. Misko. 1991. Non-responsiveness to an immunodominant Epstein-Barr virus-encoded cytotoxic T-lymphocyte epitope in nuclear antigen 3A: implications for vaccine strategies. *Proc. Natl. Acad. Sci. USA* **88**:9478-9482.
 42. Shapiro, R. S., K. McClain, G. Frizzera, K. J. Gajl-Peczalska, J. H. Kersey, B. R. Blazar, D. C. Arthur, D. F. Patton, J. S. Greenberg, B. Burke, N. K. C. Ramsay, P. McGlave, and A. H. Filipovich. 1988. Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation. *Blood* **71**:1234-1243.
 43. Simons, M. J., G. B. Wee, S. H. Chan, K. Shammugaratnam, N. E. Day, and G. B. de Thé. 1975. Probable identification of an HL-A second-locus antigen associated with a high risk of nasopharyngeal carcinoma. *Lancet* **i**:142-143.
 44. Simons, M. J., G. B. Wee, N. E. Day, P. J. Morris, K. Shammugaratnam, and G. B. de Thé. 1974. Immunogenetic aspects of nasopharyngeal carcinoma. I. Differences in HL-A antigen profiles between patients and control groups. *Int. J. Cancer* **13**:122-134.
 45. Smith, P. R., and B. E. Griffin. 1991. Differential expression of Epstein-Barr viral transcripts for two proteins (TP1 and LMP) in lymphocyte and epithelial cells. *Nucleic Acids Res.* **19**:2435-2440.
 46. Starzl, T. E., M. A. Nalesnik, K. A. Porter, M. Ho, S. Iwatsuki, B. P. Griffith, J. T. Rosenthal, T. R. Hakala, B. W. Shaw, R. L. Hardesty, R. W. Atchison, R. Jaffe, and H. T. Bahnson. 1984. Reversibility of lymphomas and lymphoproliferative lesions developing under cyclosporin A-steroid therapy. *Lancet* **i**:583-587.
 47. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol.* **7**:601-624.
 48. Wallace, L. E., M. Rowe, J. S. H. Gaston, A. B. Rickinson, and M. A. Epstein. 1982. Cytotoxic T cell recognition of Epstein-Barr virus-infected B cells. III. Establishment of HLA-restricted cytotoxic T cell lines using interleukin 2. *Eur. J. Immunol.* **12**:1012-1018.
 49. Yao, Q. Y., P. Ogan, M. Rowe, M. Wood, and A. B. Rickinson. 1989. Epstein-Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carriers. *Int. J. Cancer* **43**:67-71.
 50. Young, L., C. Alfieri, K. Hennessy, H. Evans, C. O'Hara, K. C. Anderson, J. Ritz, R. S. Shapiro, A. Rickinson, E. Kieff, and J. I. Cohen. 1989. Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. *N. Engl. J. Med.* **321**:1080-1085.
 51. Young, L. S., C. W. Dawson, D. Clark, H. Rupani, P. Busson, T. Tursz, A. Johnson, and A. B. Rickinson. 1988. Epstein-Barr virus gene expression in nasopharyngeal carcinoma. *J. Gen. Virol.* **69**:1051-1065.
 52. Zutter, M. M., P. J. Martin, G. E. Sale, H. M. Shulman, L. Fisher, E. D. Thomas, and D. M. Durnam. 1988. Epstein-Barr virus lymphoproliferation after bone marrow transplantation. *Blood* **72**:520-529.