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Ryusuke Omiya, Chantal Buteau, Hiroya Kobayashi, Carlos V. Paya and Esteban Celis

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Inhibition of EBV-Induced Lymphoproliferation by CD4⁺ T Cells Specific for an MHC Class II Promiscuous Epitope¹

Ryusuke Omiya,* Chantal Buteau,[†] Hiroya Kobayashi,* Carlos V. Paya,[†] and Esteban Celis^{2*}

Posttransplant lymphoproliferative disorder (PTLD) and B cell lymphomas induced by EBV continue to be a major life-threatening complication in transplant patients. The establishment and enhancement of T cell immunity to EBV before transplantation and immunosuppressive therapy could help diminish these complications, but the lack of an effective vaccine has limited this prophylactic approach. We describe here the identification of a peptide epitope from the EBV EBNA2 Ag that is capable of inducing in vitro CD4⁺ T cell responses that inhibit the EBV-mediated B lymphocyte proliferation associated with PTLD. Most significantly, T cell responses to the EBNA2 epitope were found to be restricted by numerous MHC class II alleles (DR1, DR7, DR16, DR52, DQ2, and DQ7), indicating that this peptide is highly promiscuous and would be recognized by a large proportion (>50%) of the general population. These results are relevant for the design of a simple, inexpensive and widely applicable peptide-based vaccine to prevent PTLD in solid organ transplant patients. *The Journal of Immunology*, 2002, 169: 2172–2179.

Epstein-Barr virus is a lymphotropic γ -herpesvirus that infects mostly B cells and is responsible for inducing their uncontrolled cell proliferation and transformation (1). Under normal circumstances and in healthy individuals, EBV infections are not life threatening and are generally effectively controlled by the immune system through the action of Ag-specific T lymphocytes (2). Both CD8⁺ CTL and CD4⁺ helper T lymphocytes (HTL)³ can discriminate EBV-infected or EBV-transformed B cells and, as a consequence, are able to inhibit their growth. EBV-specific T lymphocytes recognize Ag as molecular complexes formed by viral peptide epitopes with MHC molecules, which are expressed on the surface of the infected/transformed B lymphocytes (2).

Although lifetime immunity to EBV is apparently achieved in normal individuals, this virus is not completely eradicated and persists in a latent infection state (3–5), which is effectively controlled by the EBV-specific T lymphocytes (2). However, in immunosuppressed individuals such as transplant patients, primary EBV infection usually results in posttransplant lymphoproliferative disorders (PTLD) that often progress into B cell lymphomas (6–8). Current prophylactic and therapeutic approaches for PTLD and lymphomas are far from optimal. The reduction of immunosuppressive therapy facilitates the immune-mediated viral elimination, but with a high risk of organ rejection, and the use of antiviral agents is of unclear effectiveness (8). Results obtained using anti-CD20 mAb therapy appear promising, but have the disadvantage of long term B cell depletion and the occurrence of CD20-negative relapses (9–11). One of the most effective ways to prevent/treat PTLD and B cell lymphomas is via adoptive immunotherapy using EBV-

specific T lymphocytes (12, 13), but this approach is labor intensive, costly, and not widely available. The risk of PTLD and lymphomas increases significantly in those patients who lack immunity to EBV before transplantation and undergoing immunosuppressive therapy (14). It is possible that this risk could be lowered if EBV-seronegative patients could be immunized to stimulate their T lymphocytes, but unfortunately such a vaccine is not yet available.

An attractive and relatively expedient approach to develop vaccines that are intended to elicit Ag-specific T cell responses is the use of synthetic peptides representing CTL and HTL epitopes. This strategy has been explored for various viral and malignant diseases (15–18). A large number of T cell epitopes derived from EBV latent and lytic cycle Ags have been identified, and some of these are being considered as potential vaccine candidates (19, 20). Many of these efforts are focusing on using MHC class I-restricted peptide epitopes to induce CD8⁺ CTL responses to EBV, since these cells are considered to be the prime effector cells that will presumably annihilate the virus-infected and transformed cells. However, there is recent evidence that CD4⁺ T lymphocytes can also function as potent effectors for inhibiting EBV-induced B cell proliferation, which would be the initial step of PTLD (21).

One of the major potential drawbacks of T cell peptide epitope vaccines is the limitation of MHC restriction. Most CTL and HTL peptide epitopes will only be useful in the limited proportion of individuals who express the appropriate MHC allele. However, in the case of MHC class II epitopes, some peptides have been found to bind “promiscuously” to more than one MHC allele (22–24). In some cases these promiscuous epitopes can bind up to 10 frequently found MHC alleles, indicating that the majority of the population would recognize these epitopes (25).

In the present study we report the identification of a highly promiscuous MHC class II epitope from the EBNA2 Ag capable of stimulating CD4⁺ T cell responses. The EBNA2 epitope described herein can be presented to T cells in the context of HLA-DR1, -DR7, -DR16, -DR52, -DQ2, and -DQ7 alleles. Most significantly, the T cells induced by the EBNA2 peptide epitope were able to recognize EBV-transformed lymphoblastoid cell lines and were efficient in inhibiting early B cell proliferation induced by EBV infection. These results indicate that the promiscuous EBNA2 epitope is an excellent candidate for a vaccine to prevent and perhaps treat PTLD in transplant patients.

*Department of Immunology and [†]Division of Infectious Diseases, Department of Internal Medicine, Mayo Clinic, Rochester, MN 55905

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² Address correspondence and reprint requests to Dr. Esteban Celis, Department of Immunology, GU421A, Mayo Clinic, Rochester, MN 55905. E-mail address: celis.esteban@mayo.edu

³ Abbreviations used in this paper: HTL, helper T lymphocytes; ARB, average relative binding; CsA, cyclosporin A; DC, dendritic cells; LCL, lymphoblastoid cell line; PTLD, posttransplant lymphoproliferative disorder.

Materials and Methods

Cell lines

EBV-transformed lymphoblastoid cells (EBV-LCL) were generated from peripheral blood mononuclear cells of HLA-typed volunteers using culture supernatant from the EBV-producing B95-8 cell line (American Type Culture Collection, Manassas, VA). Mouse fibroblast cell lines (L cells), transfected and expressing individual human MHC class II molecules, were provided by R. W. Karr (Parke-Davis, Ann Arbor, MI).

Synthetic peptides and epitope prediction analysis

Potential MHC class II promiscuous helper T cell epitopes were predicted from the amino acid sequence of the EBNA2 Ag using the algorithm tables for three HLA-DR alleles (*DRB1*0101*, *DRB1*0401*, and *DRB1*0701*) published by Southwood et al. (25). The algorithm calculates the average relative binding (ARB) value for each possible 9-mer core peptide sequence for the entire EBNA2 protein. The rationale for this approach is that the higher the ARB value of a peptide, the higher the probability that the peptide will bind to the corresponding HLA-DR allele. The selected peptides that displayed high ARB values were synthesized and purified as previously described (26). The purity (>95%) and identity of peptides were determined by HPLC and mass spectrometry.

In vitro induction of Ag-specific T cell lines using synthetic peptides

The methods used for generating tumor Ag-reactive HTL lines and clones using peptide-stimulated PBMC have been described in detail previously (26, 27). All blood donors used in the present study were seropositive for EBV. For donors M14 and M44, peptide-pulsed dendritic cells (DC) were used to stimulate purified CD4⁺ T cells. Briefly, DC were generated in tissue culture from adherent monocytes that were cultured for 7 days in the presence of GM-CSF and IL-4. A total of 1×10^4 peptide-pulsed DC ($3 \mu\text{g/ml}$ for 2 h at room temperature) were irradiated (4200 rad) and cocultured with 3×10^4 autologous purified CD4⁺ T cells (using Ab-coated magnetic beads from Miltenyi Biotech, Auburn, CA) in each well of a 96-well, round-bottom culture plate. For the remainder of the blood donors (M1, M2, M9, M15, M20, M24, M25, M69, and M83), total unfractionated PBMC were stimulated with $3 \mu\text{g/ml}$ peptide in 48-well plates at 5×10^5 cells/well. Seven days later, all cultures were restimulated individually with peptide ($3 \mu\text{g/ml}$) and autologous irradiated PBMC, and 2 days later, human rIL-2 was added at a final concentration of 10 IU/ml. After two rounds of restimulation, the microcultures were tested for their proliferative responses to peptide as described below. Those wells showing a proliferative response to peptide of at least 2.5-fold over background were expanded in 24- or 48-well plates by weekly restimulation with peptides and irradiated autologous PBMC. Flow cytometric analysis after three rounds of peptide stimulation revealed that >95% of the cells were CD4⁺/CD8⁻. Culture medium for all procedures consisted of RPMI 1640 supplemented with 5% human male AB serum, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 $\mu\text{g/ml}$ gentamicin. The institutional review board on human subjects (Mayo Foundation) approved this research, and informed consent for blood donation was obtained from all volunteers.

Analysis of Ag-specific response of T cells

T cells (3×10^4 /well) were mixed with irradiated APCs in the presence of various concentrations of Ag (peptide) in 96-well culture plates. APC consisted of PBMC (1×10^5 /well), HLA-DR-expressing L cells (3×10^4 /well), or EBV-LCL (3×10^4 /well). Cell proliferation assays were incubated at 37°C in a 5% CO₂ incubator for 72 h, and during the last 16 h, each well was pulsed with 0.5 $\mu\text{Ci/well}$ [³H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ). In some cases culture supernatants were collected before the addition of [³H]thymidine for the determination of IFN- γ production using ELISA kits (BD Pharmingen, San Diego, CA). The radioactivity incorporated into DNA, which correlates with cell proliferation, was measured in a liquid scintillation counter after harvesting the cell cultures onto glass-fiber filters. To identify the MHC restriction molecules involved in Ag presentation, inhibition of the Ag-induced proliferative response was determined by the addition of anti-HLA-DR mAb L243 (IgG2a, prepared from hybridoma supernatants obtained from American Type Culture Collection) or anti-HLA-DQ mAb SPVL3 (IgG2a; Beckman Coulter, Fullerton, CA). Both Abs were used at a final concentration of 10 $\mu\text{g/ml}$ throughout the 72-h assay. The specificity of these Abs and their capacity to specifically inhibit Th responses have been determined in our laboratory on numerous occasions. All assessments of proliferative responses were conducted at least in triplicate, and results correspond to the means. The stimulation index was calculated by dividing the mean radio-

activity (cpm) obtained in the presence of Ag by the mean radioactivity (cpm) obtained in the absence of Ag but in the presence of APC.

Cell-mediated cytotoxicity assays

Cytotoxic activity of CD4⁺ T cells was determined in a ⁵¹Cr release assay as previously described (28). Targets were prepared by incubating EBV-LCL (or T2 cells) with or without 10 $\mu\text{g/ml}$ peptides at 37°C overnight. Target cells were labeled with 300 μCi [⁵¹Cr]sodium chromate (Amersham Pharmacia Biotech, Piscataway, NJ)/ 5×10^6 cells for 1.5 h at 37°C. T cells were mixed with 2×10^5 labeled targets at different E:T cell ratios in 96-well, round-bottom plates at a final volume of 0.2 ml. After 6- to 9-h incubation at 37°C, 30 μl supernatant was collected from each well, and the percentage of specific lysis was determined according to the formula: [(cpm of the test sample - cpm of spontaneous release)/(cpm of the maximal release - cpm of spontaneous release)] \times 100. Results show the average specific lysis \pm SE of triplicate determinations.

EBV-induced B lymphocyte proliferation assays

Infectious EBV stocks were prepared from culture supernatants of the EBV-producing B95-8 cell line. B95-8 cells were seeded at 2×10^5 cell/ml in complete RPMI 1640 medium and were incubated for 14 days at 37°C without medium exchange. The culture supernatant was adjusted to contain 10 g NaCl and 8% (w/v) polyethylene glycol (PEG-8000; Sigma, St. Louis, MO) per liter. The precipitate formed after overnight incubation at 4°C was collected by centrifugation at 7500 rpm in a Sorvall centrifuge with a GS3 rotor (Beckman Coulter). The pellet was resuspended in 1 ml complete medium for every 50 ml original EBV-containing supernatant, and was stored at -80°C until further use. The EBV infectivity assays were performed as recently described (21). Briefly, T cell-depleted PBMC were produced by negative selection using anti-CD3-coated magnetic beads (Miltenyi). The T cell-depleted PBMC (4×10^5 cells) were resuspended in 1 ml complete RPMI 1640 medium, and 20 μl concentrated EBV supernatant was added in the absence and the presence of various concentrations of T lymphocytes without the addition of cytokines. After 10-15 days in culture, the cultures were harvested, and the numbers of viable cells were determined using trypan blue exclusion. The percentage of CD23⁺ B cells in each culture condition was estimated by cytofluorometric analysis using an FITC-labeled specific mAb (BD Pharmingen).

Results

Identification of potential MHC promiscuous CD4⁺ T cell epitopes for EBV

Our goal was to identify promiscuous MHC class II CD4⁺ T cell epitopes that could be used to prevent/treat EBV-induced PTLD, which is a serious complication in solid organ transplant patients. For our initial studies we selected the EBNA2 latent viral Ag as a potential target for inducing T cell responses against PTLD. Thus, we examined the amino acid sequence of EBNA2 viral protein for the presence of peptide fragments containing binding motifs for HLA-*DR*0101*, *DR*0401*, and *DR*0701* using the algorithm tables described by Southwood et al. (25). Using the cut-off values of ARB necessary to predict 75% of the HLA-DR binders, we found that of a possible 479 total peptides of a nine-amino acid length (EBNA2 has 487 residues), 35 peptides were predicted as potential binders for HLA-DR1, 23 peptides for HLA-DR4, and only six peptides for HLA-DR7 (data not shown). However, more relevant for the identification of promiscuous MHC class II binders, we observed that only four peptide sequences from EBNA2 exhibited ARB scores above the cut-off values for all three alleles (Table I). Interestingly, two of these peptide sequences, EBNA2₂₈₁ and EBNA2₂₈₂, are almost identical with peptide EBNA2₂₈₀₋₂₉₀ (TVFY NIPMPL), which was reported to function as a T cell epitope restricted by HLA-DQ2 and -DQ7 (29). In view of this finding, we reasoned that peptide EBNA2₂₈₀₋₂₉₀ would be the prime candidate to be evaluated as a highly promiscuous epitope for inducing CD4⁺ T cell responses in the context of several additional HLA-class II molecules.

Table I. Peptides predicted to be promiscuous CD4⁺ T cell epitopes for EBNA2

Ag	Position	Sequence (9-mer core)	Predicted ARB ^a		
			DR1 (>1.57) ^b	DR4 (>2.617)	DR7 (>9.106)
EBNA2	23–31	LGNPSLSVI	3.65	4.93	34.92
	135–143	WMANYIVRQ	13.17	3.75	12.88
	281–289	VFYNIPMP	4.97	285.92	26.99
	282–290	FYNIPMPL	19.02	171.01	9.28

^a The higher the number, the higher the probability that a peptide will bind to the corresponding MHC allele.

^b Numbers in parentheses represent the cutoff ARB values suggested by Southwood et al. (20) to include 75% of peptides binding to the specific allele.

Induction of T cell responses to peptide EBNA2_{280–290}

Since peptide EBNA2_{280–290} was predicted to function as a highly promiscuous MHC class II CD4⁺ T cell epitope, this peptide was synthesized and tested for its capacity to stimulate CD4⁺ T cells isolated from 11 healthy, EBV-seropositive individuals. T cell lines were prepared as described in *Materials and Methods* using two different protocols. For two blood donors (M14 and M44) purified CD4⁺ T cells were stimulated with peptide-pulsed DC, and for the remaining nine donors peptide was added to total unfractionated PBMC. All T cell cultures were restimulated three or four times weekly with autologous irradiated PBMC and peptide, after which the T cell responses to peptide were evaluated in a cell proliferation assay. The majority of the individuals (7 of the 11) were able to respond in vitro to peptide EBNA2_{280–290} in an Ag-specific manner (Fig. 1). The four T cell lines that failed to exhibit peptide-induced proliferative responses did so either because they had high background (high proliferation in the absence of peptide) or because of lack of overall proliferation.

HLA restriction analysis of EBNA2_{280–290}-reactive T cells

To define the HLA restriction alleles of the EBNA2_{280–290} peptide-reactive T cell lines, cell proliferation assays were performed using a panel of HLA-DR-transfected mouse fibroblasts (L cells) and semiallogeneic human cells that were used as APC. In addition, anti-HLA-DR or anti-HLA-DQ mAbs were tested for their capacity to inhibit the peptide-induced proliferation with autologous APC. The results from these experiments (Fig. 2) indicate that peptide EBNA2_{280–290} could be presented to the T cells in the context of HLA-DR1 (donor M1), HLA-DQ2 (M14), HLA-DR16 (M24), HLA-DR52 (M44 and M69), and HLA-DR7 (M83). Cytofluorometric analysis revealed that the T cell lines expressed the CD4 marker and did not express CD8 (data not shown), indicating that these cells behaved as typical MHC class II-restricted T lymphocytes. The T cell line from donor M9, which had previously responded to peptide EBNA2_{280–290}, could not be analyzed for MHC restriction because it was not stable, and it ceased to grow in tissue culture.

Recognition of autologous EBV-transformed LCL by peptide-reactive CD4⁺ T cells

One of the most critical attributes that peptide-induced anti-EBV CD4⁺ T cells must exhibit is their capacity to recognize the naturally processed viral Ag, which is expressed by the EBV-infected or transformed B lymphocyte. Thus, it became important to determine whether EBV-transformed lymphoblastoid cell lines (EBV-LCL), which naturally process EBNA2, would be capable of stimulating the peptide-reactive T cell lines. To carry out these studies, EBV-LCL were generated from all blood donors to be used as APC for each corresponding T cell line to be analyzed. In addition, T2 cells, which are negative for MHC class II, were used as a negative control. The results from these experiments demonstrated

that all the peptide-reactive T cell lines were effective in recognizing Ag presented directly by their respective EBV-LCL, as determined by their secretion of IFN- γ (Fig. 3). On the other hand, no apparent reactivity was observed toward T2 cells when these were used as APC. These results indicate that the T cell epitope represented by peptide EBNA2_{280–290} is processed from the EBNA2 protein and can be expressed on the MHC class II molecules of transformed EBV-LCL, enabling peptide-reactive CD4⁺ T cells to efficiently recognize them.

Inhibition of EBV-induced B cell proliferation by EBNA2_{280–290}-reactive CD4⁺ T cells

There is ample evidence that CD4⁺ Th cells are capable of exhibiting effector activity either in the form of cytotoxicity or via the production of lymphokines against viral infections. Moreover, recent studies showed that CD4⁺ T cells are capable of suppressing the early stages of B cell proliferation and transformation by EBV (21), whereas CD8⁺ T cells appear to play a more significant role in controlling EBV in the latent infection state (2). In view of this,

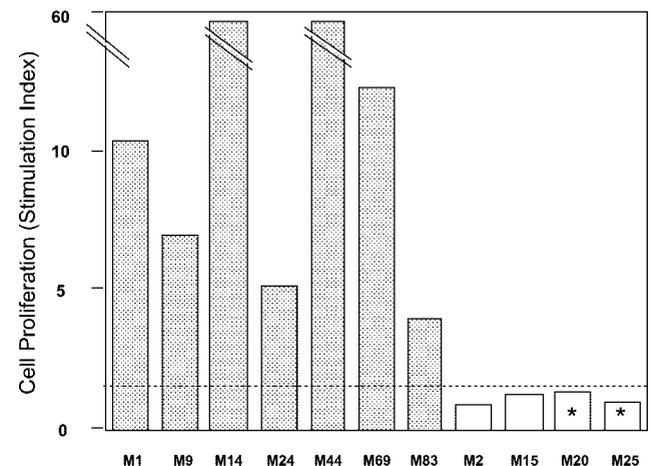
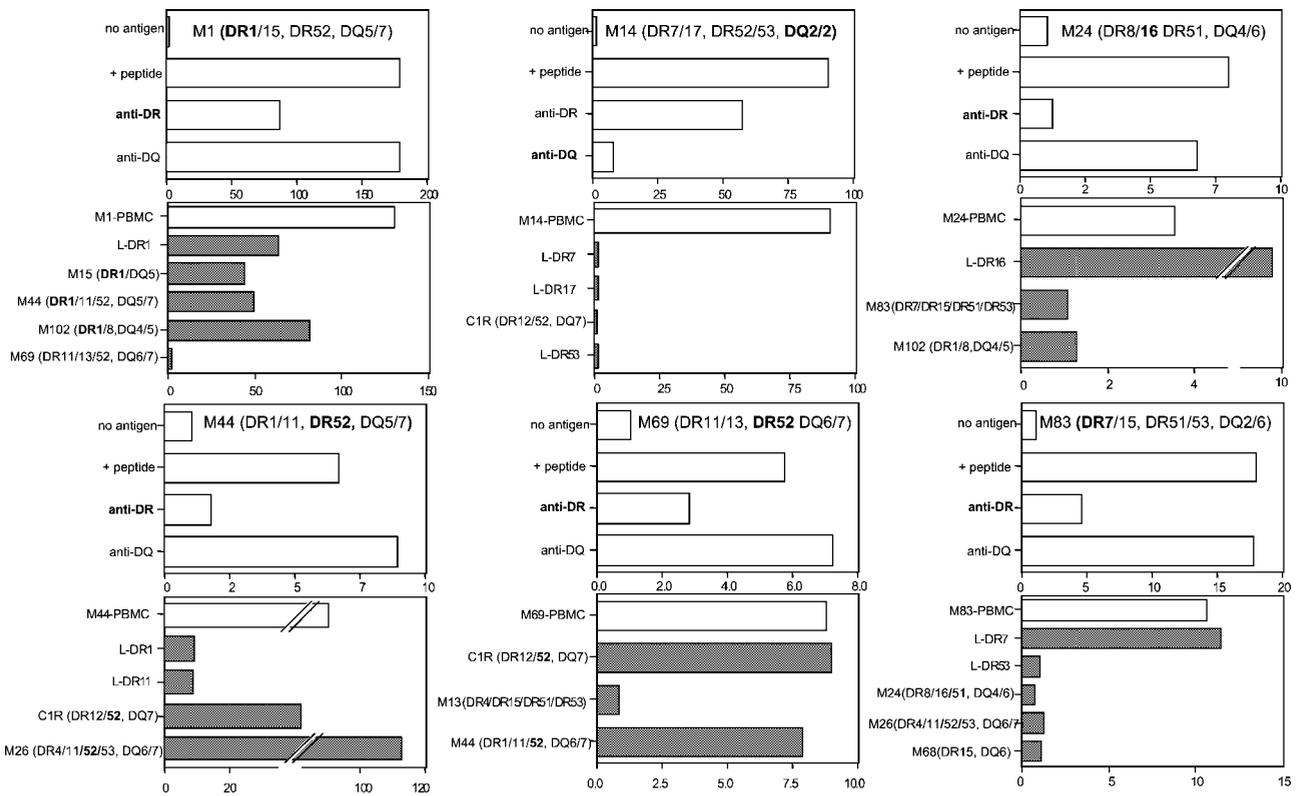


FIGURE 1. T cell proliferative responses to a predicted MHC class II promiscuous peptide from EBNA2. Eleven donors were randomly selected for preparing CD4⁺ T cell lines against peptide EBNA2_{280–290} (TVFYNIIPMPL) using the procedures described in *Materials and Methods*. The proliferative responses were measured with and without peptide (3 μ g/ml) in the presence of autologous irradiated APC (PBMC) by incorporation of [³H]thymidine. The stimulation index (SI) was calculated by dividing the mean radioactivity (cpm) obtained in the presence of peptide by the mean radioactivity obtained in the absence of peptide. ■, Proliferative responses where SI is >3 (considered significant); □, SI where no significant peptide-induced proliferation was observed. Dashed line marks SI = 1, where proliferation in the presence and that in the absence of peptide were the same. Asterisks mark T cell lines that displayed a high background (nonspecific) proliferative response. All determinations were performed in triplicate, and the SDs were <10% of the mean.



Cell Proliferation (Stimulation Index)

FIGURE 2. MHC restriction analysis of EBNA₂₈₀₋₂₉₀-reactive T cell lines. MHC class II restriction molecules were identified using Ab-blocking (anti-DR or anti-DQ) experiments with autologous APC (*top* portion of each panel). Peptide-induced proliferative responses were studied using various MHC-typed APC (*bottom* portion of the panels). □, Determinations using autologous APC; ▨, determinations using semiallogeneic APC. Peptide EBNA₂₈₀₋₂₉₀ was used in all determinations at 3 μg/ml. Values shown are the means of triplicate determinations; error bars show the SDs.

we explored whether EBNA₂₈₀₋₂₉₀-reactive CD4⁺ T cells would be capable of exerting antiviral activity to control the early events of EBV-induced B cell proliferation that ultimately lead to malignant transformation. Various numbers of EBNA₂₈₀₋₂₉₀-reactive CD4⁺ T cells derived from three separate donors (M1, M14, and M24) were evaluated for their ability to inhibit EBV-mediated B cell proliferation in a 10- to 15-day culture assay. For donor M14, a T cell clone specific for an irrelevant non-EBV Ag (MAGE3) was

included as a negative control. In all cases the EBV-reactive T cells were highly effective at inhibiting the proliferation of CD23⁺ B lymphocytes, which was induced by EBV infection (Fig. 4). In contrast, the MAGE3-reactive T cell clone did not have a significant effect on the proliferation of B cells (Fig. 4, *middle panel*, □), indicating that the inhibitory effect of the EBV-reactive T cells was Ag specific.

The above results suggest that MHC class II-restricted CD4⁺ T cells specific for the EBNA₂₈₀₋₂₉₀ epitope would be able to prevent or control PTLN in solid organ transplant patients. However, these patients usually receive immunosuppressive therapy with drugs such as cyclosporin A (CsA) to prevent the rejection of the transplanted organs. CsA and other similar immunosuppressive agents are known to inhibit some of the TCR-mediated effector functions of T lymphocytes, such as the production of lymphokines (30). Consequently, we proceeded to evaluate whether CsA would diminish the antiviral effect of EBNA₂₈₀₋₂₉₀-specific T cells. The results presented in Fig. 5, *left panel*, indicate that even in the presence of CsA (at 1 μg/ml), which was added at the initiation of the experiments, the EBNA₂₈₀₋₂₉₀-specific T cells were very effective in inhibiting the EBV-induced proliferation of B lymphocytes. On the other hand, the same concentration of CsA was capable of suppressing the production of IFN-γ by PBMC that were stimulated with antiCD3 Abs (Fig. 5, *middle panel*), indicating that this compound was active at this dose. Similarly, the production of IFN-γ by EBNA₂₈₀₋₂₉₀-specific T cells induced by Ag presented by EBV-LCL was also inhibited by CsA (Fig. 5, *right panel*). In another set of experiments performed under more

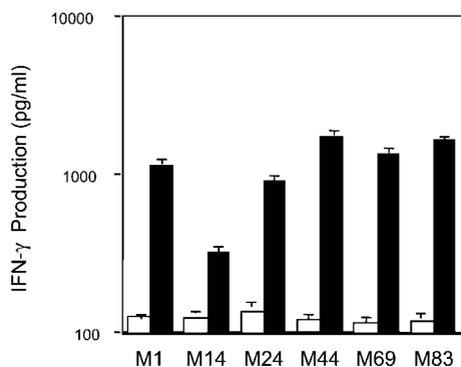


FIGURE 3. Recognition of naturally processed Ag by peptide-reactive CD4⁺ T cells. CD4⁺ T cell lines derived from six donors that could recognize peptide EBNA₂₈₀₋₂₉₀ were tested for their capacity to recognize autologous EBV-LCL, measured by the production of IFN-γ (■). The production of IFN-γ by CD4⁺ T cells was also measured using T2 cells (negative control), which do not express MHC class II molecules (□). Values are the means of triplicate determinations; error bars show the SDs.

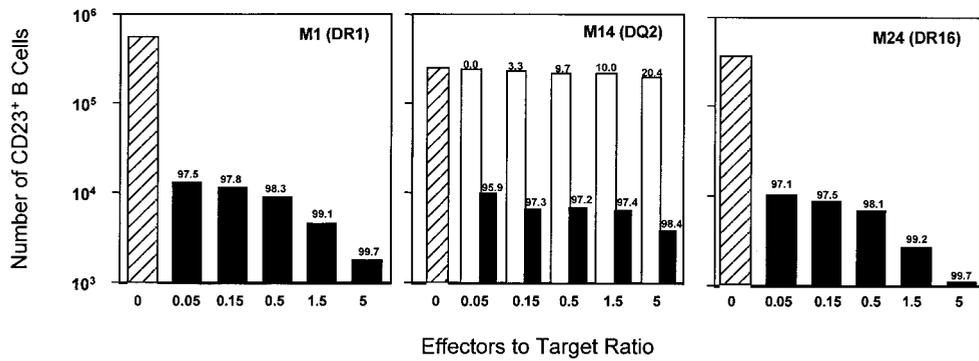


FIGURE 4. Inhibition of EBV-induced B cell proliferation by EBNA₂₈₀₋₂₉₀-specific T cells. Various numbers of T cells from three donors (M1, M14, and M24), restricted by different MHC class II alleles (DR1, DQ2, and DR16), were evaluated for their capacity to inhibit B cell proliferation induced by EBV. The numbers of CD23⁺ B cells were estimated after 10–15 days in culture in the presence of infectious EBV. ▨, Numbers of CD23⁺ B cells obtained in the absence of T cells; ■, numbers of B lymphocytes in the presence of EBNA₂₈₀₋₂₉₀-specific T cells; □, numbers of B lymphocytes (for M14 donor only) in the presence of an irrelevant T cell line, specific for the MAGE3 Ag. Numbers above black and white bars denote the percent inhibition of the yield of CD23⁺ B cells in each culture (note logarithmic scale of y-axis).

strenuous conditions, CsA was added to some of the cultures every day (to a final 1 μg/ml) to take into account the possible effects due to the decrease in the drug's potency during the 10-day experiment. Under these conditions, the EBNA₂₈₀₋₂₉₀-specific T cells were still capable of significantly inhibiting the EBV-induced B cell proliferation by >70% (Fig. 6). Overall, these results suggest that even in the presence of high doses of CsA, EBNA₂₈₀₋₂₉₀-specific CD4⁺ T cells may be effective in preventing B lymphocyte proliferation induced by EBV.

Cytolytic activity of EBNA₂₈₀₋₂₉₀-specific CD4⁺ T cells

The EBNA₂₈₀₋₂₉₀-reactive CD4⁺ T cells may use various effector mechanisms, such as cytotoxicity or the production IFN-γ, to inhibit EBV-mediated B cell proliferation. However, the results presented in Figs. 5 and 6 suggest that for T cells to inhibit EBV infection and subsequent B cell proliferation they could use a CsA-insensitive effector mechanism such as cell-mediated cytotoxicity. The cytotoxic activity of the six EBNA₂₈₀₋₂₉₀-specific T cell lines was evaluated against various target cells. As shown in Fig. 7, the CD4⁺ T cell lines from all six donors were capable of killing peptide-pulsed autologous target cells. In all cases, with the exception of donor M1, no cytotoxicity was detected against the MHC class II-negative T2 cells, indicating the requirement of TCR interaction with peptide MHC complexes on the target cells. Moreover, in the case of donors M69 and M44 (HLA-DR52-restricted)

the T cells were also effective in killing the autologous EBV-transformed LCL in the absence of exogenously added peptide, indicating that the amount of naturally processed EBNA₂₈₀₋₂₉₀ epitope in these cells was sufficient to allow the cytolytic reaction to take place. Furthermore, the EBNA₂₈₀₋₂₉₀-reactive T cell line from donor M69 was not able to kill an EBV-LCL from an HLA-DR52-negative donor, regardless of whether these cells were pulsed with synthetic peptide (Fig. 7, upper left panel). These data indicate that lysis by these cells is MHC restricted and requires the presence of the specific MHC peptide complexes on the target cells. We noted that the potency of the cytotoxic function of the M69 and M44 T cell lines was different compared with that of the other cell lines. While 30% specific lysis (50% of the maximal activity) was obtained at an E:T cell ratio of 1:1 or less in the M69 and M44 T cell lines to obtain the same level of cytotoxicity with the other T cell lines, an ~3-fold greater E:T cell ratio was required (Fig. 7, dashed lines). These results indicate that the M69 and M44 T cell lines were significantly more potent in killing their target cells than the other T cell lines, and this could help explain the inability of the latter cells in killing the EBV-LCL in the absence of exogenously added peptide.

Discussion

Here, we have described the identification of a highly promiscuous MHC class II-binding CD4 T cell epitope from the EBNA2 latent

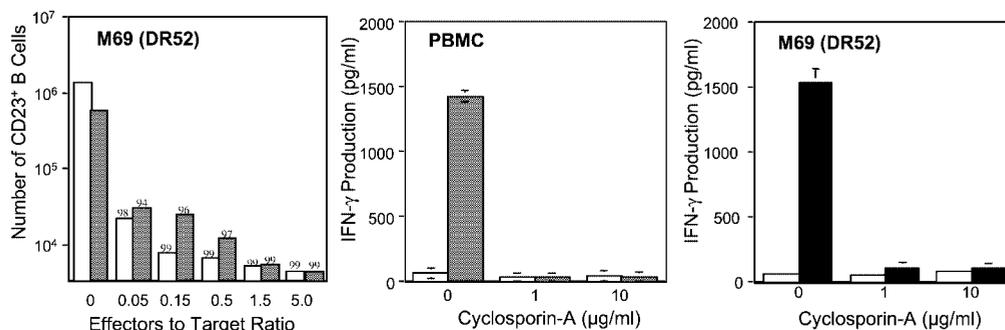


FIGURE 5. Effect of CsA on the effector function of EBNA₂₈₀₋₂₉₀-specific T cells. *Right panel*, The CD4⁺ T cell line from donor M69 (DR52 restricted) was tested for its capacity to inhibit the EBV-mediated expansion of CD23⁺ B cells in the presence of 1 μg/ml CsA (■) and in the absence of CsA (□). Numbers above each bar represent the percent inhibition of B cell expansion produced by various numbers of T cells. *Middle panel*, Production of IFN-γ of PBMC from donor M69 stimulated with antiCD3 Ab (OKT3 at 10 ng/ml) in the presence CsA (0, 1, or 10 μg/ml). *Right panel*, The production of IFN-γ by M69 EBNA₂₈₀₋₂₉₀-specific T cells induced by autologous EBV-LCL (■) or T2 cells (□) was determined in the presence of several concentrations of CsA (0, 1, and 10 μg/ml). Values are the means of triplicate determinations; error bars show the SDs.

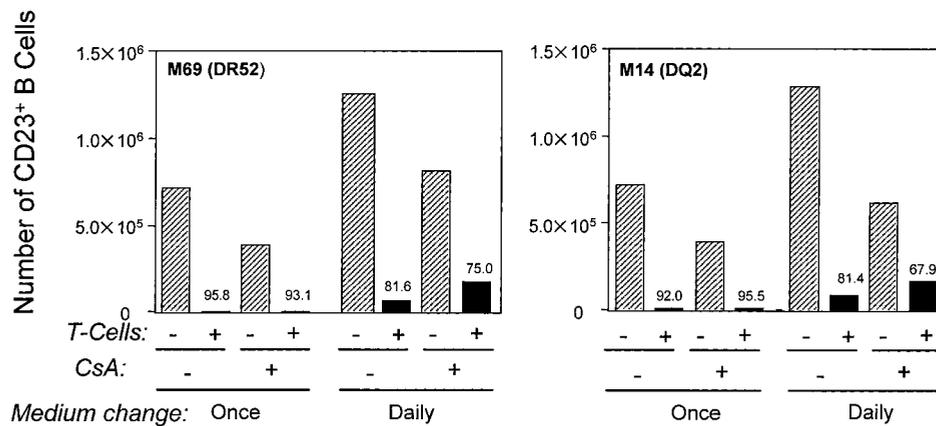


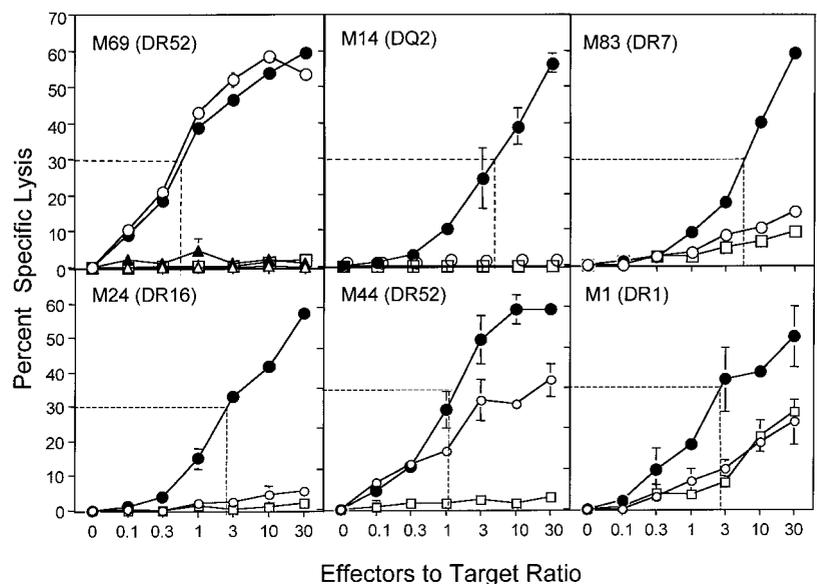
FIGURE 6. Inhibition of EBV-induced B cell proliferation by EBNA2₂₈₀₋₂₉₀-specific T cells in the continuous presence of CsA. The CD4⁺ T cell lines from donors M69 (DR52 restricted) and M14 (DQ2 restricted) were evaluated for their capacity to inhibit the EBV lymphoproliferative effect. In some cultures CsA was added only once (day 0); in other cultures medium containing fresh CsA was replaced on a daily basis. ▨, Cultures without T cells; ■, cultures with T cells at an E:T cell ratio of 3:1. Numbers above each black bar represent the percent inhibition of B cell expansion induced by T cells related to the same condition, but in the absence of T cells. Values represent the means of triplicate determinations.

viral Ag. On the basis of a computer-based algorithm (25), three peptide sequences of EBNA2 were predicted to function as promiscuous HLA-DR-binding peptides (Table I). Interestingly for one of the candidate peptides, an almost exact sequence (EBNA2₂₈₀₋₂₉₀) had been previously described to function as a T cell epitope restricted by the HLA-DQ2 and -DQ7 MHC class II alleles (29). Thus, we reasoned that peptide EBNA2₂₈₀₋₂₉₀ could constitute a typical MHC class II promiscuous epitope, capable of being presented to CD4⁺ T cells by multiple HLA-DR and -DQ alleles. Indeed, our data support this prediction, since we were able to elicit T cell responses to peptide EBNA2₂₈₀₋₂₉₀, which were restricted by HLA-DR1, -DR7, -DR16, -DR52, and -DQ2 (Fig. 2). The computer-based algorithm that we used predicted that this peptide sequence would serve as an epitope restricted by DR1, DR4, and DR7. However, we and others have observed that the many of the peptides predicted by this algorithm can also bind to other MHC class II alleles, such as DR9, DR13, DR15, DR51, DR52, DR53, and DQ6 (25, 26, 31). Although the predicted ARB of peptide EBNA2₂₈₀₋₂₉₀ for HLA-DR4 was very high (Table I), we have not yet observed T cell responses restricted by this allele. Of all the responders, M9 expressed the DR4 allele, but unfortun-

nately the EBNA2₂₈₀₋₂₉₀-reactive T cell was not stable and could not be tested for its MHC restriction. Of the four nonresponders (Fig. 1), M2 and M20 expressed DR4, but in neither case we were able to establish an Ag-reactive T cell line. Additional experiments will be required to determine whether HLA-DR4 is capable of presenting peptide EBNA2₂₈₀₋₂₉₀ to CD4⁺ T lymphocytes.

Our results indicate that although peptide EBNA2₂₈₀₋₂₉₀ is promiscuous with regard to its capacity to interact with various MHC class II molecules, the presentation of the MHC/peptide complexes to the TCR itself is not degenerate. For example, our data show that T cells recognizing peptide EBNA2₂₈₀₋₂₉₀ in the context of DR1 did not become stimulated by APC expressing DR52 and DQ7 (M69 donor in Fig. 2, top left panel), which are alleles capable of presenting this epitope to T cells restricted to their own alleles. Similarly, DQ2-restricted T cells from M14 did not recognize peptide in the context of DR7, DR52, or DQ7 (Fig. 2, top middle panel); DR52-restricted T cells from M44 did not respond to the peptide presented by DR1 (Fig. 2, bottom left panel); and DR7-restricted T cells from M83 failed to recognize peptide presented by DR16, DR52, and DQ7 (Fig. 2, bottom right panel). In addition, in the cytotoxicity assays DR52-restricted T cells from

FIGURE 7. Cytolytic activity of EBNA2₂₈₀₋₂₉₀-specific T cell lines. CD4⁺ T cells were tested at various E:T cell ratios for their capacity to kill several target cells: autologous EBV-LCL (○), peptide-pulsed autologous EBV-LCL (●), and MHC class II-negative T2 cells (□). Additional negative controls for donor M69 (upper left panel) were allogeneic EBV-LCL from donor M35 (DR4/15, DR51/53, DQ6/7) without exogenous peptide (△) and pulsed with peptide (▲).



M69 did not kill peptide-pulsed target cells that expressed DQ7 (Fig. 7, upper left panel). These results suggest that the TCR from the various T cell lines that we isolated interact not only with residues of peptide EBNA2_{280–290}, but also with polymorphic residues of the MHC class II molecules.

The lack of success in establishing EBNA2_{280–290}-reactive cell lines from the four nonresponding individuals (Fig. 1) could be due to one of several possibilities. One possible explanation could be the absence of an MHC allele capable of binding this peptide to present it to the CD4⁺ T cells. However, this was not the case in the present study, since all nonresponding donors had at least one MHC class II allele capable of presenting peptide EBNA2_{280–290}. Donor M2 expressed DQ7, donor M15 expressed DR1, M20 expressed DR7 and DQ2, and donor M25 expressed DR1 and DQ7 (data not shown). Another likely explanation for not being able to isolate EBNA2_{280–290}-reactive T cell lines from these individuals could be the presence of a low precursor frequency of T cells specific for this epitope in peripheral blood at the time of sampling. With some of these donors (M20 and M25), we had technical difficulties in isolating the peptide-reactive T cell lines, because their cells proliferated significantly in the absence of Ag (high background), making it impossible to select the EBNA2_{280–290}-specific T cell lines. Regardless of the cause(s) for not responding to the EBNA2_{280–290} epitope in some donors, it is clear from our results and previous findings that a large segment of the general population (~50%) would be able to respond to this epitope, since the probability of expressing at least one of the MHC class II alleles capable of presenting this peptide is quite high.

Our selection of EBNA2 as a target Ag to induce T cell responses to prevent PTLD appears appropriate, since EBNA2_{280–290}-reactive T cell lines were shown to be effective at inhibiting the proliferation of B lymphocytes induced by live EBV (Fig. 3). Although EBNA2 is considered to be one of EBV's latent cycle Ags, which is expressed on transformed EBV-LCL (1, 4), this protein is also one of the first to be produced after viral infection (32) and before immortalization (33). Most importantly, EBNA2 functions as a viral transcription factor that is essential for the initiation and maintenance of the B lymphocyte growth and transformation states (34, 35). Thus, T cell responses to EBNA2 would be beneficial for controlling both the early infection of EBV (to prevent PTLD) and the EBV-transformed B cells that may result in lymphomas. The latter is supported by the data demonstrating that some of the EBNA2_{280–290}-reactive T cell lines were capable of recognizing EBV-transformed LCL (Fig. 3), and in some cases the T cells were also capable of killing the EBV-LCL (Fig. 7).

The effector mechanism(s) by which the EBNA2_{280–290}-specific CD4⁺ T cells are able to inhibit EBV-induced B cell proliferation (Figs. 4–6) may be numerous. The production of lymphokines such as IFN- γ by Ag-stimulated T cells could have an antiviral effect, inhibiting infection and virus-induced B cell proliferation. However, this mechanism would not be operative in the presence of some immunosuppressive compounds such as CsA (Fig. 5, right panel). Another more likely effector mechanism that CD4⁺ T cells might employ to inhibit EBV-mediated B cell proliferation is via cytolysis of the newly-infected cells. It has been reported that CD4⁺ T cells can exhibit a high degree of cytotoxicity via either the perforin or Fas/Fas ligand pathway (36–40). Indeed, our results show that EBNA2_{280–290}-reactive CD4⁺ T cells displayed significant levels of Ag-specific cytotoxicity (Fig. 7). In the case of the DR52-restricted T cells from M69 and M44, significant cytotoxicity was observed to the autologous EBV-LCL in the absence of exogenous peptide, indicating the presence of optimal amounts of naturally produced peptide/MHC complexes on these targets. However, with other T cell lines the cytolytic activity against au-

tologous EBV-LCL was only observed with the addition of exogenous peptide (Fig. 7). The cytotoxicity results contrast with the data obtained in the IFN- γ release assay (Fig. 3), where all T cell lines were capable of recognizing the naturally processed epitope presented by autologous EBV-LCL. This discrepancy could be explained by differences in the amount of time required to generate a signal in these assays. In the cytotoxicity assays the T cells and targets were allowed to interact with each other for 6–9 h, while in the cytokine release assay this interaction lasted for 48 h. Thus, it is likely that only those T cells with high avidity for their Ag will be able to recognize autologous EBV-LCL in a short period of time (cytotoxicity assay). The avidity of the T cells for their APC (or their targets) may be influenced by numerous factors such as 1) the density of specific MHC/peptide complexes on the APC, 2) the intrinsic affinity of the TCR for the Ag, and 3) the level of expression of adhesion molecules and their ligands, which help to stabilize cell to cell interactions. It is possible that newly EBV-infected B cells could serve as better targets for EBNA2_{280–290}-reactive T cells than EBV-LCL, which would help explain the strong, CsA-resistant antilymphoproliferative effects observed (Figs. 4–6). This possibility would exist if the recently infected B cells were to express higher levels of EBNA2_{280–290}/MHC complexes (or adhesion molecules) than the EBV-LCL.

In conclusion, our results indicate that the EBNA2_{280–290} epitope would be effective at eliciting potent anti-EBV CD4⁺ T cell responses capable of preventing PTLD in solid organ transplant candidates. Vaccination of patients with this epitope before the transplant and subsequent immunosuppressive therapy would allow the establishment of T cells capable of inhibiting B cell proliferation induced by EBV infection, which leads to PTLD. Alternatively, in vitro generated T cell lines reactive with the EBNA2_{280–290} epitope could be used for adoptive immunotherapy in patients with ongoing PTLD. Most importantly, our results suggest that once primed by Ag, the resulting effector EBNA2_{280–290}-specific CD4⁺ T cells would be able to control PTLD even in the presence of some immunosuppressive drugs, such as CsA. Finally, because the EBNA2_{280–290} peptide is highly promiscuous toward various MHC class II alleles, it could be used as a vaccine in a large proportion of patients. A peptide vaccine clinical study in pretransplant patients using this epitope that is underway at our institution may help to answer some of these important questions.

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