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Direct Immunosuppressive Effects of EBV-Encoded Latent Membrane Protein 1

Danny F. Dukers,[1] Pauline Meij, Marcel B. H. J. Vervoort, Wim Vos, Rik J. Scheper, Chris J. L. M. Meijer, Elisabeth Bloemena, and Jaap M. Middeldorp

In neoplastic cells of EBV-positive lymphoid malignancies latent membrane protein (LMP1) is expressed. Because no adequate cellular immune response can be detected against LMP1, we investigated whether LMP1 had a direct effect on T lymphocyte activation. In this study we show that nanogram amounts of purified recombinant LMP1 (rLMP1) strongly suppresses activation of T cells. By sequence alignment two sequences (LALLFWL and LLLLAL) in the first transmembrane domain of LMP1 were identified showing strong homology to the immunosuppressive domain (LDLLFL) of the retrovirus-encoded transmembrane protein p15E. The effects of rLMP1 and LMP1-derived peptides were tested in T cell proliferation and NK cytotoxicity assays and an Ag-induced IFN-γ release enzyme-linked immunospot assay. LMP1 derived LALLFWL peptides showed strong inhibition of T cell proliferation and NK cytotoxicity, while acetylated LALLFWL peptides had an even stronger effect. In addition, Ag-specific IFN-γ release was severely inhibited. To exert immunosuppressive effects in vivo, LMP1 has to be excreted from the cells. Indeed, LMP1 was detected in supernatant of EBV-positive B cell lines (LCL), and differential centrifugation in combination with Western blot analysis of the pellets indicated that LMP1 is probably secreted by LCL in the form of exosomes. The amount of secreted LMP1 in B cell cultures is well below the immunosuppressive level observed with rLMP1. Our results demonstrate direct immunosuppressive properties of LMP1 (fragments) and suggest that EBV-positive tumor cells may actively secrete LMP1 and thus mediate immunosuppressive effects on tumor-infiltrating lymphocytes. Moreover, we demonstrate, for the first time, that transmembrane protein-mediated immunosuppression is not solely restricted to RNA tumor viruses, but can also be found in DNA tumor viruses. *The Journal of Immunology,* 2000, 165: 663–670.

Epstein-Barr virus, a member of the γ-herpes virus family, is associated with various lymphoproliferative and epithelial malignancies, such as Hodgkin's disease (HD),[2] Burkitt's lymphoma, B and T cell non-Hodgkin lymphoma, and nasopharyngeal carcinoma (1–5). In EBV-positive lymphomas in immune-competent patients, a restricted set of latent viral genes is expressed, i.e., latent membrane proteins 1, 2A, and 2B (LMP1, -2A, and -2B), Epstein-Barr nuclear Ag (EBNA1) and the non-translated RNAs EBER1 and EBER2 (6, 7). A key protein in EBV-mediated oncogenesis of lymphoproliferative disorders is LMP1, a protein with transforming capacities (8). The neoplastic cells in EBV-positive HD, called Hodgkin Reed-Sternberg (H-RS) cells, express exceptionally high levels of LMP1. Although LMP1 is a target for CTL recognition in the context of different MHC class I restriction elements in vitro, it seems to be subdominant (9–11). Surprisingly, only few EBV-positive HD patients have a weak, but detectable, Ab response against LMP1 despite high Ab levels against other EBV-encoded proteins (12). This underlines the inability of the host to mount appropriate immune responses against LMP1, which is suggestive of immune escape.

Because eradication of tumor cells is largely dependent on NK cell- and activated CTL-mediated killing, their presence in the reactive infiltrate is considered to be of major importance. Indeed, we previously found that the tumor-infiltrating lymphocytes in EBV-positive cases of HD contained significantly higher numbers of activated CTLs and NK cells compared with EBV-negative cases (13–15). Although in the neoplastic cells of EBV-positive HD cases potentially immunogenic viral proteins are expressed (1, 3), MHC class I molecules and TAP are present on the neoplastic cells (13), which have been shown susceptible to T cell-mediated lysis in vitro, and they are inadequately eliminated in vivo, possibly due to local immune suppression.

In a recent study we demonstrated the presence of human IL-10 expression in H-RS cells in EBV-positive cases of HD (15). This, however, did not influence T cell phenotypical markers in infiltrating lymphocytes compared with EBV-negative cases. Furthermore, van den Berg et al. showed that TARC, a T lymphocyte-attracting chemokine, is preferentially expressed in neoplastic cells of HD, but despite the influx of activated cells (granzyme B positive), the EBV-positive H-RS cells are inadequately eliminated (16). In support of this, Frisan et al. described a lack of functional CTL activity in HD-derived tumor-infiltrating lymphocytes (17). In line with this finding we described that high numbers of phenotypically activated CTLs in HD were associated with an adverse clinical outcome (14). Therefore, current data suggest that in HD mechanisms are operational to circumvent the immune system.

The presence of high levels of LMP1 in neoplastic cells of EBV-positive cases of HD suggest a direct role for LMP1 in local immune suppression, similar to that of retrovirally transformed cells. In the early 1960s it was shown that murine retroviruses (Gross,

Friend, Rauscher, and Moloney) exerted various immunosuppressive influences during infections in vivo (18–21). These studies showed, among others, a decrease in cell-mediated immunity, reduced levels of circulating cytotoxic Abs, and the inability of cells to produce IFN-γ (15–17). Snyderman et al. showed that the transmembrane protein p15E was responsible for the immunosuppressive properties of these retroviruses (22). Subsequently, a 17-aa region named CKS 17 within the p15E protein was identified that was highly conserved among murine, feline, and human retroviruses (23). In vitro, CKS 17 was able to inhibit both the proliferation of T cells and NK cell-mediated cytotoxicity (20, 24, 25). Furthermore, Oostendorp et al. showed that the immunosuppressive effects of the 17-aa-long CKS17 could be narrowed to a conserved hexapeptide with an amino acid sequence LDLLFL (24, 25). The mechanism by which these retrovirally encoded peptides employ their effects is not completely clarified. It is suggested that these peptides directly inhibit intracellular signaling pathways in T cells by interfering with protein kinase C (PKC) and adenylate cyclase (26). Thus, this effect is both MHC class I and MHC class II independent.

In this paper we demonstrate that low concentrations of immunoaffinity-purified recombinant LMP1 (rLMP1) have strong immune suppressive properties on T cells activated by mitogen, Ag, or CD3/CD28 stimulation; NK cytotoxicity; and Ag-induced IFN-γ release. By sequence alignment we found that the first transmembrane domain of LMP1 contained two sequences, LALLFWL and LLLLAL, highly homologous to the retrovirally encoded peptide LDLLFL, part of the retroviral transmembrane protein p15E. Only LALLFWL and several derivatives containing this sequence have strong inhibitory effects on T cell proliferation and NK cytotoxicity in vitro. To exert the immunosuppressive effect in vivo, LMP1 or LMP1-derived peptides have to be excreted from the cells. Using a quantitative LMP1 ELISA,[3] LMP1 can be detected at low concentrations in the supernatants of EBV-positive lymphoblastoid cell lines, suggesting that LMP1 is actually secreted by these cells. Therefore, we next investigated the mechanism by which LMP1 is excreted, either passively by means of cell death and subsequent cell lysis or actively by secretion of LMP1 alone or in the form of LMP1-containing membrane vesicles.

Our results suggest that LMP1 may be actively secreted from EBV-positive tumor cells to mediate immunosuppressive effects on tumor-infiltrating lymphocytes surrounding the neoplastic cells in vivo.

Materials and Methods
Cell lines

EBV-positive (JY, RAJI) and -negative (BJAB, RAMOS) cell lines were cultured in RPMI 1640 (BioWhittaker, Verviers, Belgium) medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 10% FCS (Integro, Zaandam, The Netherlands), 100 IU of streptomycin, and 100 μg of penicillin (referred to as complete medium) at 37°C in 5% CO₂. Sf9 insect cells, derived from the fall army worm *Spodoptera frugiperda*, infected with either wild-type or rLMP1 baculovirus (provided by Dr. F. Grässer, Universitäts Kliniken des Saarlandes, Hamburg, Germany), were cultured in Sf900-II synthetic serum-free medium (Life Technologies, Grand Island, NY) containing 100 IU of streptomycin and 100 μg of penicillin at 27.5°C at normal air atmosphere.

Preparation of B cell lymphoblastoid cell lines was performed as described previously (27). PBMCs were isolated from heparinized blood of healthy volunteers by density centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden).

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[3] P. Meij, M. B. H. J. Vervoort, C. J. L. M. Meijer, E. Bloemena, J. M. Middeldorp. Production monitoring and purification of EBV-encoded latent membrane protein I expressed and secreted by recombinant baculovirus-infected cells. *Submitted for publication.*

Recombinant LMP1

Sf9 cells expressing the full-length LMP1 in baculovirus under the control of a polyhedrin promotor and baculo wild type were cultured to the log phase (1×10^6 cells/ml) and infected with one of the baculovirus constructs at a multiplicity of infection of 0.1. After 72 h cells were harvested and washed with PBS.

Recombinant LMP1 was extracted from the cells by a 1-h incubation in sodium acetate (pH 6.0) containing 0.22 M octyl glucoside and 3 M ureum at 4°C. Recombinant LMP1 was purified using mAb-based immunoaffinity chromatography as described previously (see Footnote 3), finally reaching a concentration of 0.41 μg/ml purified protein.

Sequence alignment

Amino acid sequence homology search and alignment were performed using FASTA and PALIGN, which were included in the PC/Gene sequence software package (IntelliGenetics, Mountain View, CA).

Peptide synthesis

Peptides representing different domains of the LMP1 protein and various control peptides were synthesized using a 433 A peptide synthesizer (Applied Biosystems, Foster City, CA). The F-moc amino acids used were purchased from Bachem (Bubendorf, Switzerland). For some peptides the N-terminus was blocked by acetylation to neutralize the N-terminal charge. The peptides were purified using reverse-phase HPLC chromatography (System Gold, Beckman, Mijdrecht, The Netherlands). Peptides were dissolved in DMSO at a stock concentration ranging from 10–40 mM. An overview of the peptides used in this study is given in Table I.

Lymphocyte proliferation assay

PBMCs were cultured in a 96-well round-bottom culture plate (Nunc, Copenhagen, Denmark) at 2×10^5 cells/well for Ag-induced proliferation and 5×10^4 cells/well for mitogen-induced proliferation suspended in complete medium containing 10% human pooled serum (CLB, Amsterdam, The Netherlands) instead of FCS. Soluble Ag (tetanus toxoid, RIVM, Bilthoven, The Netherlands) was used in a final concentration ranging from 25–6.25 μg/ml, whereas the mitogen PHA was used at 5 μg/ml, and anti-CD3/anti-CD28 were both used at 2.5 μg/ml. Peptides were added at a final concentration of 10 μM or lower. As a control, the cells were cultured in the presence of 0.1% DMSO. All cultures were performed in triplicate.

Cells were cultured for 3 days at 37°C in a humidified atmosphere of 5% CO₂ in air for mitogen-induced proliferation and 6 days for Ag-induced proliferation. During the last 4 h 14.8×10^3 Bq [³H]thymidine was added per well, and the cells were harvested onto fiberglass filters. [³H]Thymidine incorporation was determined by liquid scintillation counting, and subsequently the stimulation index was determined according to the following formula: S.I. = [(cpm$_{mitogen/Ag}$ − cpm$_{background}$)/(cpm$_{untreated}$ − cpm$_{background}$)]. Peptide-induced inhibition of proliferation was calculated as the percent inhibition in relation to the proliferation in the presence of DMSO without peptide.

NK cytotoxicity assay

The leukemia cell line K562 was used as target at a concentration of 5000 cells/well. PBMCs were washed twice, resuspended in complete medium, and seeded in 96-well round-bottom plates at different concentrations (E:C cell ratios ranging from 80:1 to 2.5:1). K562 cells were labeled with ⁵¹Cr (sodium chromate, 0.5×10^6 cells/14.8×10^6 Bq of ⁵¹Cr; Malinckrodt, Petten, The Netherlands) for 90 min at 37°C. Thereafter, the target cells were washed three times and resuspended in complete medium. To each well 50 μl of peptide solution (100 μM) or medium containing DMSO was added to correct for possible effects of DMSO in which the peptides were solubilized. Maximum ⁵¹Cr release was measured in the presence of 1% Triton X-100. Plates were centrifuged for 2 min at $100 \times g$ and incubated for 4 h at 37°C in 5% CO₂. NK cell cytotoxicity was determined by measuring the ⁵¹Cr release in the supernatant using a gamma counter (Wallac, Turku, Finland). All determinations were performed in triplicate. For each sample the percentage of specific killing was calculated as follows: % specific killing = [(cpm$_{sample}$ − cpm$_{background}$)/(cpm$_{max\ release}$ − cpm$_{background}$)] × 100. Subsequently, the peptide-induced inhibition of NK cytotoxicity was calculated with respect to control wells without peptides.

Enzyme-linked immunospot assay for single cell IFN-γ release

This assay was performed as previously described to detect Ag-specific T cells in fresh PBMCs (28). Ninety-six-well, polyvinylidene difluoride-backed plates (Millipore, Bedford, MA) were precoated with 15 μg/ml anti-IFN-γ mAb 1-DIK (MABTECH, Stockholm, Sweden). PBMCs were

Table I. *Inhibition of NK cytotoxicity and T cell proliferation by LMP1-derived peptides and appropriate controls*

Peptide Sequence[a]	Amino Acid Sequence LMP	% Inhibition of	
		NK cytotoxicity	T cell proliferation[b]
LMP1 purified protein	1–386	ND	>98%
LMP1-derived LALLFWL peptides			
H-**LALLFWL**-OH	34–40	76	47
Ac-**LALLFWL**-NH2	34–40	85	92
H-LGLALLLLL**LALLFWL**-OH (LMP1 short)	25–40	35	40
Ac-LGLALLLLL**LALLFWL**-NH$_2$ (LMP1 short)	25–40	57	63
H-LLL**LALLFWL**YIVMSDWTGGA-OH	32–51	77	62
Ac-LL**LALLFWL**YIVMSDWTGGA-NH$_2$	32–51	55	67
H-**LALLFWL**YIVMSDWTGGALLVLY-OH (LMP1 long)	34–56	31	29
Ac-**LALLFWL**YIVMSDWTGGALLVLY-NH$_2$ (LMP1 long)	34–56	43	37
H-RPPRGPPLSSSLGLALLLLL**LALLFWL**YIVMSDWTGGA-OH	14–51	28	80
Retroviral peptides[c]			
H-**LDLLFL**-OH		57	44
Ac-**LDLLFL**-NH2		52	51
H-LQNRRG**LDLLFL**KEGGL-OH		36	29
Control peptides			
H-LLLLAL-OH	31–36	9	15
Ac-LLLLAL-NH$_2$	31–36	11	16
H-LALLLL-OH	Inversion 31–36	8	3
Ac-LALLLL-NH$_2$	Inversion 31–36	12	5
H-LLWTLVVLL-OH	LMP2 329–337	7	12
H-CVAHKLMSFED-OH	PgP 328–337	7	9

[a] Peptides used in this study as described by the standard one-letter abbreviations for amino acids. Ac, acetylated peptide; PgP, P-glycoprotein.
[b] PHA-induced proliferation.
[c] Peptides according to Oostendorp et al. (24, 25).

added in duplicate wells at 5.0×10^5 cells/well in the presence of 2 μM peptide GLCTLVAML (29), A2-resticted epitope of BMLF1, one of the lytic cycle Ags of EBV, and two HLA-A2 restricted LMP1-derived epitopes, YLLEMLWRL and YLQQNWWTL (10). PHA (10 μg/ml) was used as a positive control. The plates were incubated overnight at 37°C in 5% CO$_2$. The following day, cells were discarded, and the plates were incubated with the second biotinylated anti-IFN-γ mAb 7-B6-1 (MABTECH) at a concentration of 1 μg/ml for 3 h at room temperature. This was followed by incubation with streptavidin-conjugated alkaline phosphatase (MABTECH) for an additional 2 h. Individual cytokine-producing cells were detected as dark spots after a 30-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using an phosphatase-conjugate substrate kit (Bio-Rad, Richmond, CA). The spots were counted under a dissection microscope, and the number of specific T cell responders was calculated after subtracting negative control values.

Isolation of LMP1 from supernatants of EBV-positive B cell lines

All cell lines were cultured in fresh medium for 2 days at 1×10^6/ml, and subsequently the supernatant was fractionated by differential centrifugation as described recently (30). Briefly, supernatant was centrifuged for 10 min at $300 \times g$ to remove cells. After a second centrifugation at $300 \times g$ for 10 min, the supernatant was centrifuged twice at $1,200 \times g$ (10 min each time), then at $10,000 \times g$ (30 min), $70,000 \times g$ (60 min), and $100,000 \times g$ (60 min), respectively, using a Beckman ultracentrifuge with a Ti40 rotor. Following each centrifugation step, the pellet was collected for further analysis, and the supernatant was used for subsequent centrifugation.

Gel electrophoresis and immunoblotting

The pellets obtained after differential centrifugation were solubilized and sonicated in sample buffer (0.2 M Tris-HCl (pH 6.8), 4% SDS, 18% glycerol, 2% 2-ME, and 0.004% bromophenol blue) and boiled for 5 min. Samples were separated by SDS-PAGE, using a stacking gel of 3% acrylamide and a linear separation gel of 10% acrylamide (MiniProtean II electrophoresis system, Bio-Rad, Hercules, CA). Polypeptides were transferred onto 0.22-μm pore size nitrocellulose filters (Schleicher & Schuell, Hertogenbosch, The Netherlands) by Western blotting (Mini Trans-blot cell, Bio-Rad) using standard techniques. Nonspecific binding sites on the nitrocellulose were saturated with blocking buffer (5% nonfatty milk powder and 5% FCS in PBS). Subsequently, mAbs directed against the C- and N-

terminal domains of LMP1 (OT22C and OT 22CN, respectively (Organon Teknika, Boxtel, The Netherlands)) in optimal dilution (0.1–1 μg/ml) were added and incubated for 1 h at room temperature. In addition, mAbs against CD86 (B7.2, Ancell, Bayport, MN) and HLA-DR (Dako, Carpenteria, CA) were applied to confirm the presence of membrane-associated vesicles, and EBNA1 (OT1X, Organon Teknika) was used as a control for the presence of the remaining nuclear fragments. After washing three times with 0.05% Tween in PBS, specifically bound IgG was detected with a HRP-conjugated second Ab, diluted in blocking buffer. After two washes in 0.05% Tween in PBS and two washes in PBS, HRP activity was visualized by electrochemiluminescence (Roche, Mannheim, Germany).

Statistical analysis

For statistical analysis of the data, a two-sided Mann-Whitney U test was conducted. A p value <0.05 was considered statistically significant.

Results

Effects of purified rLMP1 on T cell proliferation in vitro

Preliminary T cell proliferation experiments suggested that LMP1-containing cell extracts might affect T lymphocyte function directly. Therefore, we were interested in whether highly purified rLMP1 was able to directly influence polyclonal T cell proliferation induced by PHA-mitogen or anti-CD3/anti-CD28 mAb stimulation of PBMC in vitro. For this purpose rLMP1 was purified to near homogeneity (>95%) from Sf9 cells infected with LMP1-expressing baculovirus using an mAb-based immunoaffinity purification.

Recombinant LMP1 was added during activation of PBMC at various concentrations ranging from 0.2 to 20.5 ng/ml (4.9–490 fmol/ml). As a control, the buffer in which rLMP1 was dissolved was added. After 3 days of incubation, both PHA and anti-CD3/anti-CD28-induced proliferation was measured by [^{3}H]thymidine incorporation. In the presence of the highest concentration of rLMP1 (20.5 ng/ml) the maximal inhibition was >98%, whereas addition of buffer had no effect. Inhibition of proliferation gradually decreased when lower amounts of rLMP1 were added (Fig. 1).
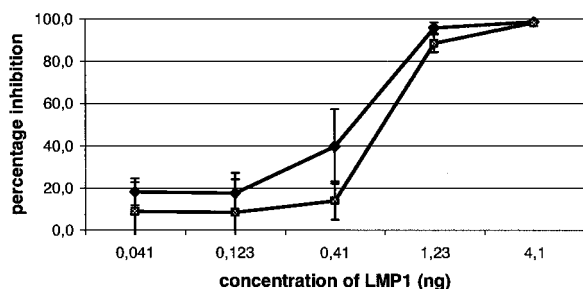
FIGURE 1. Inhibition of PHA-induced (●) and anti-CD3/antiCD28-induced (■) proliferation by rLMP1. Proliferation was measured by [³H]thymidine incorporation after 3-day culture of PBMCs with 5 μg/ml PHA or 2.5 μg/ml of both anti-CD3/antiCD28 in the presence or the absence of rLMP1. The data are the median ± SD of five different donors. The median counts per minute in controls in the absence of LMP1 were 7900 and 9700 for PHA and anti-CD3/anti-CD28, respectively.

Even in the presence of the highest concentration of rLMP1, PBMC remained viable, as evaluated visually by trypan blue exclusion. Visual evaluation of this phenomenon revealed that the increase in the number of activated cell clumps in the wells and proliferation as measured by [³H]thymidine incorporation inversely correlated with the amount of rLMP1 present in the wells (Fig. 2). Thus, LMP1 exerts a direct inhibitory effect on T cell proliferation by interfering with the cell-cell contact required during the activation stage.

Effects of rLMP1 on Ag-specific T lymphocyte activation

Tetanus toxoid-induced proliferation was analyzed showing similar suppression as described above. Subsequently, we analyzed whether rLMP1 was able to inhibit Ag-specific cytokine production of T cells. The effect of rLMP1 on IFN-γ production of T cells reactive with an immunodominant EBV-peptide GLCTLVAML

(BMLF1-derived epitope) and two HLA-A2-restricted LMP1-derived epitopes (YLLEMLWRL and YLQQNWWTL) was investigated using an enzyme-linked immunospot assay. In all experiments rLMP1 was added overnight at a final concentration of 20.5 ng/ml.

The frequencies of T cells reactive with the GLCTLVAML epitope derived from the lytic BMLF1 decreased by 70% (from 121 ± 20 spots/10⁵ PBMC to 31 ± 17 spots/10⁵ PBMC; n = 3). Furthermore, IFN-γ production in T cell precursors reactive with LMP1-derived epitopes YLLEMLWRL and YLQQNWWTL, was inhibited under the influence of rLMP1 by 59% (68 spots/10⁵ PBMC to 28 spots/10⁵ PBMC) and 62% (52 spots/10⁵ PBMC to 20 spots/10⁵ PBMC), respectively. IFN-γ production in PHA-induced PBMC was used as a positive control and was also suppressed at least 81% (>600 spots/10⁵ PBMC to 114 ± 60 spots/10⁵ PBMC) when rLMP1 was added (n = 3). These data clearly show that besides inhibition of the Ag-, mitogen-, and mAb-induced T cell proliferation, rLMP1 is also able to suppress both Ag- and mitogen-induced IFN-γ production in vitro.

Identification of potentially immunosuppressive domain in LMP1

In an attempt to find a rationale for the observed T cell effects of LMP1, we explored possible homologies with other viral proteins possessing immunosuppressive activity, such as retroviral transmembrane glycoprotein p15E. To reveal possible homologies with the p15E-derived immunosuppressive domain we used the CKS-17 sequence for alignment analysis. Surprisingly, within the first transmembrane domain of LMP1 two small domains with strong homology to the CKS-17 minimal functional domain were found (LALLFWL and LLLLAL, respectively). No homology was found with other transmembrane proteins or with LMP2 or other EBV-encoded proteins. To further test whether LMP1-derived peptides exhibited immunosuppressive properties, we investigated

FIGURE 2. Visual evaluation of rLMP1-induced inhibition of proliferation. *A–C*, Negative control (no stimulation), PHA-induced proliferation, and anti-CD3/anti-CD28-induced proliferation, respectively. *D–G*, PHA-induced proliferation; *H–K*, anti-CD3/anti-CD28-induced proliferation. *D* and *H*, Proliferation in the presence of mitogen and the buffer in which rLMP1 was dissolved. The effects of rLMP1 are depicted in *E, F*, and *G* and *I, J*, and *K* with PHA and anti-CD3/anti-CD28, respectively. Pronounced effects were seen when high and intermediate amounts of rLMP1 were added.
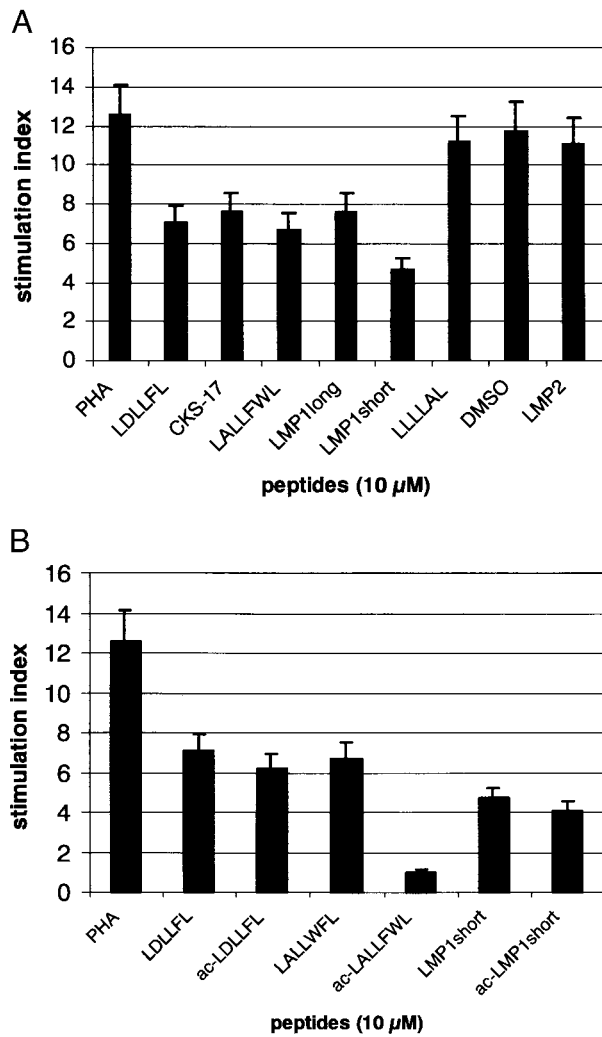
A

B
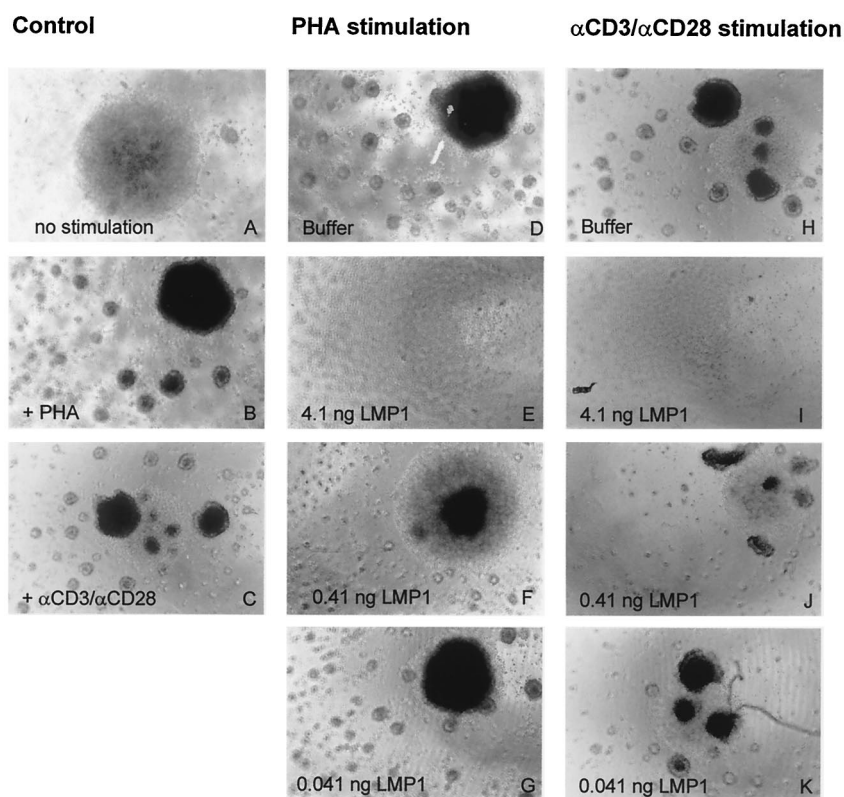
FIGURE 3. *A*, Effects of various synthetic peptides derived from LMP1 and p15E on PHA-induced T cell proliferation. T cell proliferation in the presence and the absence of peptides is represented as the stimulation index (S.I.). The data are the median ± SD of three representative experiments. LMP2 and DMSO were used as a negative control. The final concentration of peptide was 10 μM. *B*, Effects of acetylation of peptides on inhibition of PHA-induced T cell proliferation. Acetylated peptides clearly showed greater inhibition than nonacetylated peptides. This inhibitory effect is probably due to the neutralization of the N-terminal charge of the peptides.

their effects on Ag- and mitogen-induced T cell proliferation and NK cytotoxicity.

Effects of LMP1-derived peptides on T cell proliferation in vitro

To explore the effects of LMP1 on T cell function in more detail, we first investigated whether LMP1-derived peptides were able to inhibit T cell proliferation. To examine potential immunosuppressive effects we evaluated the Ag-, mitogen-, and anti-CD3/anti-CD28-induced proliferation of PBMCs in the presence and the absence of putative immunosuppressive peptides of LMP1 with homology to retrovirus-derived peptides. Retroviral peptides (CKS17 and LDLLFL), previously shown to inhibit mitogen- and Ag-induced T cell proliferation, were used as a positive control (21, 23, 24). Various irrelevant peptides, either LMP1 derived or obtained from unrelated proteins, were used as a control for T cell proliferation assays (see Table I).

PHA-induced proliferation could be inhibited up to 80% by addition of 10 μM LMP1-derived peptide. When long and short
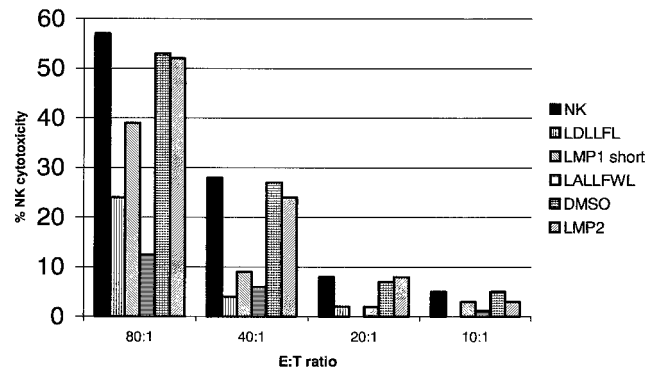

FIGURE 4. Effects of various peptides on NK cell cytotoxicity. The first transmembrane domain of LMP1 (LALLFWL) clearly shows a strong inhibition of NK cytotoxicity. An LMP2-derived peptide and DMSO were used as negative controls. The final concentration of the peptides was 100 μM.

LMP1-derived peptides were tested the latter showed a stronger inhibition of T cell proliferation (47%) compared with the longer peptides (29%; Fig. 3*A*). These peptides were also able to significantly inhibit tetanus toxoid-induced proliferation up to 70% (data not shown). When acetylated peptides were used to neutralize the N-terminal charge of the peptides to mimic, more closely, their hydrophobic character in the membrane, inhibition was even stronger, being >90% for acetylated LALLFWL (Table I and Fig. 3*B*). Again, the shorter domain of LMP1, LALLFWL, gave the strongest inhibition of proliferation compared with the longer peptides (Table I). Although both LALLFWL and LLLLAL showed strong homology with the immunosuppressive retroviral peptides CKS 17 and LDLLFL, the latter did not show immunosuppressive properties in proliferation assays (Table I and Fig. 3*A*).

Furthermore, retrovirally encoded peptides were used as a positive control for inhibition of T cell proliferation. These peptides also showed a significant inhibition of Ag- and mitogen-induced proliferation in up to 51% (Table I). In all experiments LMP1-derived peptides showed a considerable stronger inhibition of T cell activation.

Effects of LMP1-derived peptides on NK cytotoxicity

Next we were interested in the effect of LMP1-derived peptides on NK cytotoxicity (21, 22). In all experiments, peptides were continuously present during the effector phase of the NK cytotoxicity assays. No direct effect of any of the peptides on the viability of the PBMCs or K562 was observed. The results, depicted in Table I and Fig. 4, show that all LMP1-derived peptides containing the LALLFWL sequence were able to inhibit NK cytotoxicity up to 77% at a concentration of ~100 μM. In contrast, the second domain (LLLLAL), with homology to the retroviral immunosuppressive domain LDLLFL, showed no inhibition of NK cytotoxicity.

None of the control peptides (see Table I) showed inhibition of cytotoxicity. The retrovirus-derived peptides (LDLLFL and CKS17) clearly showed inhibition of cytotoxicity up to 57% comparable to the results obtained by Oostendorp et al. (22) (Table I).

Detection of LMP1 in supernatant of EBV-positive and -negative cell lines

An LMP1 capture ELISA recently developed by Meij et al. (see Footnote 3) was used to analyze whether LMP1 could be detected in the supernatant of EBV-positive cell lines. Using this assay soluble LMP1 was detected in the supernatant of EBV-positive lymphoblastoid cell lines (JY, RAJI, B95-8), whereas no LMP1
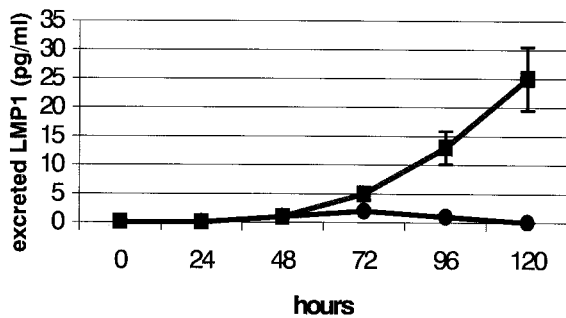
FIGURE 5. Kinetics of LMP1 excretion in EBV-positive cell line JY (■) and EBV-negative cell line Ramos (●) as detected by quantitative capture ELISA. On day 5 the highest LMP1 concentrations were found, reaching 25 pg/ml.

FIGURE 6. *A*, Distribution of LMP1 in the various fractions of EBV-positive and -negative cell lines after differential centrifugation. *Lane 1*, $300 \times g$ fraction (first time); *lane 2*, $300 \times g$ fraction (second time); *lane 3*, $1200 \times g$ fraction (first time); *lane 4*, $1200 \times g$ fraction (second time); *lane 5*, $10,000 \times g$ fraction; *lane 6*, $70,000 \times g$ fraction; *lane 7*, $100,000 \times g$ supernatant. In *lanes 2*, *5*, and *6*, clear bands are visible at ~63 kDa, confining the presence of LMP1 in the exosome-enriched fractions. *B*, Western blot analysis of the pellet of the exosome-enriched fraction for the presence of LMP1, CD86, and HLA-DR. LMP1 was detected using N-terminal-specific (OT22CN) and C-terminal-specific (OT22C) Abs in *lanes 1* and *2*, respectively.

was detected in EBV-negative cell lines. Expression of LMP1 increased during culture, and after 4–5 days the highest levels of LMP1, reaching up to 25 pg/ml (0.6 fmol), were observed, while cell viability remained >95% (see Fig. 5). Although LMP1 is detectable in supernatant of EBV-positive cell lines, the concentration seems to be insufficient to induce local T cell suppression in vitro.

To confirm the presence of intact LMP1, supernatants were analyzed by Western blotting, and LMP1 was visualized with both N- and C-terminal-specific mAbs. This indeed confirmed the appropriate molecular mass of intact LMP1 (63 kDa; data not shown).

Detection of LMP1 in exosomes

To assess whether LMP1 is secreted as single protein or in a membrane-associated form, the supernatant was fractionated by differential centrifugation. LMP1 was mainly detected in the $300 \times g$ (whole cells), $10,000 \times g$, and $70,000 \times g$ pellet fractions of the EBV-positive cell lines JY and B95-8 (Fig. 6*A*). Moreover, the presence of both MHC class II molecules and CD86, previously shown to be present in exosomes, was detected in the same fractions of EBV-positive cell lines, confirming the data reported by Raposo et al. (Fig. 6*B*) (26). In addition, the EBV-encoded nuclear Ag EBNA1 was tested to exclude the possibility of nuclear fragments during the exosome preparation and was found to be absent (data not shown). LMP1 was detected in none of the fractions of the EBV-negative cell line BJAB, whereas in these cells MHC class II molecules were present. Together these data indicate that LMP1 colocalizes with MHC class II molecules and is possibly secreted from EBV-positive B cells in the form of small vesicular structures known as exosomes.

Discussion

Despite the fact that in EBV-positive cases of HD H-RS cells express high levels of potentially immunogenic viral proteins, such as LMP1, LMP2, EBNA1, and (RK) BARF0, in addition to the nontranslated EBER1/2, these cells are not eradicated by the immune system despite intact Ag presentation function. Moreover, the presence of infiltrating activated CD8-positive lymphocytes is seen in all cases of HD. In contrast to what might be expected, high numbers of activated granzyme B-positive cells (>15%) in the reactive infiltrate are associated with an adverse prognosis (14). Therefore, the neoplastic cells must possess mechanisms to escape immune destruction. Various mechanisms to elude the immune system have been described for HD, e.g., IL-10 expression by the tumor cells, high expression of apoptosis-inhibiting proteins such
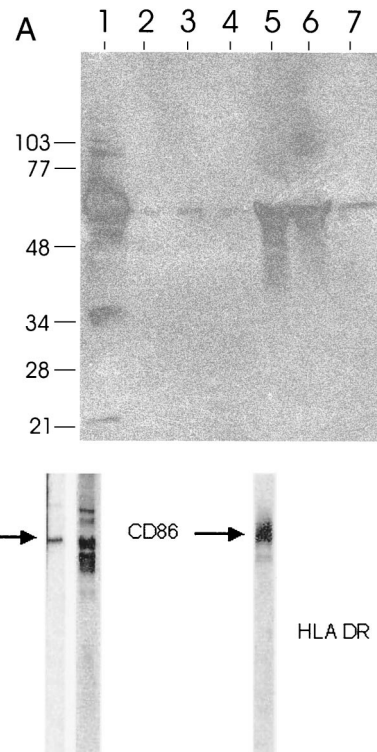
as Bcl-2, and down-regulation of MHC class I molecules on the surface of tumor cells of EBV-negative cases (13, 15, 31). Interestingly, evidence of local dysfunction of T cells has been described by Frisan et al., showing that tumor-infiltrating lymphocytes from EBV-positive HD were not able to kill in an autologous fashion (17).

In a recent study by Meij et al. (12), it was further substantiated that LMP1 is a subdominant component in the humoral immune response. In patients with EBV-associated malignancies, only a small percentage showed LMP1-directed Abs at a very low level (33% HD of cases and 8% of nasopharyngeal carcinoma patients) despite the fact that LMP1 is expressed at high levels in most of these cases. In addition, the cell-mediated immune response against LMP1 is subdominant compared with the readily detectable response against epitopes such as the EBNA3 family, since it has proven to be difficult to obtain CTL clones directed against LMP1 (27).

In this study we demonstrate, for the first time, that intact LMP1 as well as peptides derived from the first transmembrane region directly inhibit T cell activation and NK cytotoxicity in vitro. This provides a novel mechanism, namely direct immunosuppression mediated through (fragments of) LMP1, by which EBV-positive cases of Hodgkin lymphomas may circumvent the immune system. Not only is LMP1 capable of suppressing the strong mitogen and anti-CD3/anti-CD28-induced T cell activation, but it also suppresses functionally relevant Ag-specific memory T cell responses,

as indicated in this study. LMP1 contains a domain in its first transmembrane region closely homologous to retrovirally encoded immunosuppressive peptides that are known to inhibit lymphocyte functions (inhibition of T cell proliferation and NK cytotoxicity) and modulation of cytokine expression in favor of immunosuppressive cytokines (induction of IL-10 and inhibition of IL-2 and IL-12) (26). Moreover, the fact that LMP1 can be secreted from EBV-positive cell lines in vitro suggests that this mechanism might be of relevance for immune escape of EBV-positive neoplastic cells in vivo. It may be speculated that H-RS cells, which are considered to be of B cell origin and display high LMP1 expression levels, are able to secrete vesicles (exosomes) containing LMP1 in vivo.

EBV-transformed B cell lymphoblastoid cell lines are extremely potent stimulators of T cell proliferation and powerful inducers of CTL responses in vitro, despite the expression and secretion of LMP1. This phenomenon can be explained by the fact that LMP1 secretion in supernatants is only found at extremely low concentrations (<0.6 fmol), well below the immunosuppressive limit. In vivo, however, LMP1 might still exhibit its local immunosuppressive effect caused by the inability to diffuse and thereby reach much higher concentrations in close vicinity of the neoplastic cells.

Our findings indicated that at the peptide level, the amino acid sequence LALLFWL might be responsible for the observed immunosuppressive effects. The amount of peptide used to induce these effects is much greater compared with the amount of rLMP1 protein used. This phenomenon is possibly due to the hydrophobic nature of these peptides, whereby aggregation of these peptides might induce masking of the effective immunosuppressive domains.

The mechanism by which p15E, LMP1, or their related peptides exhibit their actions is not fully understood, but Haraguchi et al. (18) proposed a mechanism by which these immunosuppressive peptides directly modulate important molecules in the signal transduction pathway leading to a imbalance of cytokine production. It has been shown that upon retroviral infection, transcription of IL-12 and IL-2 is strongly decreased, whereas IL-10 transcription is augmented under the influence of CKS17 (18). The proposed model of modulation of transcription of cytokines might occur via two different pathways: 1) induction of high concentrations of cAMP via activation of adenylate cyclase, and 2) either direct inhibition of PKC activity or indirect inhibition via suppression of phospholipase C1 (18, 23). Both proposed mechanisms lead to enhanced expression of Th2-associated cytokines, IL-10 and TGF-β, with concomitant down-regulation of the Th1-like cytokines, IL-2, IL-12, and IFN-γ.

We and others have previously shown that in EBV-positive cases of HD, high numbers of cells (either neoplastic or reactive cells) express IL-10, whereas low numbers of cells showed IL-2 expression (14, 32, 33). This is supported by in vitro data showing that LMP1 is able to up-regulate the expression of IL-10 upon transfection into LMP1-negative Burkitt's lymphoma cell lines (34). Furthermore, EBV-positive cases of post-transplant lymphoproliferative disorders, expressing high levels of LMP1, are associated with elevated levels of serum IL-10 which further substantiates the hypothesis that LMP1 or LMP-derived peptides may induce local immunosuppression by induction of certain cytokines. Moreover, preliminary data show that addition of LMP1-derived peptides to activated PBMC and subsequent intracellular detection of cytokines by FACS analysis resulted in down-regulation of IL-2 expression (data not shown).

We hypothesize that LMP1 and hydrophobic transmembrane domain-derived peptides can insert directly into the membrane. This hypothesis is supported by the fact that the effective LMP1-derived peptides are very hydrophobic and, moreover, the inhibitory effects are more pronounced when charged groups on the peptides are neutralized by acetylation, thereby facilitating entry into the membrane. Recently, Busson et al. showed that in lymphoid and epithelial cells LMP1 is associated with glycosphingolipid-rich domains (GSL domains) in the plasma membrane (35, 36). These domains are clustering sites for heterodimeric G proteins, PKC, and adenylate cyclases. It is suggested that these GSL domains are important for trafficking and probably are also required for the inhibitory function of LMP1 (35, 36). LMP1 itself contains six hydrophobic membrane-spanning domains with which it is likely to penetrate the membrane at the GSL domains and subsequently can bind to and induce adenylate cyclase and inhibit PKC.

In conclusion, we have presented evidence that EBV, a human DNA tumor virus, is capable of inducing T cell anergy via a novel direct route involving LMP1, possibly mediated by secretion of EBV-encoded (fragments of) LMP1. These peptide fragments are possibly involved in circumvention of the local immune system in EBV-positive cases of HD by virtue of the induction of immunosuppressive cytokines. This is the first time that direct immunosuppression, previously thought to be restricted to RNA viruses, has been described in a DNA virus.

We recently developed a novel mAb with specificity for the N-terminal region of LMP1 (amino acid sequence 1–13) (12). Using this reagent and cloned subfragments of LMP1, in particular the first transmembrane region, the hypothesized localization and secretion as well as the role of this region in immune suppression may be further delineated.

Acknowledgments

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