Identification of HLA-DP3-restricted Peptides from EBNA1 Recognized by CD4+ T Cells

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Abstract

The EBV-encoded nuclear antigen 1 (EBNA1) is required for the maintenance and replication of the viral episome in EBV-transformed human B-lymphoblastoid cell lines. It is expressed in all EBV-associated tumors, making it a potentially important target for immunotherapy. However, this promise has not been realized, because an endogenously processed MHC class I-restricted T-cell epitope remains to be identified, and relatively little is known about MHC class II-restricted helper epitopes in the molecule. In this report, we identify a T-cell peptide derived from EBNA1 that is recognized by CD4+ T cells. More importantly, EBNA1-specific, HLA-DP3-restricted CD4+ T cells are capable of recognizing MHC class II-transduced Burkitt’s lymphoma cells, autologous peripheral blood mononuclear cells loaded with the purified EBNA1 protein, as well as target cells transfected with Ii-EBNA1 cDNA. These new findings demonstrate that EBNA1 is processed endogenously and presented to T cells by MHC class II molecules, and, hence, may be useful to incorporate into cancer vaccines to enhance antitumor immunity against EBV-associated tumors.

Introduction

EBV, a human γ herpes virus with tropism for B cells, has been implicated in the pathogenesis of a variety of human tumors, including BL, NPC, PTID, and HD (1, 2). Among the genes responsible for the growth-transforming function of EBV, EBNA1 is the only viral gene that is detected in all of the EBV-associated tumors including BL, NPC, PTID, and HD (3, 4). Other viral antigens such as the immunodominant EBNA3a, 3b, and 3c are expressed only in type 3 tumors such as PTID, whereas two other antigens, latent membrane proteins LMP1 and LMP2, are expressed in type 2 tumors such as NPC and HD, but not in BL tumor (type 1 tumor). Thus, it appears that EBNA1 is a potentially important immune target for cancer immunotherapy.

Studies from animal models and human clinical trials have demonstrated that CD4+ T cells play a central role in orchestrating host immune responses against cancer and infectious diseases (5–7). Indeed, CD4+ T cells consistently respond to the EBNA1 antigen in healthy donors and are capable of recognizing EBV-transformed B-LCLs (8, 9). To evaluate immune responses against EBNA1, EBNA3C, LMP1, and LMP2, several CD4+ T-cell lines were generated from human PBMCs after in vitro stimulation with dendritic cells pulsed with the corresponding purified proteins (10). Among the viral antigens tested, EBNA1 elicited the strongest CD4+ T-cell response, but these peptide-specific CD4+ T cells were not capable of recognizing naturally processed EBNA1 peptides on LCLs (10).

Adoptive therapy of EBV-positive HD patients with EBV-specific CTLs has shown evidence of immune function and antitumor activity, but the overall immune responses were not sufficient to eradicate tumor cells (11, 12). Effective immunotherapy against EBV-associated malignancies should be aided by identifying MHC class II-restricted peptides from EBNA1 or other EBV-tumor associated antigens for use in cancer vaccines (13, 14). In this study, we describe the identification of an EBNA1-specific T-cell peptide by stimulation of human PBMCs in vitro with a set of 13–15-mer peptides.

Materials and Methods

Cell Lines, Reagents, and Antibodies. BL cell lines AG876, Akata, and Eli; EBV-transformed LCLs 1–7, 1359, and EBV-DG75; melanoma cell line 1359mel; and HEK 293 cell lines were maintained in RPMI 1640 supplemented with 10% FCS growth medium. Antibodies used in this study were described previously (13). EBNA1 protein was expressed in SF-9 cells and purified as described previously (15).

HLA Typing of Donor PBMCs. The HLA serotypes and DNA genotypes of PBMCs from healthy human were determined by the NIH HLA Laboratory. The HLA genotype of PBMCs from donor P was HLA-A*0201, 32, B*4001, 51, DRB1*0401, 0801, DQB1*0204, 02, DRB4*0101; for donor Q it was HLA-A*01, 6802, B*15, 53, DRB1*0401, 1302, DQB1*0301, 0501, DRB3*0301, DRB4*0101; for donor S it was HLA-A*0301, 29, B*44, 4501, DRB1*0401, 0701, DQB1*0201, 0301, DRB4*0101; and for 1359mel cell line it was HLA-A*01, B*8, 40, CW*03, 07, DRB1*0401, 17, DQB1*02, 03, DRB3*0101, B*40101. The molecular typing of HLA-DP molecules for the PBMCs from donor P was performed as described previously (13). DNA sequences were searched against the IMGT-HLA database to determine the HLA-DP identity.

Synthetic EBNA1 Peptides. Ten peptides encompassing B95.8 strain EBNA1 P166-178 (EGLLLARALLSHVE), P196-208 (GIFVYGGSKSTLYNL), P318-330 (VNLRRGTAIIPQ), P352-364 (GPRLESIVCYFVMV), P386-398 (ESIVCYFMVFLQT), P361-373 (YFIVMFVQTHIFAE), P357-368 (AEVLKDAIKDLVM), P360-372 (KDLVMTKAPTCN), P392-404 (NIRTVCSFDGVG), and P307-319 (PPWFPVMVEAAAG) were synthesized by standard fluorenlyl-methoxy carbonyl chemistry and dissolved in DMSO. The purity and molecular masses of peptides were determined by high-performance liquid chromatography and mass spectrometry.

Generation of Human CD4+ T-Cell Lines and Clones. PBMCs from three donors (S, P, and Q) were used for peptide stimulation in vitro in lymphocyte culture medium at 2 × 10^6 cells/well in a flat-bottomed 96-well plate, as described (13). Two weeks after stimulation, each subline was again cultured in vitro with a set of 13–15-mer peptides. To determine the restriction element in the presence of anti-HLA-A, B, and C, anti-HLA class II, HLA-DP, -DQ, and -DR mAb at a 20 μg/ml of antibody concentration. T-cell clones were generated from bulk T-cell lines by the limiting dilution method, as described previously (16).

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4 The abbreviations used are: BL, Burkitt’s lymphoma; PTID, post-transplant lymphoproliferative disorder; NPC, nasopharyngeal carcinoma; HD, Hodgkin’s disease; LCL, lymphoblastoid cell line; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; aa, amino acid; FACS, fluorescence-activated cell sorter; EBV, Epstein-barr virus; EBNA1, EBV-encoded nuclear antigen 1; GFP, green fluorescent protein.

5 Internet address: http://www3.ebi.ac.uk/Services/imgt/hla.
Transfection of EBNA1 Expression Constructs. Full-length *EBNA1, EBNA1-GFP,* and GA-deleted *EBNA1-GFP* constructs (17) were obtained from Judy Tellam and Rajiv Khanna, University of Queensland, Brisbane, Australia. We also constructed an expression vector pβiEBNA1(aa275–686) that expresses EBNA1 as a fusion protein with a targeting sequence (aa 1–80) of invariant chain (li). HEK293 cells were transfected with LipofectAMINE reagent (Invitrogen, Carlsbad, CA). Transfection and T-cell activity assay were described previously (13).

**Results**

**Generation of Human T Cells Specific for EBNA1.** Human CD4 + T cells consistently and predominantly respond to EBNA1 (8, 10), suggesting that this antigen may be an important target for immunotherapy. Because each of the dominant HLA-DR alleles accounts for only 10–20% of the general population, we reasoned that T-cell peptides presented by different MHC class II molecules, including DP and DQ molecules, are present within the EBNA1 protein. To date, only three EBNA1 peptides presented by HLA-DR1, DR11, and DR15 have been identified, but these peptide-specific CD4 + T cells failed to recognize naturally processed epitopes on autologous LCLs or BL tumor cells (10, 18). To identify T-cell peptides presented by a HLA-DR4 molecule, we made 10 13–15mer EBNA1 peptides predicted to have an HLA-DRB1*0401 binding motif by a computer-assisted algorithm, and used them to stimulate human PBMCs *in vitro* (19). After three cycles of stimulation, T cells generated from PBMCs of each donor were tested for their ability to recognize HLA-DRB1*0401-matched 1359mel target cells pulsed with 10 individual peptides. A single 13mer peptide corresponding to EBNA1 P518–530 (YNLRRGTALAIQP) elicited substantial secretion of IFN-γ from the T-cell line P3-W4, which was obtained from the PBMCs of donor P (Fig. 1A). No peptide-specific T-cell recognition was detected among the other T cell lines, nor among target cells pulsed with control peptides.

To verify whether T-cell recognition of the EBNA1 P518–530 peptide was restricted by HLA-DR4 molecule, we tested the T-cell recognition in the presence of antibodies against HLA class I, or class II, HLA-DR, -DQ, and -DP molecules. These mAbs were purified from culture supernatants of hybridoma cells and used previously for blocking T-cell recognition of various T-cell lines/clones (13, 20). T-cell recognition of the EBNA1 P518–530 peptide by T-cell line P3-W4 was specifically blocked by mAb against HLA class II and HLA-DR-DQ molecules, but not by mAb against HLA-DR, HLA-DQ, or anti-HLA class I molecules (Fig. 1B). Furthermore, we showed that anti-HLA class II and HLA-DR-DQ mAb did not inhibit recognition of the EBNA1 peptide by CD8 + M1-B9 T cells. Anti-HLA class II mAb blocked recognition of target cells by CD4 + N-F6 and PCS-B6 T-cell clones, whereas anti-HLA-DR or anti-HLA-DQ mAb could inhibit T-cell recognition of antigens presented by the corresponding HLA-DR or HLA-DQ molecules (Fig. 1B). These results suggest that human T-cell line P3-W4 recognizes a peptide derived from EBNA1 presented by HLA-DR molecules.

**Characterization of T-Cell Clones and Their Antigenic Peptides.** To additionally characterize the P3-W4 T-cell line, we generated CD4 + T-cell clones by the limiting dilution method. Twelve CD4 + T-cell clones specific for EBNA1-P518–530 were successfully cloned and expanded (data not shown). Although T-cell clones were initially identified based on T-cell activity of the peptide presented by 1359mel cells, 100-fold higher T-cell activity was observed when autologous PBMCs were pulsed with the EBNA1-P318–530 peptide compared with peptide-pulsed 1359mel cells (data not shown), suggesting that the antigen presenting molecules expressed on 1359mel cells are not right restriction molecules. T-cell recognition of BL cell line (AG876) by different T-cell clones was demonstrated (Fig. 2A). We chose T-cell clones with a high tumor reactivity rather than peptide reactivity as our selection criteria for additional study. One of the T-cell clones, designated P3-B7, was chosen, and FACS analysis showed that the P3-B7 T cells were CD4 + T cells (Fig. 2B). Recognition of EBNA1 P518–530 peptide by P3-B7 CD4 + T cells was blocked by antibody against HLA-DR molecules (data not shown), suggesting that the T-cell clone closely resembles the original T-cell line from which it was derived.

To determine the minimum concentration of the EBNA1 P518–530 peptide required for T-cell recognition, we pulsed the autologous donor P PBMCs with the EBNA1 P518–530 peptide and used them as antigen-presenting cells. After four washes with serum-free RPMI 1640 to remove residual peptides, P3-B7 CD4 + T cells were colu-
tured with peptide-pulsed target cells overnight. Culture supernatants were collected, and T-cell activity was determined on the basis of IFN-γ release from T cells. P3-B7 CD4⁺/H11001 T cells recognized the EBNA1 P 518–530 peptide at concentrations as low as 1 nM and the T-cell reactivity increased with increasing peptide concentrations (Fig. 2C). No T-cell reactivity was observed against the control peptide, even at a concentration of 1 μM.

Recognition of LCLs and EBV⁺ BL Cells by P3-B7 CD4⁺ T Cells. Although CD4⁺ T cells have often been generated from human PBMCs against putative tumor antigens or peptides, in many cases tumor reactivity could not be demonstrated attributable to either the low affinity of the T cells or the failure of tumor cells to present naturally processed peptides on their surface (14). Indeed, EBNA1 peptide-specific CD4⁺ T cells have been generated from human PBMCs after in vitro stimulation, but have failed to recognize autologous LCLs. T-cell reactivity was found only when autologous LCL cells were preloaded with EBNA1 protein or pulsed with EBNA1 peptides (10, 18). To test whether CD4⁺ T cells generated in this study were capable of recognizing naturally processed peptides on LCLs and BL cells, we chose several LCLs and BL tumor cell lines as target cells. As shown in Fig. 3A, P3-B7 CD4⁺ T cells were capable of recognizing LCLs 4, 5, 6, and 7, as well as AG876 BL tumor cells. Recognition of AG876 BL tumor cells by P3-B7 CD4⁺ T cells could be blocked by antibodies against MHC class II and HLA-DP molecules (Fig. 3B). Taken together, these results suggest that CD4⁺ T cells recognize a naturally processed peptide on the surface of EBV⁺ BL cells in the context of HLA-DP molecules.

Recognition of EBNA1 Protein by P3-B7 CD4⁺ T Cells in the Context of HLA-DP3. To determine the restriction element for P3-B7 CD4⁺ T cells, we amplified the HLA-DP alleles from autologous PBMCs by reverse transcription-PCR, using HLA-DP-specific primers, and subcloned them into a pcDNA3.1/neo expression vector as described previously (13). DNA sequence analysis revealed that HLA-DPA cDNA had 100% sequence homology to that published for HLA-DPA (DPA1*01031) and that HLA-DPB was almost identical to HLA-DPB1*0301 with a single nucleotide change from T to C at position 112 resulting in a substitution of histidine for tyrosine. To test whether both HLA-DPA and HLA-DPB1*0301 are capable of presenting a peptide to T cells, DPA and DB1*0301 cDNA were transfected into HEK293 cells together with II-EBNA1 or
full-length EBN1 cDNA. As shown in Fig. 4A, T cells responded to HEK293 cells expressing HLA-DPA, HLA-DPB, and IL-EBNA1, demonstrating that HLA-DP3 (DPA1=0103/DPB1=0301) is the restriction element for the presentation of a peptide to T cells. Interestingly, the T cells failed to recognize HEK293 cells transfected with HLA-DPA1=01031, full-length EBN1 cDNA, or HEK293 cells transfected with other cDNAs. This result suggests that without targeting of EBNA1 to the MHC class II pathway, HEK293 cells expressing HLA-DP3 molecules alone are not sufficient to process and present the EBNA1 peptide to T cells. To exclude the possibility that other HLA-DP alleles can present the EBNA peptide to T cells, we cotransfected HLA-DP1, HLA-DP3, and HLA-DP4 cDNAs into 293 cells with IL-EBNA1, respectively. Fig. 4B shows that CD4⁺ T cells could recognize 293 cells transfected with HLA-DP3 and IL-EBNA1 cDNAs, but did not respond to IL-EBNA1 expressing 293 cells transfected with either HLA-DP1 or HLA-DP4 cDNAs. These results clearly demonstrate that HLA-DP3 is the antigen-presenting molecule for CD4⁺ P3-B7 T cells.

To additionally test whether EBNA1-specific CD4⁺ T cells were capable of recognizing the full-length EBNA1 protein, autologous PBMCs were pulsed with the purified EBNA1 protein overnight, and CD4⁺ T cells were then cocultured with protein-pulsed target cells for 18 h. An irrelevant protein, BSA, was used as a control. As shown in Fig. 4C, P3-B7 CD4⁺ T cells specifically recognized autologous PBMCs pulsed with the full-length EBNA1 protein, but not BSA.

**Discussion**

The results reported here demonstrate that the EBNA1 protein of EBV is processed and presented to CD4⁺ T cells in the context of a HLA-DP3 molecule. EBNA1-specific CD4⁺ T cells recognize both the EBNA1 P518–530 peptide and full-length EBNA1 protein pulsed on autologous PBMCs. Importantly, these T cells can recognize several HLA-DP3-matched LCLs and AG876 EBV⁺ BL tumor cells, suggesting that the EBNA1 P518–530 peptide can be endogenously processed and then presented by DP3 molecules to T cells. The observation that CD4⁺ T cells recognize only HEK293 cells transfected with HLA-DPA, HLA-DPB, and IL-fused EBNA1, but not full-length EBNA1, indicates that DMA, DMB, and other molecules are required for efficient antigen presentation through the MHC class II pathway. In a previous study we showed that IL-targeting can significantly enhance MHC class II antigen processing and presentation (20), and may override the requirement for other components in antigen presentation. This may explain why CD4⁺ T cells can recognize HEK293 cells transfected with DPA, DPB, and IL-fused EBNA1, but not full-length EBNA1. It is likely that the full-length EBNA1 is effectively processed and presented to T cells by professional antigen-presenting cells such as B and dendritic cells, as illustrated in Fig. 3A. However, it should be noted that whereas tumor antigen MAGE-3 was identified recently as a MHC class II epitope presented by HLA-DR13 molecules, CD4⁺ T cells recognized only DR13⁺ B cells transfected with IL-fused MAGE-3 cDNA, not DR13⁺ B cells transfected with the full-length MAGE-3 cDNA (21).

Although our initial intention was to identify HLA-DR4-restricted EBNA1 peptides based on computer-assisted DR4 peptide-binding algorithm, the CD4⁺ T cells generated in this study recognized peptides presented by HLA-DP3 molecules. We have reported previously a CD4⁺ T-cell line that can recognize NY-ESO-1 peptides in the context of HLA-DP4 molecules, although this peptide contains an HLA-DP3-restricted peptides (22). Subsequent studies using autologous PBMCs showed much higher T-cell activity than that when the same peptide was pulsed on 1359mel cells (data not shown; Fig. 4C). A possible explanation is that this peptide can bind to the HLA-DR4 molecule, but HLA-DR4 is not the right restriction element for the CD4⁺ T cells generated in vitro. The EBNA1 P518–530 peptide bound on HLA-DR4 molecules on 1359mel cells may dissociate from the MHC/peptide complexes and bind to HLA-DP3 molecules on T cells for recognition. The relative binding affinity of a peptide to different MHC class II molecules and the frequency of antigen-specific CD4⁺ T-cell precursors in human PBMCs may determine the outcome of T-cell stimulation in vitro with peptides.

Our EBNA1 peptide-specific HLA-DP3-restricted CD4⁺ T cells recognize BL tumor cells. By contrast, the previously reported EBNA1 peptide-specific, HLA-DR1-, DR11-, or DR15-restricted
CD4+ T cells failed to recognize EBV-positive autologous LCLs or BL tumor cells, limiting their potential therapeutic value (10, 18). However, Munz et al. (8) reported that CD4+ T cells generated in vitro could recognize EBV-positive LCL cells. These CD4+ T cells were later shown to recognize an HLA-DR1-restricted EBNA1 P514–527 peptide (23), although this peptide is identical to the one described previously by Khanna et al. (18). Whereas the HLA-DP3-restricted EBNA1 P518–530 peptide presented here overlaps with the DR1restricted EBNA1 P514–527 peptide, this is a new HLA-DP-restricted peptide. Thus, our data together with the results of Paludan et al. (23) indicate that CD4+ T cells are capable of recognizing EBV-positive LCLs and BL tumor cells. Whether the discrepancy between these and previous studies (10, 18) reflects differences in T-cell avidity for MHC/peptide complexes is not clear. Indeed, in many cases, T cells generated from PBMCs stimulated in vitro with peptides or proteins tend to recognize peptides but not tumor cells (14). Given the role of CD4+ T cells in maintaining CD8+ T-cell responses, we suggest that the HLA-DP3-restricted EBNA1 P518–530 peptide described in this report would aid in the development of effective immunotherapy for EBV-associated malignancies.

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