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Immunology

Generation of specific cytotoxic T cells with a fragment of the Epstein–Barr virus-encoded p63/latent membrane protein

(target structure/immunity/herpesvirus)

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ABSTRACT **Human B lymphocytes, transformed by the herpesvirus Epstein–Barr virus, are known to express a characteristic antigen(s) recognized by the cellular immune response. This structure has been termed lymphocyte-determined membrane antigen. Because of the significance of this structure in controlling Epstein–Barr virus infection *in vivo*, the molecular nature of lymphocyte-determined membrane antigen has been long sought. In this paper, we show that a sequence of 10 amino acids (residues 43–53) from the Epstein–Barr virus-encoded membrane protein p63/latent membrane protein can induce Epstein–Barr virus-specific cytotoxic T cells and, therefore, bears at least one of the lymphocyte-determined membrane antigenic determinants.**

Epstein–Barr virus (EBV) is a human herpesvirus that infects the majority of the human population. This infection, which persists for life, has been associated with a number of clinical disorders, including infectious mononucleosis, Burkitt lymphoma, and nasopharyngeal carcinoma. One of the sites for maintenance of the persistent infection is within peripheral blood B cells. These B cells may be considered oncogenically transformed, as they will proliferate indefinitely *in vitro* (1–3) and will give rise to lymphoproliferative disorders including lymphoma *in vivo* in the presence of appropriate immunosuppression (4–6). As these lymphoproliferative disorders occur in the face of a strong antibody response, it has been assumed that cell-mediated rather than humoral immunity plays a critical role in controlling the persistently infected, transformed B cells *in vivo*. Support for this idea has been obtained by the demonstration *in vitro* that T cells from seropositive individuals suppress the outgrowth of EBV-infected B cells (7, 8). Subsequently, it was shown that classical, major histocompatibility complex-restricted, virus-specific, cytotoxic T cells (CTL) were a major component of this response (9–12). Although this EBV-specific, cell-mediated immunity has been known for several years, the target structure, the so-called LYDMA (lymphocyte-defined membrane antigen), on the transformed B cell, has remained elusive. However, it has been shown that EBV encodes an intrinsic membrane protein termed LMP (latent membrane protein) that is present in the plasma membrane of transformed B cells (13, 14). A structure has been proposed for this protein (15, 16) based on the deduced amino acid sequence. This has been confirmed experimentally and is shown in Fig. 1 (refs. 13, 14, and 17; K. P. Mann and D.A.T.-L., unpublished data).

Attempts have been made to demonstrate the recognition by CTL of specific EBV antigens after transfection and expression of the genes in appropriate human cell lines. Such experiments have to date proved universally negative and have led to the suggestion that EBV CTL may recognize B-cell activation antigens (18).

We have attempted to demonstrate a role for LMP as a CTL target using a different approach that is based on work originally described for the influenza system (19, 20). These studies indicated that specific CTL could be generated in a secondary stimulation *in vitro* with synthetic peptides derived from the target epitope on the hemagglutinin molecule. This approach has the advantage that it readily allows the exact mapping of the amino acid sequence that is recognized by the CTL. Although CTL target epitopes have been mapped to external structures of the hemagglutinin (21), experiments suggest that a substantial part of the CTL response recognizes internal structures, including nuclear proteins (22, 23). It has been suggested that this is a consequence of the processing and presentation of fragmented antigen at the cell surface. As it is unclear how to predict which fragment of which EBV antigen could be processed in this way, and since evidence for such processing in the EBV system is, at present, lacking, we thought it appropriate to begin our search for EBV-specific CTL targets among sequences already shown to be present at the cell surface. We have, therefore, tested peptides equivalent to the three reverse-turn loops shown in Fig. 1 [LMP-(43–53), LMP-(98–107), and LMP-(158–167)] and found that one of them [LMP-(43–53)] is capable of stimulating EBV-specific cytotoxic T cells.

MATERIALS AND METHODS

Cells. EBV-transformed lymphoblastoid cell lines (LCLs) were derived by *in vitro* transformation with the B95-8 strain of virus as described (7). All lines were derived in this laboratory with the exception of the HLA-B14 typing line that was the kind gift of E. Yunis (Dana–Farber Cancer Institute). HLA typing was performed in the laboratory of E. Yunis. The K562 line is the standard natural killer cell (NK)-sensitive human line and was obtained from the American Type Cell Culture Collection. To prepare B- and T-cell blasts, whole peripheral blood lymphocytes were purified over Ficoll/Hypaque and then fractionated by two rounds of sheep erythrocyte rosette formation. The rosette-positive (T-cell-enriched) population was incubated with phytohemagglutinin (0.2 μg/ml, Burroughs Wellcome, Research Triangle Park, NC), and the rosette-negative (B-cell-enriched) population was incubated with pokeweed mitogen (10 μg/ml, GIBCO) overnight. The mitogens were then washed out, and the cells were cultured for a further 6 days before use in the assay. After culturing, the populations were >80% T or B cells, respectively, as judged by staining with either CD3 (anti-T3) or CD20 (anti-B1) antibodies.

Peptides. Peptides were purchased from Janice Young of The Children's Hospital Medical Center (Boston). The pep-

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Abbreviations: EBV, Epstein–Barr virus; CTL, cytotoxic T lymphocyte; LMP, latent membrane protein; NK, natural killer cell; EBNA, EBV-encoded nuclear antigen; LCL, lymphoblastoid cell line.

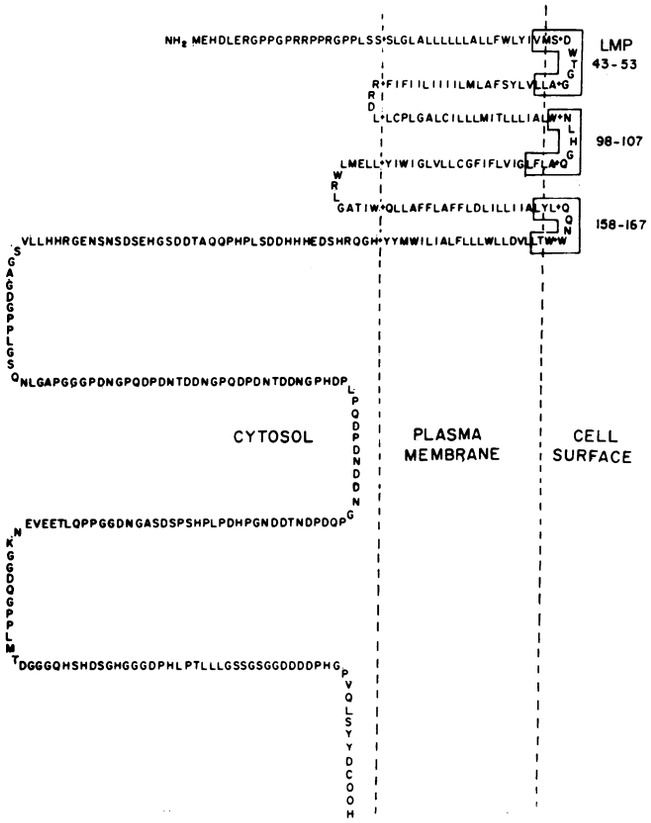

FIG. 1. Schematic representation of the structure of p63/LMP in the B cell membrane. The one-letter amino acid code sequence and original prediction of the structure are taken from Fennewald *et al.* (15) and Hudson *et al.* (16). The boxed-off areas conform to the sequences used to generate the synthetic peptides used in this study.

tides were repurified by gel filtration on a G-25 column and then lyophilized. Amino acid analysis was used to confirm the composition and purity of each peptide. In all, two independently synthesized lots of the peptide LMP-(43–53) were used in this study. The sequences of the peptides in the single-letter code were as follows:

LMP-(43–53)	V M S D W T G G A L L C*
LMP-(98–107)	W N L H L H G A L F L C*
LMP-(158–167)	C*L Y L Q Q N W W T L
Control	L G F Y P A E I T C*

Derivation of T-Cell Clones. T-cell clones were derived essentially as described by Meuer *et al.* (24). Ficoll/Hypaque-purified whole peripheral blood lymphocytes (1.5×10^6 cells per ml, 5 ml) were cultured in 10% (vol/vol) human type AB serum in RPMI 1640 and peptide at the concentration shown in the tables. After 5 days, the surviving cells were cloned in V-bottom microtiter wells. Usually, a 96-well plate was established containing either 100, 10, or 1 cell(s) per well with a feeder layer of 75×10^4 irradiated autologous peripheral blood lymphocytes in 0.1 ml of medium [10% (vol/vol) human type AB serum, 6–12% (vol/vol) phytohemagglutinin-conditioned medium prepared as described by Meuer *et al.* (24), RPMI 1640, and peptide]. Generally, proliferating colonies were visible within 2–3 weeks. In all experiments the plates were kept at least 1 month and in some cases 2 months; however, very few colonies arose after 1 month. The colonies were propagated by transferring first to round-bottom microtiter plates and subsequently to 2-ml flat-bottomed plates. Cultures were fed weekly with medium and were given a fresh feeder layer at each transfer. Once established in multiple wells of a 24-well plate, colonies were tested in cytotoxicity assays and were frozen in liquid nitrogen.

^{51}Cr Release Assay. A standard ^{51}Cr release microassay was used as described (12). The length of assays and effector/target ratio used varied from experiment to experiment as noted in Table 2. The percent specific lysis was calculated as follows: (cpm in experimental sample − cpm spontaneously released/cpm from cells in 1% $NaDodSO_4$ − cpm spontaneously released) × 100%.

RESULTS

Induction of T-Cell Proliferation by Synthetic Peptides. We have analyzed various synthetic peptides in interleukin 2-containing medium, for their ability to stimulate the outgrowth of EBV-specific T cells. We chose to measure proliferation by limiting dilution cloning so that individual T cells could be further characterized in functional assays. The results of these studies are presented in Table 1.

We chose three peptides derived from sequences of LMP [LMP-(43–53), LMP-(98–107), and LMP-(158–167)] that are predicted to be exposed on the outer surface of infected cells, and a fourth, randomly selected control peptide of similar length and hydrophobicity. In Table 1 (experiment 1) is shown the result of a cloning experiment performed with the three LMP peptides. The data indicate that LMP-(43–53) was able to reproducibly stimulate the proliferation of T-cell clones and that the frequency of responding cells was ≈1 in 50. By comparison, only sporadic clones were detected with LMP-(98–107) or LMP-(158–167) (frequency 1 or 2 in 10^4). As shown in Table 1

Table 1. Peptide LMP-(43–53) induces T-cell proliferation from EBV-immune individuals over a range of concentrations

Donor	Peptide	Wells with proliferating cells, no. per plate 100*	10*	1*
Experiment 1				
Seropositive 1	LMP-(43–53) (10 nM)	75	19	2
Seropositive 1	LMP-(98–107) (10 nM)	1	2	0
Seropositive 1	LMP-(158–167) (10 nM)	2	1	0
Experiment 2				
Seropositive 1	LMP-(43–53) (10 nM)	37	8	3
Seropositive 1	Control (10 nM)	0	0	0
Seropositive 1	LMP-(43–53) (20 nM)	39	12	2
Seropositive 1	Control (20 nM)	0	0	0
Seropositive 1	LMP-(43–53) (100 nM)	16	9	0
Seropositive 1	Control (100 nM)	0	0	0
Experiment 3				
Seropositive 1	LMP-(43–53) (10 nM)	56	10	1
Seropositive 2	LMP-(43–53) (10 nM)	81	13	3
Seropositive 3	LMP-(43–53) (10 nM)	56	29	10
Seronegative	LMP-(43–53) (10 nM)	0	0	0

Whole peripheral blood lymphocytes were incubated in the presence of the peptide alone, at the concentration indicated, for 5 days in RPMI 1640 supplemented with 10% (vol/vol) human type AB serum. After this time the cells were cloned in 96-well V-bottom microtiter plates containing 100, 10, or 1 cell(s) per well in the presence of an irradiated feeder layer of autologous peripheral blood lymphocytes in RPMI 1640 supplemented with peptide, 10% (vol/vol) human type AB serum and phytohemagglutinin-conditioned medium [6–12% (vol/vol)]. Proliferating colonies were observed after 2–3 weeks, and plates were generally kept for 2 months. The presence of proliferating cells was confirmed microscopically.
*Number of cells plated per well.

*A terminal cysteine was added to facilitate coupling to carrier for experiments not presented in this paper. The location of the LMP-derived peptides is shown in Fig. 1.

(experiment 2) LMP-(43–53) was effective in stimulating the growth of clones with a frequency of ≈1 in 100 over a range of concentrations from 10 to 100 nM. By comparison a frequency of <1 in 10^4 was obtained with the control peptide over the same range of concentrations. We conclude from these experiments that one peptide from LMP-(43–53) is capable of stimulating the outgrowth of T-cell clones.

Specificity of the Response. The observation that only LMP-(43–53) stimulated proliferation of T cells suggests that this peptide may represent a structure recognized by LMP-specific T cells. An alternate explanation would be that LMP-(43–53) is in some way capable of delivering a nonspecific growth signal. To test this possibility, we performed a study where we compared the response of T cells from three different EBV-seropositive individuals with those of an EBV-seronegative individual. As may be seen in Table 1 (experiment 3), proliferating T-cell clones were readily obtained from the lymphocytes of all three seropositives tested. The frequency with which clones arose was again in the range of 1 to 3 in 100 whereas no clones were obtained from the seronegative donor (frequency <1 in 10^4). The results provide evidence that the proliferation of T cells due to LMP-(43–53) requires prior exposure to EBV and, therefore, has an immunological basis. This is consistent with LMP-(43–53) itself being recognized by EBV-specific T cells.

Specificity and Function of the T-Cell Clones. The experiments described above suggest that LMP-(43–53) is stimulating the proliferation of immune T cells through a specific mechanism. As a further specificity test of the response, we assessed the T-cell clones for an EBV-specific function, namely cytotoxicity for the autologous EBV-infected lymphoblastoid cell line (EBV/LCL). Of the hundreds of clones derived with LMP-(43–53) through the course of our experiments, we screened a total of 56 for cytotoxicity against the autologous EBV/LCL and the highly sensitive NK target K562 line. In all, 11 of the clones lysed the EBV/LCL but not the K562 line. The clones were derived in three independent experiments, as summarized in Table 2. The frequency with which the EBV-specific clones arose was similar in all three cases (6 of 19 clones, experiment 1; 1 of 6 clones, experiment 2; 4 of 31 clones, experiment 3). We have characterized the specificity of 5 of these clones in more detail as summarized in Table 2, experiments 4, 5, and 6. From these studies, it is clear that the T-cell clones reproducibly lysed the autologous EBV/LCL, whereas lysis of autologous EBV-negative lymphoblasts, allogeneic EBV-positive lymphoblasts, or K562 was never detected. Thus, they all behaved as self-restricted EBV-specific cytotoxic T cells. In the case of clone 6, we were able to map the restricting element to the HLA A1 type. Interestingly, the rest of the clones that were tested (45 in all) showed no specific lysis for the EBV/LCL but exhibited a variety of activities. Some had no detectable lytic activity, whereas others were cytotoxic for K562 alone, and others lysed both the EBV/LCL and the K562 line. These latter two groups of clones resemble classical NK cells and lymphokine-activated killer cells, respectively.

As discussed above, one interpretation of our findings could be that LMP-(43–53) acts nonspecifically to stimulate the proliferation of T cells. The failure of T cells from an EBV-seronegative individual to respond is evidence against this explanation. Additionally, if LMP-(43–53) were acting nonspecifically, then the frequency with which we detected clones, cytotoxic for the autologous EBV/LCL (≈1 in 5), should be reflected in a collection of T-cell clones derived from the same seropositive individual in the absence of LMP-(43–53). In all our cloning experiments, we derived a total of 35 such clones in contrast to the many hundreds that we obtained with LMP-(43–53) in parallel cultures. All 35 clones were tested for cytotoxicity. Whereas none lysed the autologous EBV/LCL detectably, several did lyse K562 targets, suggesting that they may possess NK activity (data not shown). Thus, we conclude that T-cell clones cytotoxic for the autologous EBV/LCL occur at a frequency of <1 in 35 in these T-cell clones, as compared to the 1 in 5 frequency detected in T-cell clones derived in the presence of LMP-(43–53).

DISCUSSION

In this paper we describe a series of experiments conducted on the LMP of EBV to determine if any part of it could

Table 2. Cytotoxicity of LMP-(43–53)-induced clones for a variety of target cells

Exp.	Target cell	% specific lysis											Effector/target ratio	Assay time, hr
		Cl. 1	Cl. 2	Cl. 3	Cl. 4	Cl. 5	Cl. 6	Cl. 7	Cl. 8	Cl. 9	Cl. 10	Cl. 11		
1	Aut EBV/LCL	92	90	49	85	52	84						20:1	6
	K562	1	2	12	1	6	6							
2	Aut EBV/LCL							58					20:1	6
	K562							1						
3	Aut EBV/LCL								37	40	45	37	20:1	4
	K562								11	9	3	6		
4	Aut EBV/LCL	28	26		29		28						5:1	4
	Aut B-blasts	−2	−1		−1		4							
	Aut T-blasts	−3	−4		−2		−3							
	K562						−1							
5	Aut EBV/LCL*	27	25		41		38						5:1	4
	Allo EBV/LCL 1	0	1		1		0							
	Allo EBV/LCL 2	0	0		−1		0							
6	A1B8†						27						5:1	4
	A1†						21							
	B8†						0							
	B14†						2							

Eleven T-cell clones (clones 1–11) were used as indicated to lyse target cells. The clones shown in experiments 1, 2, and 3 were derived from three entirely separate cloning experiments. All experiments were performed on separate occasions. The variation in percent lysis from experiment to experiment generally reflects the effector/target cell ratio and the length of the assay used. Spontaneous release was <15% of total for all cell types with the exception of the autologous B blasts used in experiment 4, where it was 18%. All experiments were performed in triplicate. Aut, autologous; allo, allogeneic; Cl., clone.

*HLA type of the donor was HLA A1, B8, B14. In experiment 5, the HLA types were LCL 1 HLA A3, A11, B27, B40; LCL 2 HLA A3, AW31, B7, BW51.

†Allogeneic EBV/LCL sharing HLA determinants with donor.

stimulate EBV-specific CTL. We show that one peptide, LMP-(43–53), was able to do so. Thus, this represents the first demonstration that an EBV-encoded protein, which is present in transformed cells, can stimulate CTL. The effect appears to be specific for LMP-(43–53) as other peptides of similar length and hydrophobicity, either chosen at random (Table 1, experiment 2) or from other parts of the LMP molecule (Table 1, experiment 1), fail to stimulate the outgrowth of the large number of T-cell clones obtained with LMP-(43–53). The critical question is whether LMP-(43–53) is specifically recognized by CTL or whether it acts as a nonspecific stimulator or activator of T-cell proliferation. The evidence for specificity is 2-fold. First, LMP-(43–53) stimulated the outgrowth of clones from the T cells of three different EBV-seropositive donors but not from a seronegative individual, suggesting an immunological basis for the response. Second, if LMP-(43–53) were a nonspecific stimulator then EBV-specific cytotoxic clones should be obtained at the same frequency in the presence or absence of LMP-(43–53). In fact such clones were obtained at a frequency of 1 in 5 in the presence of LMP-(43–53), a value too high to have occurred by chance, whereas none of 35 clones obtained in the absence of LMP-(43–53) was cytotoxic for the autologous EBV/LCL.

The amino acid sequences that we have used for our peptides were picked on the basis of both theoretical (15, 16) and experimental evidence (13, 14, 17) indicating that the three reverse-turn loops containing the LMP-(43–53), LMP-(98–107), and LMP-(158–167) sequences are the only parts of the LMP molecule exposed on the outer surface. Further experimental support for this conclusion is that only these sequences in the native LMP molecule can be digested when intact cells are digested with nonspecific proteases such as Pronase (K. P. Mann and D.A.T.-L., unpublished data). Of the three loops, LMP-(43–53) is the more readily digested, consistent with it having the most hydrophilic sequence of the three. Additionally, the first studies in the influenza system, using peptides or transfection techniques, initially identified CTL target epitopes as being on structures on the outside of the infected cell (21). For these reasons we decided to begin our search for EBV-specific CTL targets with sequences that gave the best evidence for their being exposed on the outer surface of the cell, namely the LMP-(43–53), LMP-(98–107), and LMP-(158–167) sequences of LMP. Data in the influenza system, however, suggest that most of the CTL response to that virus is to sequences of membrane proteins not exposed on the outer surface of the cell or to nuclear proteins (22, 23).

These experiments suggest that there may be specific mechanisms for the internal degradation of influenza proteins and subsequent transport of the fragments to the cell surface, analogous to the processing of antigen by antigen-presenting cells. The possibility that CTL can recognize processed internal viral proteins, in addition to the expected external membrane proteins, has also been raised by studies with cytomegalovirus (25). To date, it has not been possible to detect CTL effector activity directed against target cells expressing the transfected EBV-encoded nuclear antigen (EBNA) genes *EBNA 1* and *2* (G. Klein and A. Rickinson, personal communication), the original technique used to identify influenza nucleoprotein-specific CTL. Now that we have developed a technique that has successfully allowed the mapping of at least one lymphocyte-defined membrane antigen epitope to the LMP molecule, it will be of great interest to apply this approach in a more extensive study with various EBNA peptides.

The failure to detect anti-EBNA CTL by transfection techniques could simply be due to technical difficulties. Alternatively, it is conceivable that in the EBV system such CTL do not exist. This is because EBV is a transforming virus, whereas influenza and cytomegalovirus are lytic viruses. Thus, influenza CTL *in vivo* will be generated against cells that are replicating and are in the process of being lysed by the virus. It is not unreasonable to assume that cells have internal mechanisms for the destruction and degradation of viral products in lytically infected cells. This in turn could generate peptides that would then move to the surface to act as CTL targets. For EBV, on the other hand, the viral products in the latently infected B cell are presumably an integral part of the growth requirements of that cell and may be less likely to be degraded. Thus, EBV-specific CTL would need to recognize a structure on the outside of the cell. It is conceivable that, subsequent to this CTL-mediated lysis, a fraction of the CTL generated against EBV-infected cells may recognize internal structures released as a consequence of lysis. The immunological significance of these CTL is unclear, however, if they are unable to recognize the intact transformed B cell. It could be that *in vivo* most of these CTL are irrelevant. This would be analogous to the humoral response, where much of the elicited antibody is directed to internal structures such as EBNA, presumably due to release after cellular lysis. Such antibodies, presumably, play no protective role *in vivo*.

The alternative scenario would be that the internal processing suggested by the influenza experiments may be a normal mechanism for the turnover of cellular proteins. Then only peptides from viral protein would be seen as foreign when they reach the surface. In this case LMP-(43–53) may be recognized by CTL, not because of its location in the intact LMP molecule but because it is readily detected as a product of proteolytically processed LMP. It also follows that one should also expect to detect nucleoprotein (EBNA)-specific CTL in the EBV system. A continued and careful search for such T cells is, therefore, still warranted. In our view a more critical question is, what role does cellular immunity play in controlling the lytic infection of epithelial cells by EBV. It is apparent that increased viral shedding occurs in the face of high anti-viral antibody titer in immune-suppressed individuals. Interestingly, and unexpectedly, the EBV-related lymphoproliferative disorders that occur in these individuals are frequently responsive to treatment with acyclovir (26), an inhibitor of the viral lytic cycle (27). These observations suggest that cellular immunity directed against the lytically infected cell plays a critical role in controlling viral replication and lymphoproliferation as a consequence of viral spread. Nothing is known about target structures on cells lytically infected with EBV, a situation that would be more closely analogous to that of influenza and cytomegalovirus than the latently EBV-infected B cell.

In conclusion, the present study defines, to our knowledge for the first time, a single target structure for eliciting human EBV-specific CTL. Nevertheless, a large amount of work remains to be done. The failure of other peptides from LMP to stimulate CTL precursors does not mean that they are not targets but could imply that they fail to achieve the right configuration in solution or when presented. Furthermore, we cannot rule out the possibility that other EBV-encoded proteins, such as the EBNAs, or even cellular activation antigens could also be targets. A careful analysis of precursor frequencies in a number of donors will be needed to assess the strength of the response to LMP-(43–53).

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