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Epstein-Barr Virus-Specific Cytotoxic T-Cell Recognition of Transfectants Expressing the Virus-Coded Latent Membrane Protein LMP

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Cytotoxic T cells from Epstein-Barr virus (EBV)-immune individuals specifically kill EBV-transformed B cells from HLA class I antigen-matched donors even though the latently infected cells express only a restricted set of virus genes. The virus-induced target antigens recognized by these immune T cells have not been identified. In our experiments, EBV DNA sequences encoding the virus latent gene products Epstein-Barr nuclear antigen (EBNA)1, EBNA 2, and EBNA-LP and the latent membrane protein (LMP) were individually expressed in a virus-negative human B-lymphoma cell line, Louckes. Transfected clones expressing LMP were killed by EBV-specific cytotoxic T-cell preparations from each of three virus-immune donors HLA matched with Louckes through HLA-A2, B44 antigens; control transfectants or clones expressing one of the EBNA proteins were not recognized. Expression of LMP in a second virus-negative B-cell line, BL41, sensitized these cells to EBV-specific cytolysis restricted through the HLA-A11 antigen. To distinguish between the viral protein and an induced human B-cell activation antigen as the target for T-cell recognition, LMP was then expressed in a murine mastocytoma cell line, P815-A11, which was already carrying an introduced HLA-A11 gene and which was capable of antigen presentation to HLA-A11-restricted human T cells. The LMP-expressing P815-A11 transfectants were susceptible to lysis by EBV-specific cytotoxic T cells from three HLA-A11-positive individuals. Both Louckes and P815-A11 cells were also transfected with constructs capable of encoding a truncated form of LMP (Tr-LMP) which lacks the N-terminal 128 amino acids of the full-length protein. Tr-LMP-expressing transfectants were not recognized by the above T-cell preparations. The results suggest that LMP, and, in particular, epitopes derived from the N-terminal region of the protein, provides one of the target antigens for the EBV-induced human cytotoxic T-cell response.

Epstein-Barr virus (EBV) infection of normal B lymphocytes in vitro leads to the outgrowth of permanent B lymphoblastoid cell lines (LCLs). Virus-induced cell growth transformation appears to be achieved through the coordinate action of a limited set of viral proteins, the "latent" EBV gene products, which are constitutively expressed in all LCLs (reviewed in reference 6). These proteins include the Epstein-Barr nuclear antigens (EBNA) EBNA1, encoded by the BKRF1 reading frame (13, 37) of the viral genome (1); EBNA 2, encoded by BYRF1 (5, 7, 14); EBNA 3, encoded by a BLRF3/BERF1 splice (15); and EBNA-LP, encoded by spliced exons from the *Bam*WY region (8, 33, 48). In addition, latently infected EBV-transformed cells express a cytoplasmic/plasma membrane protein, LMP (BNLF1 encoded) (12, 18, 19), which associates with the cytoskeleton (17).

When the peripheral blood mononuclear cells of EBV-immune donors are exposed to the virus in culture, EBV-infected B cells begin to proliferate, but their outgrowth to an LCL is prevented through the in vitro reactivation of a cytotoxic T-cell response (21, 22). The reactivated effector T cells are operationally specific for EBV-infected target B lymphocytes (23, 24) expressing HLA class I molecules in common with the effectors (20, 28). One or more "lymphocyte-detected membrane antigens," presented on the surface of EBV-infected B cells in association with HLA class I molecules, are postulated to form the targets for this cytotoxic T-cell recognition (27). The identity of these antigens has still not been resolved, but clearly they could be EBV encoded. In this regard, since major histocompatibility complex-restricted cytotoxic T cells recognize intracellularly processed forms of viral proteins rather than the proteins in their native form (11, 41, 42), any of the EBV-encoded latent proteins (nuclear or cytoplasmic) could contain target epitopes. Alternatively, others have proposed that cellular "activation" antigens, whose expression is induced specifically by EBV as part of the process of B-cell growth transformation (30, 39, 47), might provide targets for the "EBV-specific" T-cell response (40).

The present experiments were designed to test these possibilities. First, cloned EBV DNA sequences encoding EBNA 1, EBNA 2, EBNA-LP, and LMP were individually expressed in a human B-lymphoma cell line. These transfected cells were then used as targets for effector T cells generated from the peripheral blood of HLA-matched EBV-immune donors. Transfected cells expressing LMP were specifically lysed by these effectors. To counter the possibility that an LMP-induced human B-cell activation antigen might be providing the target structure, the LMP-coding sequence was expressed in a nonlymphoid cell line of murine origin which was already carrying a transfected HLA class I gene. Again, the LMP-positive transfectants were sensitive to EBV-specific T-cell recognition.

MATERIALS AND METHODS

Cell lines. The Louckes cell line (44) (HLA antigen types A1, A2, B8, and B44) and the BL41 cell line (25) (HLA antigen types A11, B35, and B49), both derived from cases

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of EBV-negative sporadic Burkitt's lymphoma, were routinely maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 10% (vol/vol) fetal calf serum from selected batches (Flow Laboratories, Inc.). EBV-transformed LCLs were established from HLA antigen-typed donors, using the B95.8 strain of EBV as already described (23). The EBV-negative human leukemic cell lines K562 and HSB2 were also maintained as a source of targets sensitive to natural killer cells.

The mouse mastocytoma cell line P815-HTR (43) is a thymidine kinase-negative subclone of P815 with high transfection efficiency. It was maintained in Dulbecco modified Eagle medium supplemented with glutamine, antibiotics, and fetal calf serum as above and containing 100 μg of bromodeoxyuridine per ml. The P815-A11 cell line (10), generated by cotransfection with a human HLA-A11 genomic clone and the neomycin phosphotransferase gene allowing selection in the neomycin analog G418, was maintained in Dulbecco modified Eagle medium supplemented with glutamine, antibiotics, and fetal calf serum as above and containing 500 μg of G418 per ml.

Production of EBV gene transfectants. Fragments of EBV DNA encoding EBNA 1 (nucleotides 107930 to 110176 of the EBV genome), EBNA 2A (nucleotides 48475 to 50306), EBNA-LP (cDNA clone T65 [33]), or LMP (nucleotides 169581 to 166614) were introduced into Louckes cells by recombinant retroviral transfer (47). Clones underwent two cycles of limiting dilution cloning and were maintained by plating at 5×10^4 cells per ml in medium containing 3 mg of G418 per ml.

In further experiments, Louckes BL41, and P815-A11 cells were transfected by electroporation with psV2gpt-based expression plasmids carrying either the full-length (plasmid psV2.gpt.LMP) or the truncated (plasmid psV2.gpt.DI-LMP) LMP-coding sequence linked to the human metallothionein type 2 gene promoter as fully described elsewhere (46, 46a). Clones were then selected and maintained in the original parental cell medium now supplemented with 150 μg of xanthine, 10 μg of hypoxanthine, and 0.5 μg of mycophenolic acid per ml.

Immunoblotting. Protein samples were prepared from parental cell lines and from derived transfectants, separated by discontinuous polyacrylamide gel electrophoresis, and blotted onto nitrocellulose as described previously (32, 47). Expression of EBNA 1, EBNA 2, and EBNA-LP was determined by immunoblotting with selected human sera having defined reactivities against one or more of these proteins (47). Expression of LMP was determined by immunoblotting with monoclonal antibodies (MAbs) S12 (19) and CS1-4 (29), again as described previously (29, 46).

Immunofluorescence. Expression of the EBNA 1, EBNA 2, and EBNA-LP antigens was determined by anticomplement immunofluorescence; expression of LMP was determined by indirect immunofluorescence as described before (29).

Preparation of cytotoxic T cells. Cytotoxic T-cell preparations with activity against autologous EBV-infected B cells were generated from virus-immune donors by cocultivating blood mononuclear cells with the autologous EBV (B95.8)-transformed LCL and were maintained in interleukin-2-conditioned medium (25, 45). The donor cells used in these experiments were CMc (HLA-A2,All;B8,B44), RM (HLA-A2,A24;B7,B44), MS (HLA-A2,A2;B18,B44), KS (HLA-A2,A11;B35,B40), SW (HLA-A24,A11;B7,B35), CG (A25, A28;B39,B62), and IB (A2,A2;B7,B7).

Alloreactive T-cell preparations were generated by cocultivating blood mononuclear cells from selected donors with allogeneic LCLs (25). This protocol favors the induction of cytotoxicity against HLA-A,B alloantigens on the stimulator LCL (34). The particular combinations used in these experiments were ST (A3,A24;B7,B7) anti-RM (A2,A24;B7,B44), giving activity against HLA-A2,B44; LY (A1,A24;B27,B35) anti-Louckes (A1,A2;B8,B44), giving activity against HLA-A2,B8,B44; ST (A3,A24;B7,B7) anti-SW (A24,A11;B7,B35), giving activity against HLA-A11,B35; and JD (A1,A29; B37,B44) anti-PB (A1,A11;B22,B37), giving activity against HLA-A11,B22.

Cytotoxicity and MAb blocking assays. Cytotoxic T-cell preparations from interleukin-2-dependent T-cell lines were washed, cultured in interleukin-2-free medium overnight, and then used as effectors in 5-h chromium release assays (45). In MAb blocking assays, the anti-HLA class I framework MAb W6/32 (2) was included in the chromium release assay medium at a final dilution of 1/200 of ascitic fluid (Sera Lab, Crawley Down, U.K.), a concentration sufficient to saturate HLA class I molecules on the target cell surface. Lack of toxicity of the MAb itself at this concentration was confirmed by including control wells in each assay which contained target cells and MAb but no effector T cells.

RESULTS

Cytotoxicity assays on human B cells expressing EBNA 1, EBNA 2, or EBNA-LP. Independent clones of the Louckes BL cell line (HLA-A1,A2;B8,B44) with stable expression of EBNA 1 (Lo E1.1, 1.2, 1.3), EBNA 2 (Lo E2.1, 2.2, 2.3), or EBNA-LP (Lo T65.1, T65.2, T65.3) were obtained by infection with recombinant retrovirus and selection in G418-containing medium (47). Expression of the relevant nuclear antigen was verified by immunofluorescence and immunoblot (Fig. 1 and data not shown). Some of the EBNA 1 transfectants expressed a lower-molecular-weight EBNA 1 (Fig. 1; see also reference 47) presumably as a consequence of a deletion of portions of the glycine-alanine-encoding repeats within the BKRF1 sequence (13). EBNA 2 and EBNA-LP were consistently expressed as proteins of the expected size. The level of expression of the EBNAs was comparable to that in IB4, a reference EBV-transformed LCL (Fig. 1).

Three Lo E1, three Lo E2, and three Lo T65 clones with confirmed expression of EBNA 1, EBNA 2, and EBNA-LP, respectively, were repeatedly tested for recognition by EBV-specific T-cell preparations from donor cells CMc (HLA-A2,B8,B44-matched with Louckes) and MS and RM (both HLA-A2,B44-matched with Louckes). Louckes parental cells, and also clones carrying the retrovirus expression vector alone (Lop Z), served as controls in these same assays. Representative results obtained with three separate CMc effector preparations and two separate MS effector preparations are shown in Table 1. No significant lysis was observed with any of the Louckes transfectants, even though in the same assay these effectors did kill HLA-A2,B44-matched EBV-transformed LCL targets (Table 1). On the other hand, the various transfectants were susceptible to T-cell cytolysis when incubated with allospecific effectors generated against HLA class I antigens expressed on the Louckes cell line (HLA-A2,B44 in the case of ST anti-RM effectors; HLA-A2,B8,B44 in the case of LY anti-Louckes effectors).

Cytotoxicity assays on human B cells expressing LMP or Tr-LMP. Retrovirus-mediated introduction of the LMP gene into Louckes cells resulted in lower LMP expression than that usually detected in EBV-transformed LCLs, and expression tended to be lost with serial passage (47). Stronger and more stable expression was achieved by linking the

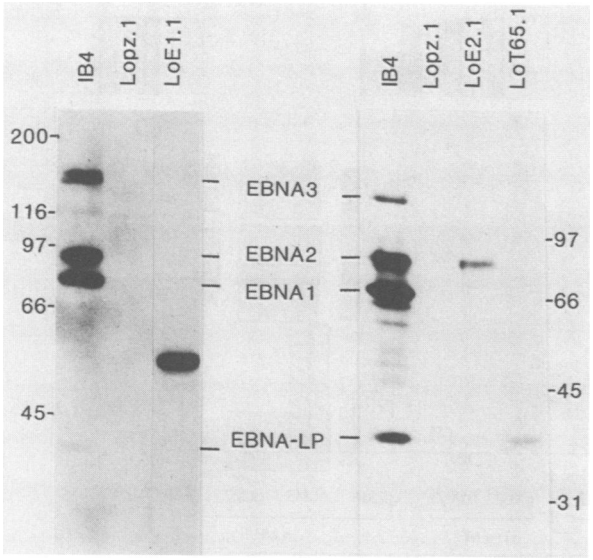

FIG. 1. Immunoblots of EBNA expression in transfected Louckes clones. IB4 is a latently infected EBV-transformed LCL expressing EBNAs 1, 2, 3, and LP. Clones of Louckes cells expressing the vector control plasmid (LopZ.1), EBNA 1 (LoE1.1), EBNA 2 (LoE2.1), and EBNA-LP (LoT65.1) are shown. EBV-immune human sera were used to detect the viral proteins on immunoblot.

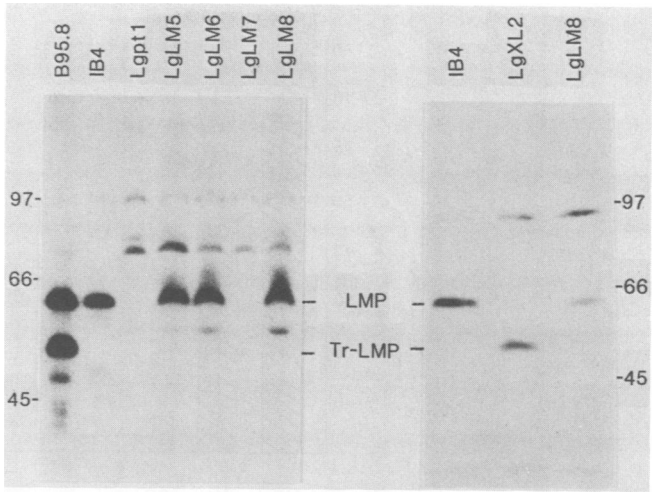

FIG. 2. Immunoblots of LMP and Tr-LMP expression in transfected Louckes clones, using MAb S12. Louckes cells were transfected with vector control plasmid (Lgpt 1), full-length LMP coding sequences (LgLM5 to -8), and Tr-LMP coding sequences (LgXL-2). Full-length LMP (60 kilodaltons) is detected in clones LgLM5, -6, and -8, and Tr-LMP (54 kilodaltons) is expressed in clone LgXL-2. The partially permissive B95.8 cell line expresses both full-length LMP and Tr-LMP, and only the full-length LMP is detected in the latently EBV-infected IB4 cell line. The slight reactivity in the higher-molecular-weight region of the gel is nonspecific.

LMP coding sequence to the human metallothionein type II promoter (D. Wang, D. Liebowitz, F. Wang, C. Gregory, A. Rickinson, R. Larson, T. Springer, and E. Kieff, submitted for publication). Of four clones transfected with the metallothionein type II-promoted gene in pSV2gpt, three (gLM5, -6, and -8) expressed the 60-kilodalton LMP at levels comparable to that seen in the EBV-transformed LCL, IB4 (Fig. 2). A fourth clone (gLM7) did not appear to express the protein (Fig. 2).

The four gLM transfectants, and control clones established by transfecting with the pSV2gpt plasmid alone (gpt1 and -2), were tested for susceptibility to EBV-specific T-cell cytolysis. In all, these assays used four separate effector T-cell preparations from each of the three virus-immune donors, CMc, RM, and MS; CMc effectors (HLA-A2,B8, B44-matched with Louckes) were used in a total of seven experiments, and RM and MS effectors (HLA-A2,B44-matched with Louckes) were each used in a total of four experiments. On each occasion of testing (as in the earlier assays; Table 1) lysis of the autologous LCL and of a relevant HLA-matched LCL, in the absence of any significant killing of HLA-mismatched LCLs or of natural killer-sensitive targets such as K562 and HSB2, confirmed the EBV-specific and HLA class I-restricted nature of these effector preparations (see Fig. 3 and legend). As illustrated by the results of two representative experiments (Fig. 3), such effectors caused significant lysis of the LMP-positive Louckes clones gLM5, and gLM6, and gLM8 but not of the control clones or of gLM7. This pattern of results was consistently observed; in the assays overall, lysis of the LMP-positive clones gLM5, gLM6, and gLM8 was in the 15 to 60% range of specific isotope release, while the corresponding values for control clones and gLM7 were almost always <10% specific release.

TABLE 1. T-cell cytotoxicity assays on Louckes transfectants

Target cells	Latent genes expressed	% Specific lysis by:						
		EBV-specific effectors (CMc)			EBV-specific effectors (MS)		Allospecific effectors	
		CMc-T1	CMc-T2	CMc-T3	MS-T1	MS-T2	ST-1	LY-1
Louckes	–	1	3	4	6	2	36	39
Lop Z	–	3	2	6	3	0	40	39
Lo E1.1	EBNA 1	2	0	5	3	0	58	26
Lo E1.2	EBNA 1	NT[a]	2	3	NT	1	NT	24
Lo E2.1	EBNA 2	1	2	2	2	5	28	26
Lo E2.2	EBNA 2	2	0	0	0	1	35	26
Lo T65.1	EBNA-LP	NT	1	4	2	2	46	NT
Lo T65.2	EBNA-LP	1	3	6	1	2	NT	25
Autologous LCL	A11	57	39	41	44	NT	NT	NT
A_2B_{44}-matched LCL	A11	35	NT	33	23	23	34	50
Mismatched LCL	A11	6	0	0	4	0	7	7

[a] NT, Not tested.

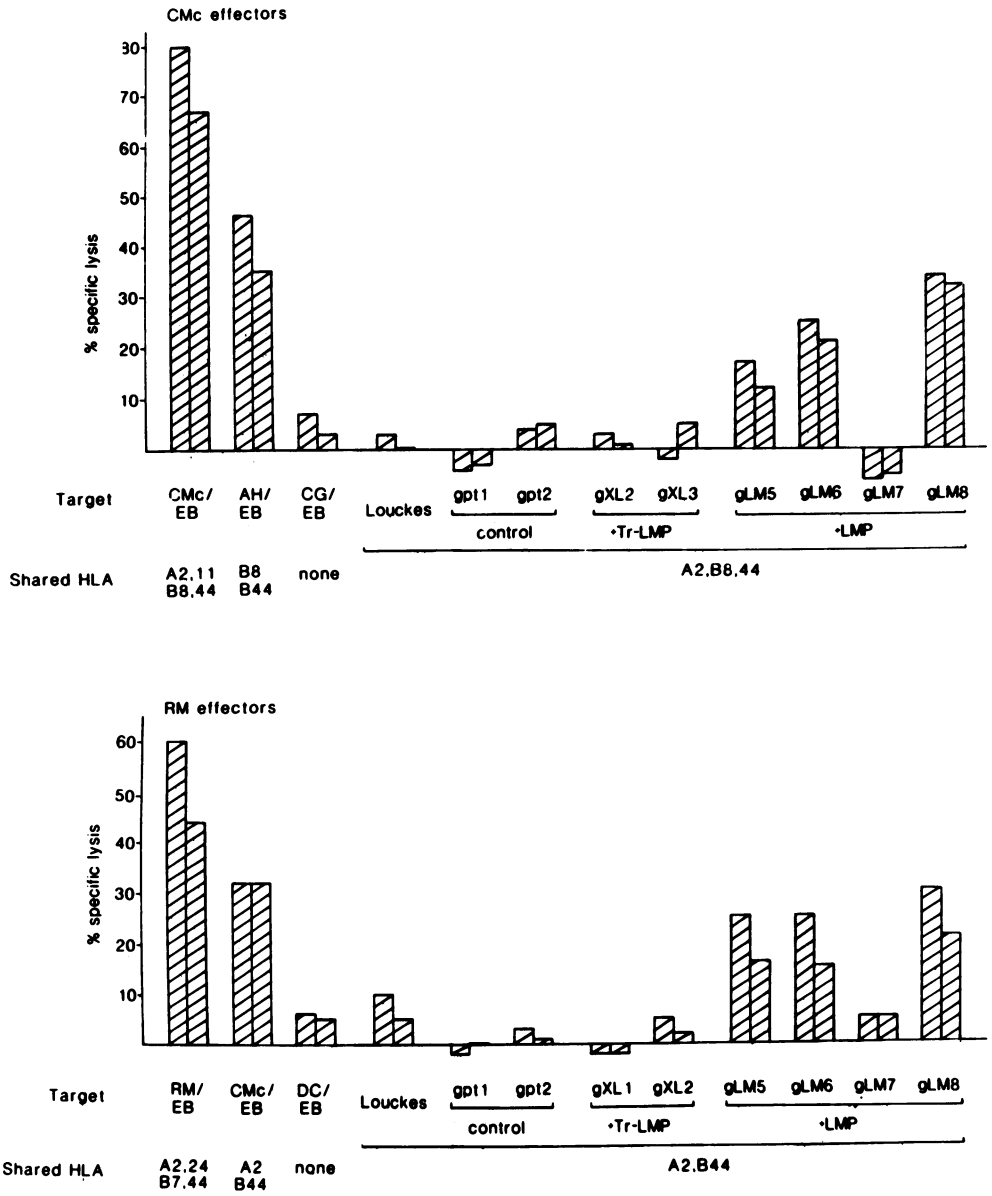

FIG. 3. EBV-specific T-cell cytolysis of LMP- and Tr-LMP-transfected Loukes cells by effector T-cell preparations from virus-immune donors CMc (upper panel) and RM (lower panel). Target cells included the Loukes parental cell line and Loukes clones transfected with vector control plasmid (gpt1 and -2), with the Tr-LMP coding sequences (gXL1, -2, and -3), or with the full-length LMP coding sequences (gLM5- to 8); additional targets serving as specificity controls included the autologous EBV-transformed LCL, a partially HLA-matched LCL, an HLA-mismatched LCL, and (not shown) the natural killer-sensitive K562 and HSB2 cell lines. The degree of HLA class I antigen matching between effectors and targets is indicated. Results are expressed as percent specific lysis of the target cells in a standard chromium release assay at effector/target ratios of 85:1 and 25:1 for donor CMc (upper panel) and 11:1 and 6:1 for donor RM (lower panel). Lysis of K562 and HSB2 targets in the same assays did not exceed 2%. In this experiment, specific lysis of gLM5, -6, and -8 targets by CMc effectors was reduced by 24 to 47% in the presence of the anti-HLA class I MAb W6/32 compared with a 43% reduction in the lysis of the autologous EBV-infected LCL, and specific lysis of gLM5, -6, and -8 by RM effectors was reduced by 32 to 50% compared with a 50% reduction in autologous EBV-infected LCL lysis.

Lysis of the LMP-positive Loukes transfectants was reduced in the presence of the anti-HLA class I MAb W6/32. Thus, in the particular experiments illustrated in Fig. 3, there was a reduction of 25 to 50% (Fig. 3 legend). This indicated an involvement of HLA class I molecules in such target cell recognition, although the level of blocking tended to be less marked than that observed for autologous LCL target lysis in the same assays. The identity of the HLA class I molecule mediating this lysis could not be determined unequivocally since the effector populations shared more than one antigen with Loukes cells. However, since these populations had a greater component of their EBV-specific cytotoxicity restricted through HLA-B44 than through HLA-A2 (or -B8) (data not shown), their recognition of LMP-positive Loukes clones is more likely to be via HLA-B44.

In additional control experiments designed to check that the lysis of LMP transfectants was indeed dependent on HLA-restricted recognition, we also prepared equally potent EBV-specific effector cells from two donors (SW and CG) who were HLA antigen mismatched at all class I loci with

Louckes cells. In a total of four experiments with such effectors, we noted that the gLM5, gLM6, and gLM8 targets suffered levels of lysis which were slightly higher than the background levels observed with the gpt controls. However this unscheduled lysis of LMP-expressing targets was distinct from that observed with HLA-matched effectors in that it did not exceed 15% and was not reduced in the presence of MAb W6/32 (data not shown).

Further experiments were undertaken to see whether transfectants expressing only a part of the LMP molecule were sensitive to EBV-specific cytolysis. A truncated form of LMP (Tr-LMP) is expressed late in the virus replication cycle in productively infected lymphocytes (16, 46). DNA sequence and mRNA mapping studies have suggested that translation of this protein begins at a methionine situated in the fourth LMP transmembrane domain and continues through to the LMP carboxy terminus (16). Expression of this truncated protein in Louckes cells therefore offered a means of determining the relative importance of the short amino terminus and first four transmembrane domains of LMP in T-cell recognition. Louckes cells were transfected with the DNA sequence capable of encoding Tr-LMP in the same vector and with the same promoter as used to express the full-length protein. Four transfected clones (gXL 1 to -4) expressed significant amounts of a 54-kilodalton Tr-LMP, similar in size to the truncated protein found in the productively infected B95.8 cell line (Fig. 2). When tested as targets for EBV-specific T-cell cytolysis, the Tr-LMP-expressing clones never showed lysis above the background levels suffered by control gpt transfectants (Fig. 3).

Selective lysis of the LMP transfectants, and not of Tr-LMP or gpt transfectants, by EBV-specific T cells (Fig. 3) could not be explained by any obvious difference between the clones in their display of HLA class I antigens or in their inherent susceptibility to cell-mediated killing. Thus, in each of the above assays (as in earlier work, Table 1), allospecific effectors directed against HLA class I antigens present on Loucks cells lysed all of the transfectant clones to a similar extent. The allospecific nature of this lysis was confirmed by the fact that it was markedly reduced (>50% blocking) in the presence of the anti-HLA class I antibody W6/32 (data not shown).

By using the pSV2.gpt.LMP construct, expression of full-length LMP in a second virus-negative human B-cell line, BL41 (HLA type A11; B35,B49), allowed similar T-cell detection experiments to be conducted with EBV-specific HLA-A11-restricted effectors. HLA-A11 is a "preferred" restricting determinant for the EBV-induced cytotoxic response (24), and we had access to cytotoxic T-cell preparations from three virus-immune donors (CMc, KS, and SW), each with a strong HLA-A11-restricted component. Figure 4 gives the results of representative assays with CMc and with KS effectors on two control (gpt) and two LMP-positive (gLM) clones of the BL41 cell line. Clearly, LMP expression sensitized the cells to high levels of EBV-specific cytolysis. A similar pattern of results was also observed with SW effectors. As a control in the same experiments, allospecific effectors directed against HLA-A11 as an alloantigen (see Materials and Methods) lysed the same gpt and gLM transfectants equally well (data not shown).

Cytotoxicity assays on mouse mastocytoma cells expressing A11 and LMP or Tr-LMP. Further experiments sought to determine whether the above lysis of LMP-positive targets by EBV-immune T cells was occurring through recognition of LMP epitopes or through recognition of an LMP-induced human B-cell activation antigen. LMP and Tr-LMP were expressed by transfection in a mouse mastocytoma cell line,

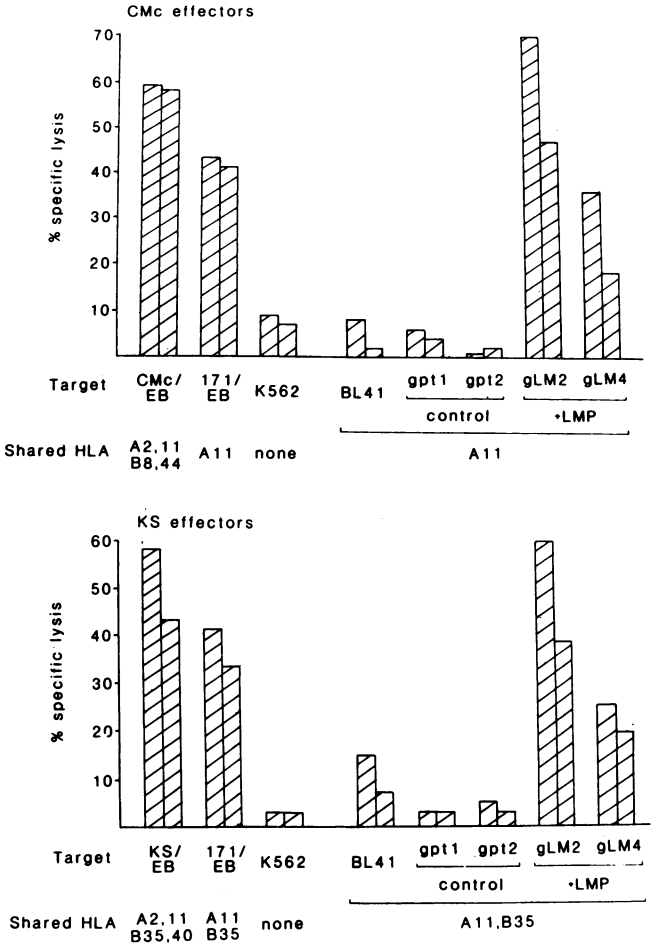

FIG. 4. EBV-specific T-cell cytolysis of LMP-transfected BL41 cells by effector T-cell preparations from the virus-immune donors CMc (upper panel) and KS (lower panel). Target cells included the BL41 parental cell line and BL41 clones transfected with the vector control plasmid (gpt1 and -2) or with the full-length LMP coding sequences (gLM2 and -4); additional targets serving as specificity controls included the autologous EBV-transformed LCL, an LCL derived by EBV transformation of normal B cells from the original BL41 patient, and the natural killer-sensitive K562 cell line. The degree of HLA class I antigen matching between effectors and targets is indicated. Note that, although KS effectors share both HLA-A11 and -B35 with BL41 cells, the dominant component of KS T-cell cytotoxicity is HLA-A11 restricted (see also Fig. 7). Results are expressed as percent specific lysis of the target cells in a standard chromium release assay at effector/target ratios of 10:1 and 5:1 for donor CMc (upper panel) and 8:1 and 2:1 for donor KS (lower panel).

P815-A11, already expressing an introduced human HLA-A11 gene (10). Figure 5 shows immunoblots of the transfected P815/A11 clones showing expression of LMP in the gLM series of clones and of Tr-LMP in the gXL series. The two proteins were equivalent in size to the corresponding gene products detectable in the latently infected IB4 LCL, which expresses LMP, and in the partially permissive B95.8 cell line, which expresses both LMP and Tr-LMP.

A series of assays with two alloreactive cytotoxic T-cell preparations (ST anti-SW with specificity for HLA-A11,B35 and JD anti-PB with specificity for HLA-A11,B22) confirmed that all of the above transfectants had retained expression of the HLA-A11 molecule and were indeed susceptible to A11-directed lysis by human T cells. Figure 6 shows the relevant results from one such assay, in which

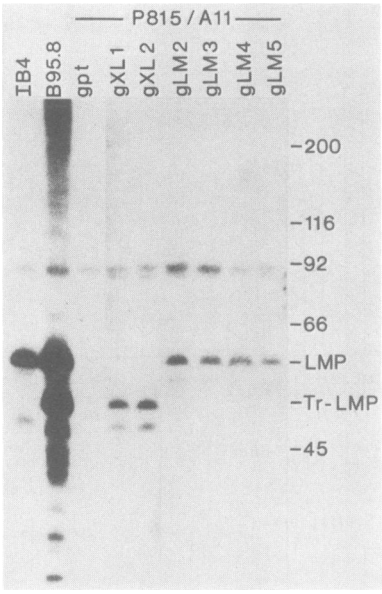

FIG. 5. Immunoblots of LMP expression in a mouse mastocytoma cell line expressing human HLA-A11 (P815-A11). IB4 and B95.8 are EBV-infected LCLs described in the legend to Fig. 2. Clones of B815-A11 are transfected with vector control plasmid (gpt), Tr-LMP coding sequences (gXL 1 and 2), and the full-length LMP coding sequences (gLM2 to -5).

strong killing of all P815-A11 transfectants and of the P815-A11 parent line was observed in the absence of any effect on mouse P815 cells themselves.

The same series of transfectants was then tested for lysis by the relevant EBV-specific cytotoxic T-cell preparations containing a strong HLA-A11-restricted component. Figure 7 (upper panel) shows the results of one such experiment, using KS effector T cells. Specific lysis of the LMP-positive clones was observed, with no significant killing of the vector control transfectants or of clones expressing Tr-LMP. This pattern of results was obtained on six separate occasions, using four independent preparations of KS effectors; overall, in these assays, lysis of LMP-positive clones was always in the range of 15 to 55% specific isotope release, whereas lysis of the other transfected clones was always <10%. Lysis of the LMP-positive targets was reproducibly inhibited by 40 to 60% in the presence of the anti-HLA class I MAb W6/32 (Fig. 7 legend). Similar results were obtained with CMc effectors, while SW effectors tended to give lower absolute levels of LMP-positive target lysis (10 to 25% specific isotope release versus <5% for control transfectants). Representative results from one of six assays with SW effectors are shown in Fig. 7 (lower panel). Lysis of the LMP-positive clones was again specifically inhibited with MAb W6/32 (Fig. 7 legend). When EBV-specific T-cell preparations from two HLA-A11-negative donors, CG and IB, were used as control effectors in the same experiments, lysis of the LMP-expressing cells was at background levels (data not shown).

DISCUSSION

Immune T-cell surveillance of EBV infection appears to play a crucial role in the prevention of virus-associated lymphoproliferative disease. Thus, immunosuppressed allograft recipients, whose EBV-specific T-cell function is severely impaired (4, 9), show a greatly increased susceptibility to EBV-positive lymphomas (3). Moreover, these lesions will regress if the patients' T-cell responses are restored (36). The present series of experiments begins a systematic evaluation

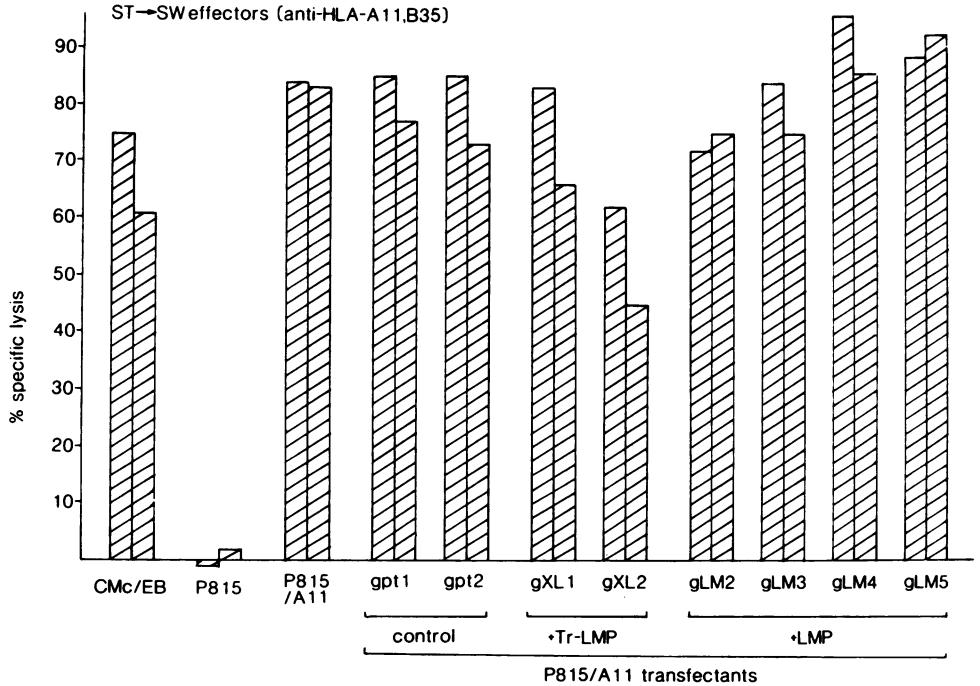

FIG. 6. Allospecific T-cell cytolysis of P815-A11 cells and P815-A11 transfectants. Allospecific T cells from donor ST directed against HLA-A11,B35 (ST→SW effectors [anti-HLA-A11,B35]) were used at effector/target ratios of 8:1 and 4:1. Targets included the EBV-transformed LCL (CMc/EB) which expresses A11 but not B35, the parental mouse mastocytoma cell line P815, the same mouse cell line expressing transfected HLA-A11 (P815-A11), and clones of P815-A11 cells transfected with vector control (gpt 1 and -2), the Tr-LMP coding sequence (gXL 1 and -2), or the full-length LMP coding sequence (gLM2 to -5). In this experiment, lysis of the P815-A11 cell line and of its transfectants was reduced by 30 to 60% in the presence of the anti-HLA class I MAb W6/32.

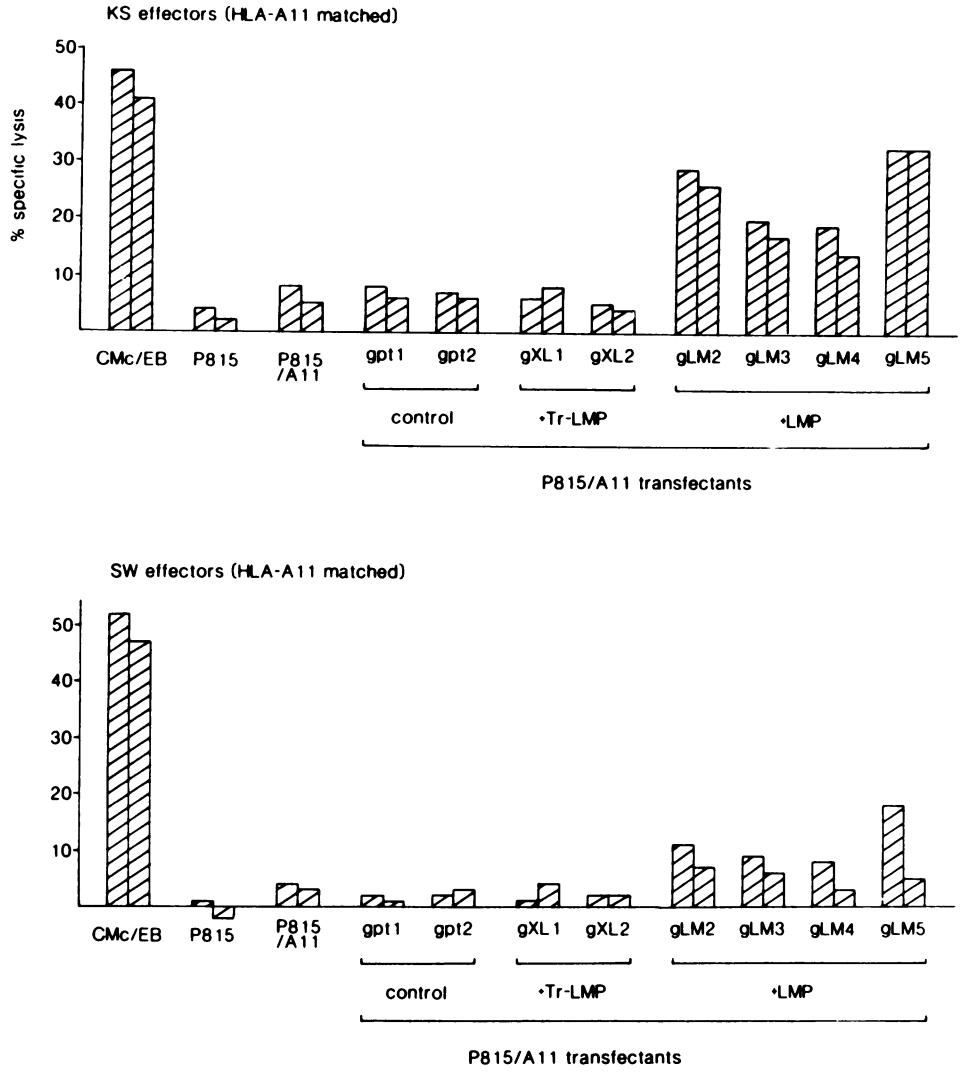

FIG. 7. EBV-specific T-cell cytolysis of LMP- and Tr-LMP-expressing P815-A11 cells by effector T-cell preparations from the HLA-A11-positive virus-immune donors KS (upper panel; effector/target ratios, 16:1 and 10:1) and SW (lower panel; effector/target ratios, 15:1 and 8:1). Reference targets included the HLA-A11-matched EBV-transformed LCL CMc/EB, an HLA-mismatched LCL (CG/EB; not shown), and the P815 mouse cell line. Lysis of the HLA-mismatched LCL by KS effectors did not exceed 10%, and that by SW effectors did not exceed 4%. In this experiment, specific lysis of the gLM2 to -5 targets by KS effectors was reduced by 40 to 60% in the presence of the anti-HLA class I MAb W6/32 compared with an 85% reduction in CMc/EB lysis, and specific lysis of gLM2 to -5 by SW effectors (at the 15:1 effector/target ratio) was reduced by 50 to 65% compared with a 79% reduction in the CMc/EB lysis.

of the individual EBV latent proteins as possible target antigens for the human cytotoxic T-cell response. A recent report has shown that one of the latent proteins, LMP, when expressed in murine cells, can elicit a cytotoxic T-cell response in the mouse (26). Here we show that in the naturally infected host, humans, LMP epitopes are recognized by components of the cytotoxic T-cell response from five different virus-immune donors, and in three cases HLA-A11 could be unequivocally identified as the restricting element.

In the design of these experiments we were mindful of the fact that EBV is a powerful activator of cellular gene expression in human B cells. Thus, the expression of individual EBV latent proteins in a virus-negative B-cell line could have dramatic effects on the cellular phenotype; in consequence, EBV-specific T-cell recognition of virus gene transfectants might be mediated through some indirect effect of the viral protein rather than through the protein itself. In this context we noted that the LMP-expressing Loucks and BL41 clones used as targets in this work tended to grow in clumps, rather than as single cells like the control transfectants, and showed increased surface expression of the intercellular adhesion molecules LFA1, ICAM1, and LFA3 (49; Wang, Liebowitz, Wang, et al., submitted unpublished observations). Because adhesion molecules are known to facilitate the initial phase of effector/target conjugation before immunologically specific recognition occurs (35), we were concerned that LMP expression might be associated with a generally increased susceptibility to T-cell-mediated cytolysis. In fact, there was no obvious difference between control and LMP-positive transfectants in their sensitivity to the allospecific effector populations used in this work. On the other hand, compared with control clones, the LMP-positive targets did show slightly higher levels of "background" killing when exposed to EBV-specific but HLA-mismatched effectors. This rise in background lysis may indeed be a consequence of LMP-induced changes in adhesion molecule expression. However, the effect is small when compared with the differences observed when the control

and LMP-positive targets were exposed to EBV-specific, HLA-matched T-cell preparations (Fig. 3 and 4). Here there was very significant killing of the LMP-positive clones at levels well above background and which could be reduced by MAb blockade of HLA class I antigens. We infer, therefore, that the lysis of LMP-positive clones by HLA-matched effectors is largely the result of immunologically specific recognition and not some nonspecific consequence of altered adhesion molecule expression.

Our experiments also examined a second mechanism by which a viral protein such as LMP might indirectly sensitize cells to EBV-specific recognition. Thus, it is possible that the T-cell response is directed against cellular target antigens whose expression by human B cells is selectively induced by EBV infection (27, 40). The various B-cell activation antigens which characterize the LCL cell surface phenotype (30, 31, 39) are obvious candidates in this respect (40). Indeed, we know that expression of individual EBV genes in the human B-lymphoma cell line Louckes can elicit particular facets of the LCL phenotype, the best example of which is the EBNA 2-induced upregulation of the B-cell activation antigen CD23 (47). Such changes appear to be exclusive features of the EBV–human B-cell interaction, however, and have never been experimentally reproduced in any other type of recipient cell. We therefore sought to establish EBV gene transfectants of a nonlymphoid mouse cell line, P815-A11, to examine EBV-specific T-cell recognition away from the human B-cell environment.

The LMP transfectants of P815-A11 cells showed no obvious change in growth phenotype, remained sensitive to HLA-A11-directed allospecific lysis, and did not suffer any unscheduled lysis when incubated with EBV-specific, HLA-mismatched effector preparations. Three different sources of EBV-specific, HLA-A11-restricted cytotoxic T cells lysed the LMP-expressing clones. This strongly implies T-cell recognition of the viral protein rather than of some induced cellular change (unless the latter were conserved across species). Furthermore, just as in the Louckes experiments, Tr-LMP-expressing clones were not killed. Assuming that the truncated form of LMP is not protected from processing in any way, the results raise the possibility that HLA-A11-restricted recognition involves target epitopes derived from the N-terminal 128 amino acids of LMP, i.e., that segment which is not present in Tr-LMP.

These findings are interesting in view of a recent report (38) showing that a synthetic peptide representing amino acids 43 to 53 of the LMP sequence could reactivate apparently EBV-specific cytotoxic T-cell activity when directly cocultivated with the peripheral lymphocytes of certain virus-immune donors. Unfortunately, the converse experiment, testing whether this peptide could also sensitize EBV-negative target cells to lysis by the reactivated effectors (c.f. reference 42), was not described in the above paper. Our own observations to date with this type of approach suggest that the LMP 43 to 53 peptide is not the dominant target antigen for the LMP-specific effectors described here (R. J. Murray, unpublished observations). Such findings are not necessarily contradictory, however, since they refer to T-cell responses restricted through different HLA determinants. Likewise, EBV-specific T-cell clones have recently been isolated which kill autologous cell lines transformed by type A EBV isolates, and therefore expressing the EBNA 2A protein, but not autologous lines transformed by type B isolates and expressing the antigenically distinct EBNA 2B protein (20a). These T-cell clones, which are restricted through HLA determinants other than those involved in the present work, therefore appear to be recognizing epitopes derived from either EBNA 2A or possibly some other protein exclusively expressed in type A virus-transformed lines. Much remains to be done, therefore, before the list of EBV lymphocyte-detected membrane antigens can be considered complete.

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