Published in final edited form as:

Oncol Res. 2009 ; 18(5-6): 287–292.

Dynamic CD8 T-Cell Responses to Tumor-Associated Epstein-Barr Virus Antigens in Patients With Epstein-Barr Virus-Negative Hodgkin's Disease

Holbrook Kohrt^{*,†,1}, Alexandre Johannsen^{*,1}, Richard Hoppe[‡], Sandra J. Horning[†], Saul A. Rosenberg[†], Ranjana Advani[†], and Peter P. Lee^{*}

^{*}Division of Hematology, Stanford University School of Medicine, Stanford, CA, USA

[†]Division of Oncology, Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA

[‡]Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA, USA

Abstract

In almost half of patients diagnosed with Hodgkin's disease (HD), the malignant Reed-Sternberg (RS) cells express Epstein-Barr virus (EBV) antigens. Multiple translational efforts are actively investigating antitumor immune strategies by stimulating cytotoxic T lymphocytes (CTL) against tumor-associated EBV antigens. It has previously been believed that this therapeutic strategy and presence of EBV-specific CTLs are limited to EBV-positive HD. In an effort to explore the EBV-specific immune response, here we characterize EBV-specific CTL responses to lytic and latent EBV antigens in 12 consecutive EBV carriers with EBV-negative HD. Compared to healthy donors, we detected weak, baseline EBV-specific responses to both lytic and latent antigens by IFN- γ ELISPOT in patients with EBV-negative HD at diagnosis. Chemoradiotherapy was associated temporally with a decrease EBV-specific responses. At final follow-up (24 months), recovery of EBV-specific CTL responses was observed with robustness of lytic-specific response equivalent to healthy controls. We confirm evidence of EBV-specific CTLs in patients with EBV-negative HD and provide the first report of dynamic variance in this population during treatment. Our observation challenges prior belief that patients with HD remain immunodeficient following therapy and argues that the clinical significance of the EBV-specific immune response in EBV-negative HD should be further investigated.

Keywords

Epstein-Barr virus; Hodgkin's disease; Lymphocyte; Immune response

Address correspondence to: Peter P. Lee, M.D., CCSR Room 1155, 269 Campus Drive, Stanford, CA 94305, USA. Tel: 650-498-7942; Fax: 650-736-0974; ppl@stanford.edu or Ranjana H. Advani, M.D., Stanford Cancer Center, 875 Blake Wilbur Drive, MC5151, Stanford, CA 94305, USA. Tel: 650-724-8372; Fax: 650-724-5203; radvani@stanford.edu.

¹Authors contributed equally to this work.

INTRODUCTION

A strong association of Epstein-Barr virus (EBV) in the pathogenesis of cancer has been well described in Burkitt lymphoma, peripheral NK/T-cell lymphoma, posttransplant lymphoproliferative disorder (PTLD), and nasopharyngeal carcinoma (NPC) (1). This is also seen in almost half of the patients with Hodgkin's disease (HD), where malignant Reed-Sternberg (RS) cells express EBV-related antigens (2). The pattern of EBV antigen expression (latent and lytic) differs in healthy carriers and in patients with EBV-associated malignancies. In the latter, two type II latency membrane proteins (LMP1 and LMP2) and a virally encoded nuclear protein (EBNA1) are expressed, while among healthy carriers of EBV latent antigen expression is restricted to type I latency (EBNA1) (3). EBV lytic antigen expression may also be seen in healthy individuals and patients with EBV-associated tumors. In healthy immune-competent individuals, a quiescent state of EBV infection is maintained by cytotoxic T lymphocytes (CTL) specific for EBV-type I latency and lytic antigens (3).

Based on these observations, novel immunotherapies against EBV-associated malignancies targeting type II latency-specific antigens are being investigated. While this approach has been successful in EBV-related PTLD and peripheral NK/T-cell lymphomas (4,5), the majority of patients with HD are not potential candidates for these immunologic maneuvers as a significant proportion have EBV-negative disease (6). Additionally, patients with HD have been recognized to have impaired cell-mediated immune function, limiting their ability to respond to either tumor-associated or naturally encountered antigens (7).

The objective of this study was to examine EBV-specific immune responses among patients with EBV-negative HD and, if demonstrated, follow these responses serially from diagnosis through completion of therapy. We postulated that among patients with EBV-negative HD, due to the lack of EBV antigen expression on tumor cells and systemic immune dysregulation in the setting of cancer, no evidence of EBV-specific CTLs would be observed. In contrast to our assumptions, we observed populations of EBV-specific, CD8⁺ lymphocytes from 12 patients with EBV-negative tumors.

MATERIALS AND METHODS

In order to compare EBV responses in HD patients to healthy controls, peripheral blood samples were also obtained from 15 healthy individuals from the Stanford Blood Bank. Patients with pathologically confirmed HD, according to the World Health Organization (WHO) criteria (8), receiving treatment at Stanford Cancer Center were screened for enrollment under a protocol reviewed and approved by the Institutional Review Board and conducted in accordance with the Helsinki declaration. All patients were treated with Stanford V chemotherapy for 8 or 12 weeks followed by radiotherapy as previously described (9).

Formalin-fixed, paraffin-embedded baseline biopsy samples were tested for EBV by immunofluorescence to detect viral capsid antigen and confirmed by in situ hybridization. Only cases with EBV-negative staining were included. All patients were confirmed positive EBV carriers by positive IgG and negative IgM serologic status. Baseline peripheral blood

samples were obtained from all patients to perform HLA typing by PCR-based DNA typing. EBV status was confirmed by IgM and IgG serologies as well as EBV viral load by PCR if available. Due to HLA-specific-CD8⁺ T-cell tetramers used for the assays, only HLA-A*201⁺ cases were included. Peripheral blood was collected pretreatment, during therapy, and follow-up, and analyzed for response to a control peptide (G209), EBV lytic peptides (BRLF1 and BMLF1), and EBV type II latency peptides (LMP2 and LMP2A) with CD8⁺ T-cell tetramers to demonstrate antigen specificity and IFN- γ ELISPOT as well as CD107 mobilization to assay T-cell function.

EBV peptides were synthesized by the Stanford Protein and Nucleic Acid Facility and included lytic peptides BRLF1 (109–117) YDLHLIVV and BMLF1 (280–288) GLCTLVAML, latent peptides LMP2 (329–337) LLWTLVVLL and LMP2A (426–434) CLGGLL TMV, and control peptide G209 (ITDQVPFSV). Peripheral blood mononuclear cells were stained with CD3, CD4, CD8, CD19, and EBV-specific peptide/MHC (pMHC) tetramers purchased from Immunomics (San Diego, CA) or obtained from the NIAID Tetramer Core Facility (Atlanta, GA). IFN- γ ELISPOT was performed as previously described with anti-h-IFN- γ Ab obtained from Mabtech (Cincinnati, OH). CD107 mobilization assay was performed as previously described. Briefly, peripheral blood mononuclear cells (effectors) were incubated with T2 cells pulsed with EBV peptides (targets) at a ratio of 1:2 (E/T) for 4 h at 37°C in 7% CO₂. T2 cells were pulsed with the appropriate peptide in 10-fold dilutions from 1 to 10⁻⁶ mg/ml for 1 h at 37°C in 7% CO₂. Samples were stained with CD107a-APC and CD107b-APC before incubation and CD8, CD19, and tetramer after incubation. Tetramer-positive CD8⁺CD19⁻ cells were gated and plotted for CD107 versus CD8 to determine the level of CD107⁺ population.

RESULTS

Twelve patients with EBV-negative HD and HLA-A*201⁺ status were enrolled for CTL analysis of EBV-specific responses as described above. Patient characteristics are listed in Table 1. The median age was 31 years and at a median follow-up of 4.3 years (range 2–16 years) no relapses or deaths were observed (Table 1).

CTL responses to control, lytic, and latent EBV-specific peptides from patients with EBV-negative HD and healthy individuals are shown in Fig. 1. CD107 mobilization by CTLs from healthy subjects as well as patients with EBV-negative HD at diagnosis was directly correlated with IFN- γ ELISPOT when exposed to targets expressing latent ($r^2 = 0.967$, $p = 0.033$) or lytic peptides ($r^2 = 0.937$, $p = 0.001$). Responses to control peptide, G209 (CD8 tetramer and IFN- γ ELISPOT assays) were not observed in healthy controls or in patients with EBV-negative HD at diagnosis or throughout follow-up. No changes in EBV serologic status or viral load were observed throughout follow-up.

For lytic peptide responses prior to the initiation of therapy, the magnitude of response was more robust by IFN- γ ELISPOT among healthy individuals than among patients with EBV-negative HD (BMLF1, 199.8 ± 49.7 and 27.9 ± 9.7 , $p < 0.001$; BRLF1, 48.8 ± 11.45 and 16.9 ± 3.4 , $p < 0.05$). The same pattern was observed for CD8 tetramer staining with BMLF1 pMHC tetramers and IFN- γ ELISPOT (0.50 ± 0.16 and 0.07 ± 0.03 , $p < 0.01$). In

patients, CTL responses to EBV-specific lytic peptides were followed throughout treatment. In comparison to responses observed at baseline, 4 weeks after initiation of chemotherapy, circulating CTL responses against lytic peptides decreased by 45–70% without significant change in response to control peptide. At 8 months lytic peptide responses increased beyond pretreatment levels by approximately 20–30% for BMLF1 and BRLF1. This trend continued at 16 months (26–82% increase in response by IFN- γ ELISPOT to lytic peptides), and at 2 years response to lytic peptide BMLF1 was 2.6-fold greater compared to pretreatment IFN- γ release. A slight decrease in the response to lytic peptide BRLF1 was observed at 2-year follow-up, although it remained approximately 25% greater than pretreatment and 225% greater than nadir activity at 4 weeks.

For latent peptide responses prior to initiation of therapy, populations of CTLs to LMP2A by CD8⁺ T-cell tetramer analysis were significantly greater among patients with EBV-negative HD compared to healthy donors (0.02 ± 0.01 to 0.13 ± 0.05 , $p < 0.05$). A similar pattern was seen when assayed by IFN- γ ELISPOT to LMP2A (16.1 ± 3.9 to 49.4 ± 8.6 , $p < 0.05$). Responses to LMP2 did not significantly differ between healthy donors and patients with EBV-negative HD pretreatment (33.0 ± 6.8 to 30.7 ± 6.0). CTL responses to latent peptides in patients mirrored the dynamic changes observed to lytic peptides during therapy and follow-up. Initial responses, measured at 4 weeks, to LMP2 and LMP2A decreased by 43% and 35%, respectively. Recovery of latent-antigen specific CTLs to levels observed pretreatment lagged behind that seen against lytic peptides, requiring 16 months for response recovery to LMP2 and 2 years for LMP2A. At final assessment, CTL response to latent antigen, LMP2 was 2.4-fold greater than pretreatment and over fourfold greater than the nadir at 4 weeks.

DISCUSSION

We observed dynamic changes to EBV-specific immune responses in patients with EBV-negative HD for both lytic and latent antigens by IFN- γ ELISPOT as well as CD8 tetramer flow cytometry. The transient suppression of response to lytic and latent antigens recovered in 8 months to 2 years to pretreatment levels. Responses to lytic antigen, BMLF1, and latent antigen, LMP2, at 2 years were more robust (2.6- and 2.4-fold) compared to pretreatment responses. These findings suggest that at diagnosis patients with EBV-negative HD demonstrate suppressed responses to lytic antigens while maintaining a latent antigen-specific population of CTLs with preserved function and IFN- γ release. The pattern of CTL responses we observed has previously been described among patients with EBV-positive HD in which circulating CTL responses to BMLF1 by IFN- γ ELISPOT were up to 17-fold reduced compared to those of healthy individuals (10). In our series, treatment with combination chemoradiotherapy resulted in a transient decrease in LMP2A- and BMLF1-specific responses, which became more robust 20–50 weeks after completing therapy. This suggests that systemic chemotherapy resulted in a period of transient immunosuppression followed by increased EBV activity and thus a reactivated immune response following completion of treatment. This phenomenon has also been reported in hepatitis B, in which a posttreatment flare in HBV virus and immune response can result in fulminant hepatic failure (11).

At 2 years among patients with EBV-negative HD, lytic peptide responses were similar to healthy controls, suggesting that the reported persistent dysregulation of the immune response following completion of HD therapy was not seen in our series (7). This intriguing finding argues that EBV-specific CTL responses change dynamically in EBV-negative HD and supports further study of their clinical and therapeutic significance. Whether recovery of immune function is unique to our series of patients or is due to the chemotherapy regimen (Stanford V) deserves additional investigation.

The inciting event that triggers malignant transformation and a shift in EBV antigen expression in HD is unknown. Immune escape, acquisition of secondary mutations, and immunologic insult such as concurrent infection have all been postulated as possible triggers (12). Our observation suggests that EBV may be involved in the oncogenesis of EBV-negative HD. Despite patients lacking EBV RNA at the time of initial diagnosis, EBV may still have contributed to tumor development and later have become undetectable, as supported by the “hit and run” theory (13). Despite no direct evidence of EBV within the tumor, the augmented type 2 latency responses we observed in circulating CTLs indirectly argue that a background population of EBV-infected cells stimulates this response either prior to diagnosis or exist below a detectable threshold with current testing modalities.

The presence of EBV-specific immune responses may have therapeutic implications, if confirmed by other series of EBV-negative HD. Infusion of EBV-specific CTLs among patients with EBV-positive HD results in improvement in B symptoms with either stabilization of disease or a decrease in EBV viral load (14,15). Adoptive T-cell transfer’s capacity to inhibit EBV infection in vitro was first translated therapeutically among transplant recipients with EBV-positive PTLN (14,16,17). Stabilization as well as resolution of PTLN was observed with decreased or eliminated immunosuppression posttransplant, allowing expansion of the existing EBV-specific immune response. A clinically effective, EBV-specific CTL population could also be created by ex vivo augmentation. This includes selection of patient CTLs, activation and expansion during culture with autologous EBV-transformed lymphoblastoid cell lines, followed by adoptive transfer of EBV-specific T cells targeting antigens including EBNA1 or 2, and LMP1 or 2. EBV-specific T cells could also be transferred in a prophylactic fashion into transplant recipients resulting in lower rates of PTLN. In 1998, a similar approach was applied in EBV-positive HD (14). EBV-specific CTL lines were successfully generated from 9 of 13 patients, three of whom received autologous, expanded EBV-specific CTLs for multiply relapsed HD resulting in prolonged disease control. In 2004, infusion of EBV-specific CTLs among 14 patients with EBV-positive HD resulted in improvement in B symptoms with stabilization of disease or a decrease in EBV viral load (15). Five patients experienced a durable complete remission, one had a partial response to treatment, and five had stable disease at up to 40 months after therapy. In a patient with multiply relapsed HD who had received prior autologous and allogeneic hematopoietic cell transplant, infusion of autologous EBV-specific CTLs produced disease stabilization for 6 months, with evidence of gene-marked EBV-specific CTLs persisting for 12 months after infusion (15).

Analogous to the antitumor immune activity of EBV-specific CTLs in EBV-positive HD, immunotherapy may also be an effective treatment for patients with previously recognized,

EBV-negative HD. Further, our findings have implications for all patients with HD as we observe dynamic variation in immune function with transient immune suppression and gradual immune recovery. If efficacy of immunotherapy in HD is dependent upon presence of a functional immune response, delayed EBV-targeted immunotherapy as a consolidative maneuver may improve clinical outcomes. Ongoing study of EBV antigen expression by tumor cells and the corresponding dynamic, temporal changes in host CTL response will improve our understanding of the role for EBV-targeted immunotherapy for patients with HD.

References

1. Levine PH, Ablashi DV, Berard CW, Carbone PP, Waggoner DE, Malan L. Elevated antibody titers to epstein-barr virus in Hodgkin's disease. *Cancer*. 1971; 27(2):416–421. [PubMed: 4322464]
2. Weiss LM, Movahed LA. In situ demonstration of epstein-barr viral genomes in viral-associated b cell lymphoproliferations. *Am J Pathol*. 1989; 134(3):651–659. [PubMed: 2538060]
3. Rickinson AB, Moss DJ. Human cytotoxic t lymphocyte responses to epstein-barr virus infection. *Annu Rev Immunol*. 1997; 15:405–431. [PubMed: 9143694]
4. Merlo A, Turrini R, Dolcetti R, Zanolello P, Amadori A, Rosato A. Adoptive cell therapy against ebv-related malignancies: A survey of clinical results. *Expert Opin Biol Ther*. 2008; 8(9):1265–1294. [PubMed: 18694349]
5. Cho HI, Hong YS, Lee MA, Kim EK, Yoon SH, Kim CC, Kim TG. Adoptive transfer of epstein-barr virus-specific cytotoxic t-lymphocytes for the treatment of angiocentric lymphomas. *Int J Hematol*. 2006; 83(1):66–73. [PubMed: 16443556]
6. Klein E, Kis LL, Klein G. Epstein-barr virus infection in humans: From harmless to life endangering virus-lymphocyte interactions. *Oncogene*. 2007; 26(9):1297–1305. [PubMed: 17322915]
7. King GW, Yanes B, Hurtubise PE, Balcerzak SP, LoBuglio AF. Immune function of successfully treated lymphoma patients. *J Clin Invest*. 1976; 57(6):1451–1460. [PubMed: 932190]
8. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J. Lymphoma classification—from controversy to consensus: The r.E.A.L. And who classification of lymphoid neoplasms. *Ann Oncol*. 2000; 11(Suppl 1):3–10.
9. Horning SJ, Hoppe RT, Breslin S, Bartlett NL, Brown BW, Rosenberg SA. Stanford v and radiotherapy for locally extensive and advanced Hodgkin's disease: Mature results of a prospective clinical trial. *J Clin Oncol*. 2002; 20(3):630–637. [PubMed: 11821442]
10. Chapman AL, Rickinson AB, Thomas WA, Jarrett RF, Crocker J, Lee SP. Epstein-barr virus-specific cytotoxic t lymphocyte responses in the blood and tumor site of hodgkin's disease patients: Implications for a t-cell-based therapy. *Cancer Res*. 2001; 61(16):6219–6226. [PubMed: 11507075]
11. Rossi G. Prophylaxis with lamivudine of hepatitis b virus reactivation in chronic hbsag carriers with hemato-oncological neoplasias treated with chemotherapy. *Leuk Lymphoma*. 2003; 44(5):759–766. [PubMed: 12802911]
12. Thorley-Lawson DA, Gross A. Persistence of the epstein-barr virus and the origins of associated lymphomas. *N Engl J Med*. 2004; 350(13):1328–1337. [PubMed: 15044644]
13. Gan YJ, Razzouk BI, Su T, Sixbey JW. A defective, rearranged epstein-barr virus genome in eber-negative and eber-positive Hodgkin's disease. *Am J Pathol*. 2002; 160(3):781–786. [PubMed: 11891176]
14. Roskrow MA, Suzuki N, Gan Y, Sixbey JW, Ng CY, Kimbrough S, Hudson M, Brenner MK, Heslop HE, Rooney CM. Epstein-barr virus (ebv)-specific cytotoxic t lymphocytes for the treatment of patients with ebv-positive relapsed Hodgkin's disease. *Blood*. 1998; 91(8):2925–2934. [PubMed: 9531603]
15. Bollard CM, Aguilar L, Straathof KC, Gahn B, Huls MH, Rousseau A, Sixbey J, Gresik MV, Carrum G, Hudson M, Dilloo D, Gee A, Brenner MK, Rooney CM, Heslop HE. Cytotoxic t

- lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. *J Exp Med*. 2004; 200(12):1623–1633. [PubMed: 15611290]
16. Rooney CM, Smith CA, Ng CY, Loftin S, Li C, Krance RA, Brenner MK, Heslop HE. Use of gene-modified virus-specific t lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet*. 1995; 345(8941):9–13. [PubMed: 7799740]
17. Khanna R, Bell S, Sherritt M, Galbraith A, Burrows SR, Rafter L, Clarke B, Slaughter R, Falk MC, Douglass J, Williams T, Elliott SL, Moss DJ. Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic t cells in solid organ transplant patients with posttransplant lymphoproliferative disease. *Proc Natl Acad Sci USA*. 1999; 96(18):10391–10396. [PubMed: 10468618]

Figure 1.

Profile of the immune response to EBV peptides in EBV-negative HD patients (white bars) and healthy donors (black bars). (A) Baseline: release of IFN- γ by ELISPOT following peptide stimulation. (B) Baseline: percent of CD8⁺ cells and tetramer⁺ cells following peptide stimulation. (C) Changes in IFN- γ by ELISPOT following peptide stimulation throughout the study follow-up period.

Table 1

Characteristics of Patients With EBV-Negative Hodgkin's Disease

Characteristic	No.	%
Age (years)		
Median (range 19–56)	31	
Gender		
Male	7	58.3%
Female	5	41.7%
Ann Arbor Stage		
I/II	8	66.7%
III/IV	4	33.3%
B symptoms		
Present	2	16.7%
Absent	10	83.3%
International prognostic score		
2	8	66.7%
3	4	33.3%
Therapy: Stanford V		
12 weeks CT + 36 Gy RT	4	33.3%
8 weeks CT + 30 Gy RT	3	25.0%
8 weeks CT + 20 Gy RT	5	41.7%
Follow-up (years)		
Median (range 2–16)	4.3	
Relapse	0	0.0%

CT, chemotherapy; RT, radiotherapy; Stanford V, mechlorethamine, doxorubicin, vinblastine, vincristine, bleomycin, etoposide, prednisone.