Atypical prediagnosis Epstein-Barr virus serology restricted to EBV-positive Hodgkin lymphoma

Lynn I. Levin,[1] Ellen T. Chang,[2,3] Richard F. Ambinder,[4] Evelyne T. Lennette,[5] Mark V. Rubertone,[6] Risa B. Mann,[4] Michael Borowitz,[4] Edward G. Weir,[7] Susan L. Abbondanzo,[8] and Nancy E. Mueller[9]

[1]Department of Epidemiology, Preventive Medicine Program, Walter Reed Army Institute of Research, Silver Spring, MD; [2]Exponent Inc, Health Sciences Practice, Menlo Park, CA; [3]Division of Epidemiology, Department of Health Research and Policy, Stanford University School of Medicine, Stanford, CA; [4]Division of Hematologic Malignancies, Johns Hopkins School of Medicine, Baltimore, MD; [5]Virolab Inc, Emeryville, CA; [6]Armed Forces Health Surveillance Center, Silver Spring, MD; [7]Clinical Pathology Associates, Austin, TX; [8]Department of Hematopathology, Armed Forces Institute of Pathology, Washington, DC; and [9]Department of Epidemiology, Harvard School of Public Health, Boston, MA

An altered anti–Epstein-Barr virus (EBV) serologic profile preceding diagnosis is associated with an increased risk of Hodgkin lymphoma. It is unknown whether this atypical pattern predicts Hodgkin lymphoma risk further subdivided by determination of EBV in tumor cells. A nested case-control study of 128 incident Hodgkin lymphoma cases and 368 matched controls from active-duty military personnel with archived serum in the US Department of Defense Serum Repository was conducted to determine whether a panel of anti-EBV antibody titers differed in EBV$^+$ and EBV$^-$ Hodgkin lymphoma. Among 40 EBV$^+$ Hodgkin lymphoma cases and matched controls, statistically significant increased risks were associated with elevated anti-EBV VCA IgG antibody titers (relative risk = 3.1; 95% confidence interval [CI], 1.1-8.7), and an anti–EBNA-1/anti–EBNA-2 antibody ratio ≤ 1.0 versus > 1.0 (relative risk = 4.7; 95% CI, 1.6-13.8). In contrast, no significant associations were found among 88 EBV$^-$ Hodgkin lymphoma cases relative to their matched controls. In case-case analysis, EBV$^+$ disease was significantly associated with a low anti–EBNA-1/anti–EBNA-2 antibody ratio. This distinctive serologic response to EBV latent antigens, indicative of immune dysfunction in other clinical settings, is associated with an increased risk of developing EBV$^+$ but not EBV$^-$ Hodgkin lymphoma. (*Blood*. 2012;120(18):3750-3755)

Introduction

Early clues that the Epstein-Barr virus (EBV) may play a role in the etiology of Hodgkin lymphoma came from seroepidemiologic case-control studies showing that Hodgkin lymphoma cases had altered antibody titers against the EBV viral capsid antigen (VCA) and early antigen (EA) complex, relative to population controls.[1] In 1989, we reported that this altered serologic profile of EBV preceded the development of Hodgkin lymphoma by several years.[2] Further, we found that this pattern involved not only elevated antibodies to VCA and the EA complex, antigens expressed in the lytic cycle, but also elevated antibodies to the EBV nuclear antigen (EBNA) complex expressed in latency. These findings were subsequently confirmed in a report by Lehtinen et al.[3]

Since that time, reliable assays have been developed to detect antibodies against subcomponents of the EBNA complex,[4] which have helped to further characterize the host response to EBV. Typically, patients with primary EBV infection that results in infectious mononucleosis first develop antibodies against VCA, EA, and the latent protein EBNA-2. This pattern is followed by the appearance of antibodies to EBNA-1. Antibodies against EBNA-2 subsequently diminish, resulting in an anti–EBNA-1/anti–EBNA-2 antibody ratio of > 1.0 in healthy carriers.[5] Persistent anti–EBNA-1/anti–EBNA-2 antibody ratios of ≤ 1.0 have been described in patients with inherited immunologic disorders, severe infectious mononucleosis, chronic EBV infection, rheumatoid arthritis, chronic renal failure, and AIDS.[4-9] Recently, it has been shown that an anti–EBNA-1/EBNA-2 ratio ≤ 1.0 is associated with Hodgkin lymphoma risk, independent of a history of infectious mononucleosis.[10] Henle et al have suggested that this atypical pattern is indicative of inadequate host regulation of latent EBV infection.[5]

In 20%-50% of Hodgkin lymphoma cases overall, EBV nucleic acids and proteins are detected in the diagnostic Hodgkin and Reed-Sternberg cells.[11,12] These cells express several viral proteins that have been implicated in transformation in various model systems.[13-15] The detection of EBV in a variable proportion of cases worldwide raises several alternative models for Hodgkin lymphoma pathogenesis. These include a model in which EBV plays a pathogenic, early role in almost all of Hodgkin lymphoma, and the genome is somehow lost from some patients' lesions in concert with a more favorable immune response to the virus, leaving no viral "footprint" behind. Alternatively, there may be 2 independent causal pathways in the etiology of Hodgkin lymphoma, with EBV involved only in the pathogenesis of EBV$^+$ Hodgkin lymphoma cases and other factors involved in EBV$^-$ cases.[16,17]

Our strategy to clarify the relative likelihood of these hypotheses was to compare the prediagnosis EBV serologic response in EBV$^+$ and EBV$^-$ Hodgkin lymphoma cases to determine whether antibody profiles against viral antigens differ based on EBV presence in tumor cells. We therefore conducted a nested-case control study within a cohort of active-duty military personnel with archived serum samples collected several years before Hodgkin lymphoma diagnosis. In addition to case-control comparisons, the EBV antibody profile of EBV$^+$ Hodgkin lymphoma cases was

Submitted December 1, 2011; accepted August 27, 2012. Prepublished online as *Blood* First Edition paper, September 12, 2012; DOI 10.1182/blood-2011-12-390823.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

compared with that of EBV$^-$ cases to determine whether there was heterogeneity in the serologic response between the 2 groups.

Methods

Study population

All patients with a new diagnosis of Hodgkin lymphoma (ICD-9 201) identified from January 1, 1990, through December 31, 1999, among active-duty military personnel with an archived serum specimen in the DoD Serum Repository drawn before the diagnosis date were eligible for inclusion in the study. Two military data sources, the Automated Centralized Tumor Registry and the Defense Medical Surveillance System, were queried to identify incident cases. Defense Medical Surveillance System capabilities include linking persons identified from various demographic and medical databases to serum specimens archived in the DoD Serum Repository.[18]

We identified 473 potential cases with confirmed pathology reports and serum specimens archived in the DoD Serum Repository. Of these, we were able to retrieve tissue blocks for 308 cases (65%) from anatomic pathology departments in 62 military hospitals worldwide and from the National Pathology Repository at the Armed Forces Institute of Pathology. Prediagnosis serum samples from 160 cases with tissue blocks were retrieved from the repository for this analysis. Of these cases, pathology review could not confirm the diagnosis of 4 cases, detection of the tumor EBV was indeterminate for 16 cases, and restriction to classic Hodgkin lymphoma eliminated 11 cases of nodular lymphocyte predominance subtype. In addition, 1 case was excluded because of inconclusive serologic results. Complete data including EBV tumor status were thus available for 128 cases.

We attempted to match each case with 3 controls by age ($\pm$ 1 year), sex, race, and ethnic group (white non-Hispanic, black non-Hispanic, Hispanic, Asian, or other), and date of serum collection ($\pm$ 30 days). To achieve risk set sampling, whereby controls were selected at random from the group at risk to become a case in a given time period,[19] controls were required to be on active duty on the matched case's diagnosis date.

Serologic specimens and assays

The DoD Serum Repository is currently the world's largest serum repository, with $>$ 40 million specimens collected from $\sim$ 9 million people since 1985.[18] The repository was originally established for the purpose of storing excess sera from the Armed Forces HIV testing program. Since that time, the repository has expanded to include specimens from service members deployed overseas.[18] Specimens are stored at -30°C in walk-in freezers. All samples that are available for study in the DoD Serum Repository are HIV-antibody negative.

Study serum specimens were shipped frozen to Virolab Inc. All serologic tests were performed in a blinded fashion. Case and control specimens were randomly mixed within each batch of serum samples sent for testing. In addition, matched case-control sets were analyzed together in the same run of any given assay. Immunoglobulin G (IgG) antibodies to EBV VCA and EA complex (diffuse [EA-D] and restricted [EA-R]) were measured using immunofluorescence.[5] If a person had detectable titers to both EA-D and EA-R, the higher value was classified as the titer against the EA complex. IgG antibodies against the EBNA complex and 2 of its subcomponents, EBNA-1 and EBNA-2, were determined using anticomplement immunofluorescence.[4] The EBV$^-$ BJAB cell line was used as a control.[4,5]

The value for a titer was considered as the highest of serial 2-fold dilutions to register a positive reaction. Cases and controls were considered as EBV-seropositive if the IgG antibody titer to VCA was $\geq$ 1:20 or if the IgG antibody titer to EBNA-1 was $\geq$ 1:5 and antibodies to BJAB were not detected. An elevated titer was defined a priori as the upper 15% (or nearest cut-off) of the distribution of each antibody among controls. The minimal values of elevated titers were as follows: anti-VCA: $\geq$ 1:2560, anti-EA complex: $\geq$ 1:40, anti-EBNA complex $\geq$ 1:1280, anti–EBNA-1: $\geq$ 1:1280, and anti–EBNA-2: $\geq$ 1:80. The anti–EBNA-1/anti–EBNA-2 ratio also was examined, with a ratio of $\leq$ 1.0 considered as serologic evidence of a deregulated immune response in other clinical settings.[4-9]

Masked repeat samples (5% of specimens) were included to enable monitoring of within-batch reproducibility of antibody titers. The within-batch coefficients of variation from the blind quality control samples were 9.9% for anti-VCA, 38.9% for anti-EA complex, 12.4% for anti-EBNA complex, 16.8% for anti–EBNA-1, and 37.5% for anti–EBNA-2.

Histopathologic classification and detection of EBV in tumor tissue

The 2001 World Health Organization Classification of Hematopoietic and Lymphoid Tumors was used to classify the following subtypes of classic Hodgkin lymphoma: nodular sclerosis, mixed cellularity, lymphocyte depletion, and classic Hodgkin lymphoma, not further classified.[20] Tumors were evaluated for EBV using in situ hybridization to detect viral transcripts (EBERs)[21,22] and immunohistochemistry to detect latent membrane protein-1 in Hodgkin and Reed-Sternberg cells and their variants.[15] Cases scored positive for either EBERs or latent membrane protein-1 were considered as EBV$^+$ Hodgkin lymphoma. Cases were defined as EBV$^-$ Hodgkin lymphoma if both assays were negative or if information from only 1 assay was available and the result was negative.[23]

Statistical analysis

Statistical analysis was performed using SAS Version 8.01 (SAS Institute). *P* values were 2-tailed, and tests of statistical significance were based on an α level of .05. Among EBV seropositives, median antibody titers were compared using the Wilcoxon rank-sum test. Conditional logistic regression for matched data was used to compute the relative risk (RR), estimated by the odds ratio (OR), of Hodgkin lymphoma associated with the prevalence of detectable antibody or an elevated EBV antibody level, the 95% confidence interval (CI) around the relative risk, and the corresponding *P* value estimated using the Wald χ^2 test. For case-case comparisons to examine the heterogeneity of the serologic response to EBV between the 2 case groups, unconditional logistic regression was performed controlling for age (18-22 years, 23-26 years, $>$ 26 years); sex; race (white, nonwhite); and year of serum collection (1988-1991, 1992-1994, 1995-1998).[24] We controlled for potential confounding by histology (nodular sclerosis, mixed cellularity, other histology) in multivariate models.

The research protocol was approved by the institutional review boards of the Walter Reed Army Institute of Research, the Harvard School of Public Health, and the Johns Hopkins School of Medicine. The study was conducted in accordance with the Declaration of Helsinki.

Results

Of the 128 cases of Hodgkin lymphoma in this analysis, 40 (31.3%) had EBV detected in tumor cells and 88 (68.7%) did not. The median age at diagnosis was 24 years (range, 18-49 years); $\sim$ 90% of the study subjects were male and 78% were white (Table 1). In general, EBV$^+$ Hodgkin lymphoma cases were more likely than EBV$^-$ Hodgkin lymphoma cases to be younger and white and less likely to be of nodular sclerosis subtype.

Based on either the earliest serum specimen or the specimen drawn at least 2 years after the first serum sample for persons who were initially seronegative, 100% of the EBV$^+$ Hodgkin lymphoma cases, 89.8% of the EBV$^-$ cases, and 92.4% of the controls were EBV-seropositive before diagnosis. As shown in Table 2, all EBV$^+$ Hodgkin lymphoma cases, but not all EBV$^-$ cases or controls, had detectable IgG antibody titers to VCA or EBNA complex, indicating an established EBV infection. The prevalence of detectable antibody titers was significantly higher in EBV$^+$ Hodgkin lymphoma cases compared with their matched controls for anti-EA complex (RR = 2.5; 95% CI, 1.1-5.8) and anti–

Table 1. Selected characteristics of EBV$^+$ Hodgkin lymphoma cases, EBV$^-$ Hodgkin lymphoma cases and all controls

Characteristic	EBV$^+$ HL cases (N = 40), N (%)	EBV$^-$ HL cases (N = 88), N (%)	Controls (N = 368), N (%)
Age at diagnosis, y			
18-21	15 (37.5)	18 (20.5)	97 (26.4)
22-25	10 (25.0)	42 (47.6)	144 (39.1)
26-29	5 (12.5)	10 (11.4)	49 (13.3)
≥ 30	10 (25.0)	18 (20.5)	78 (21.2)
Median	24.1	24.7	24.3
Interquartile range	20.9-29.7	22.5-27.3	21.9-27.9
Sex			
Male	37 (92.5)	75 (85.2)	327 (88.9)
Female	3 (7.5)	13 (14.8)	41 (11.1)
Race			
White	32 (80.0)	65 (73.9)	286 (77.7)
Black	6 (15.0)	7 (7.9)	37 (10.1)
Other	2 (5.0)	16 (18.2)	45 (12.2)
Military rank at entry			
Enlisted	33 (82.5)	75 (85.2)	332 (90.2)
Officer	4 (10.0)	8 (9.1)	36 (9.8)
Missing	3 (7.5)	5 (5.7)	0 (0.0)
Year of first serum collection			
1988-1991	23 (57.5)	52 (59.1)	225 (61.2)
1992-1994	12 (30.0)	29 (33.0)	112 (30.4)
1995-1998	5 (12.5)	7 (7.9)	31 (8.4)
Interval between first serum collection and diagnosis date, mo			
Median	22.3	33.8	32.3
Interquartile range	11.0-37.4	15.0-49.9	14.1-51.4
Histologic subtype			
Nodular sclerosis	19 (47.5)	77 (87.5)	NA
Mixed cellularity	16 (40.0)	5 (5.7)	NA
Lymphocyte depletion	1 (2.5)	0 (0.0)	NA
Classic HL, not further classified	4 (10.0)	6 (6.8)	NA

HL indicates Hodgkin lymphoma; and NA, not applicable.

EBNA-2 (RR = 2.5; 95% CI, 1.1-5.8), and the anti–EBNA-1/anti–EBNA-2 antibody ratio was significantly more likely to be ≤ 1.0 (RR = 4.7; 95% CI, 1.6-13.8). No significant differences, however, were noted in the prevalence of any of the antibody titers comparing EBV$^-$ Hodgkin lymphoma cases with their matched controls. The prevalence of an anti–EBNA-1/anti–EBNA-2 antibody ratio of ≤ 1.0 was 28.2% in EBV$^+$ Hodgkin lymphoma cases and markedly lower in both EBV$^-$ Hodgkin lymphoma cases (3.8%) and controls (9.2%).

We also examined median antibody titers in the 2 case groups and in controls (Table 2). Median titers were significantly higher among EBV$^+$ Hodgkin lymphoma cases than among their matched controls for IgG against VCA ($P = .01$) and EA complex ($P = .03$). The EBV$^+$ cases also had a significantly lower median anti–EBNA-

Table 2. Association of prevalence of EBV antibody titer and median antibody titer with risk of EBV$^+$ and EBV$^-$ Hodgkin lymphoma

Characteristic	EBV$^+$ HL cases (N = 40)	EBV$^+$ HL cases vs matched controls RR (95% CI) or *P**	EBV$^-$ HL cases (N = 88)	EBV$^-$ HL cases vs matched controls RR (95% CI) or *P**	EBV$^+$ HL cases vs EBV$^-$ HL cases RR (95% CI)† or *P**	All controls (N = 368)
Detectable antibody titer,‡ %						
VCA IgG	100.0	0.06	89.8	0.63 (0.3-1.5)	0.09	92.4
EA complex	40.0	2.5 (1.1-5.8)	30.1	1.2 (0.7-2.1)	1.2 (0.5-3.1)	25.8
EBNA complex	100.0	0.06	89.8	0.60 (0.3-1.4)	0.09	92.7
EBNA-1	90.0	1.1 (0.3-3.6)	87.5	0.66 (0.3-1.5)	1.8 (0.5-7.2)	90.0
EBNA-2	80.0	2.5 (1.1-5.8)	60.2	0.69 (0.4-1.2)	2.6 (0.9-7.6)	66.0
EBNA-1/EBNA-2 ratio ≤ 1.0	28.2	4.7 (1.6-13.8)	3.8	0.35 (0.1-1.3)	14.0 (2.7-72.5)	9.2
Median antibody titer						
VCA IgG	1:1280	0.01	1:640	0.48	0.03	1:640
EA complex	< 1:5	0.03	< 1:5	0.47	0.16	< 1:5
EBNA complex	1:160	0.89	1:320	0.80	0.27	1:320
EBNA-1	1:80	0.20	1:320	0.17	0.02	1:160
EBNA-2	1:20	0.11	1:10	0.58	0.10	1:10
EBNA-1/EBNA-2 ratio	8	0.03	32	0.15	0.004	16

HL indicates Hodgkin lymphoma; RR, relative risk; and CI, confidence interval.

*Fisher exact test.

†Unconditional logistic regression adjusted for age, sex, race, year of serum collection, and histology.

‡Based on either the first serum specimen or the specimen 2 years after the first, if the first was EBV-seronegative.

Table 3. Association of elevated EBV antibody titer and low antibody ratio of EBNA-1/EBNA-2 with Hodgkin lymphoma risk overall and EBV$^+$ and EBV$^-$ Hodgkin lymphoma

Antibody	Referent	All cases vs matched controls (N = 128 sets)	EBV$^+$ HL cases vs matched controls (N = 40 sets)	EBV$^-$ HL cases vs matched controls (N = 88 sets)	EBV$^+$ HL cases vs EBV$^-$ HL cases (N = 40 vs 88)*
VCA (IgG ≥ 1:2560)†	< 1:2560	2.1 (1.2-3.7)	3.1 (1.1-8.7)	1.7 (0.9-3.5)	1.4 (0.5-3.8)
EA complex (≥ 1: 40)†	< 1:40	1.7 (1.0-2.9)	2.4 (0.9-6.3)	1.4 (0.7-2.8)	1.5 (0.5-4.2)
EBNA complex (≥ 1:1280)†	< 1:1280	1.2 (0.7-2.1)	1.7 (0.6-4.7)	1.0 (0.5-2.0)	1.7 (0.5-6.3)
EBNA-1 (≥ 1:1280)†	< 1:1280	1.2 (0.6-2.3)	1.4 (0.4-4.6)	1.1 (0.5-2.5)	0.93 (0.2-4.8)
EBNA-2 (≥ 1: 80)†	< 1:80	1.6 (0.9-2.8)	1.3 (0.5-3.3)	1.8 (0.9-3.5)	0.81 (0.3-2.5)
EBNA-1/EBNA-2 ratio (≤ 1.0)	> 1.0	1.4 (0.7-2.7)	4.7 (1.6-13.8)	0.4 (0.1-1.3)	14.0 (2.7-72.5)

Data are RR (95% CI).
*Unconditional logistic regression adjusted for age, sex, race, year of serum collection, and histology.
†Elevated titer levels.

1/anti–EBNA-2 antibody ratio ($P = .03$). No significant differences in any of the median antibody titer levels were found for EBV$^-$ Hodgkin lymphoma cases compared with their matched controls. Overall, median titers were strikingly similar in EBV$^-$ Hodgkin lymphoma cases compared with all controls, and both groups had lower median levels than EBV$^+$ cases. The notable exception was for anti–EBNA-1, for which EBV$^-$ cases had the highest median titer of the 3 groups. In case-case comparisons, EBV$^+$ cases also had a significantly lower median anti–EBNA-1/anti–EBNA-2 antibody ratio compared with EBV$^-$ cases ($P = .004$).

In analyses of the 40 EBV$^+$ Hodgkin lymphoma cases and matched controls, a statistically significant increased risk was associated with elevated anti-VCA IgG (RR = 3.1; 95% CI, 1.1-8.7) and the low anti–EBNA-1/anti–EBNA-2 ratio (RR, 4.7; 95% CI, 1.6-13.8). Analyses of 88 EBV$^-$ Hodgkin lymphoma cases relative to their matched controls revealed a different pattern, as null associations were found with all antibody titers and with a low antibody ratio (Table 3).

Case-case comparisons were performed in addition to EBV-stratified case-control comparisons to determine whether the risk associated with an altered EBV antibody profile differed between EBV$^+$ and EBV$^-$ Hodgkin lymphoma cases, controlling for age, sex, race, year of serum collection, and histology. The only antibody titer with a case-case RR that differed statistically from the null was a low anti–EBNA-1/anti–EBNA-2 antibody ratio (RR = 14.0; 95% CI, 2.7-72.5 for EBV positivity; Table 3). This association remained the same with additional mutual control of antibodies to the other antigens (RR = 14.2; 95% CI, 2.7-74.2 for EBV positivity; Table 4).

We performed exploratory analyses using serial samples (N = 11 EBV$^+$ cases with ≥ 2 serum samples) and stratified by time between serum collection and diagnosis, but results were statistically unstable (data not shown).

Discussion

Our findings, the first to be based on prediagnosis serology, indicate that the EBV antibody profile differs by the detection of EBV in tumor cells of Hodgkin lymphoma cases, with EBV$^+$ cases displaying a distinctly atypical pattern compared with either EBV$^-$ cases or controls. Among the EBV$^+$ Hodgkin lymphoma cases compared with matched controls, we found evidence of elevated IgG antibody titers against VCA and EA complex and a higher prevalence of a low anti–EBNA-1/anti–EBNA-2 antibody ratio. The low antibody ratio was the indicator most strongly associated with EBV positivity, controlling for the other EBV antibody titer levels. EBV$^-$ cases, however, had a serologic pattern that closely resembled that of controls without Hodgkin lymphoma.

The determinants of humoral immunity to viral antigens remain incompletely understood. EBV infection, however, is lifelong, and patterns of viral gene expression differ among cell and tissue types, as well as among EBV-associated tumors. VCA and EA are antigens that are expressed in lytic but not in latent EBV infection. Thus, one possible interpretation of higher IgG titers to these antigens in patients with EBV-associated Hodgkin lymphoma is that these patients have increased exposure to lytic antigens, perhaps as a result of increased viral lytic replication (in normal or neoplastic tissues). Similarly, low anti–EBNA-1/anti–EBNA-2 antibody ratios may reflect the presence of a cell population in vivo expressing EBNA-1 and EBNA-2. EBV-immortalized lymphocytes in vitro express both of these antigens; however, in vivo EBNA-2 expression in peripheral blood lymphocytes has not been documented except in the setting of recent primary infection or in immunocompromised patients. EBV-associated Hodgkin lymphoma expresses EBNA-1 but not EBNA-2, so it seems unlikely

Table 4. Multivariate analyses of elevated EBV antibody titer and low antibody ratio of EBNA-1/EBNA-2 and risk of EBV$^+$ vs EBV$^-$ Hodgkin lymphoma

Antibody	Referent	EBV$^+$ HL cases vs EBV$^-$ HL cases*	EBV$^+$ HL cases vs EBV$^-$ HL cases†
VCA (IgG ≥ 1:2560)‡	< 1:2560	1.3 (0.4-4.2)	0.95 (0.3-3.3)
EA complex (≥ 1: 40)‡	< 1:40	1.6 (0.5-5.1)	1.2 (0.4-4.2)
EBNA-1 (≥ 1:1280)‡	< 1:1280	1.1 (0.2-6.5)	NA
EBNA-2 (≥ 1: 80)‡	< 1:80	0.61 (0.2-2.4)	NA
EBNA-1/EBNA-2 ratio (≤ 1.0)	> 1.0	NA	14.2 (2.7-74.2)

Data are RR (95% CI).
NA indicates not applicable.
*Unconditional logistic regression with mutual control for antibodies to VCA, EA complex, EBNA-1, EBNA-2, and adjusted for age, sex, race, year of serum collection, and histology.
†Unconditional logistic regression with mutual control for antibodies to VCA, EA complex, EBNA-1/EBNA-2 ratio ≤ 1.0, and adjusted for age, sex, race, year of serum collection, and histology.
‡Elevated titer levels.

that Hodgkin tumor cells per se account for this humoral response. Given that these tumors do express EBNA-1, however, it is notable that EBV$^+$ Hodgkin lymphoma cases were more likely than EBV$^-$ cases to have detectable and elevated EBV antibody titers, except against the latent nuclear antigen EBNA-1.

In a population-based case-control study of Hodgkin lymphoma with blood specimens obtained after diagnosis, a marginally statistically significant OR of 1.9 (95% CI, 0.9-4.0) was found with a low anti–EBNA-1/anti–EBNA-2 antibody ratio, comparing EBV$^+$ with EBV$^-$ cases.[25] In another case-case comparison of 27 EBV$^+$ Hodgkin lymphoma cases relative to 80 EBV$^-$ cases, Enblad et al found that the prevalence of detectable titers and elevated titers for anti-VCA IgG and anti–EBNA-2 were associated with EBV positivity,[26] although none of the associations were statistically significant.

The strengths of our study stem from our ability to assess subcomponents of the EBNA complex in prediagnosis serology in EBV$^+$ and EBV$^-$ Hodgkin lymphoma cases. A limitation is the relatively small sample size, which precluded a statistically robust analysis stratified by time to diagnosis or based on serial samples. Analyses stratified by histologic subtypes of classic Hodgkin lymphoma rather than tumor EBV status also were not robust as there were only 21 cases of mixed cellularity subtype. Moreover, because our study population was composed mainly of young men, these results may not be generalizable to other age groups or to women. Of note, 31% of our Hodgkin lymphoma cases were EBV$^+$, a prevalence similar to that found in other comparable populations.[12]

The hypothesis that EBV may be involved in all of Hodgkin lymphoma and is somehow lost in a subset of cases was suggested by reports of the loss of EBV in Burkitt lymphoma cell cultures.[27,28] Sixbey has proposed that immune selection may lead to the loss of the EBV episome in Burkitt lymphoma that is seen in economically developed populations, implicating a hit-and-run role for the virus.[29] It also has been speculated that the hit-and-run model may explain the pathogenesis of EBV$^-$ Hodgkin lymphoma.[30,31] Our findings show that people who will develop EBV$^-$ Hodgkin lymphoma do not differ from control populations in their EBV serology. Although this does not exclude the possibility that the virus might be lost from tumor cells, our results do not provide any support for this hypothesis.

Our findings, however, do suggest a distinctive immune response to EBV infection in patients destined to develop EBV$^+$ Hodgkin lymphoma. There are a number of other reports in the literature that may bear on the immune response to EBV in patients with EBV$^+$ Hodgkin lymphoma. It has been established that the increased risk associated with symptomatic primary EBV infection (manifested as infectious mononucleosis) is found only in EBV$^+$, but not EBV$^-$ Hodgkin lymphoma.[32] Genetic variations in the HLA class I region appear to be specifically associated with EBV$^+$ Hodgkin lymphoma.[33-35] Increased copy number of EBV-DNA in blood of patients with EBV$^+$ Hodgkin lymphoma has been documented.[36] And, provocatively, a decreased frequency of T cells expressing IL-15 receptor α after symptomatic infectious mononucleosis has been reported,[37] although a subsequent investigation called the observation into question.[38] Our novel finding that a prediagnosis abnormal serologic response to EBV latent antigens is restricted to EBV$^+$ Hodgkin lymphoma may provide further clues to the character of the immune dysregulation associated with the pathogenesis of this disease.

Acknowledgments

The authors thank Dr Patrick Kelley who originated this study, Drs Donna Spiegelman and Chung-Cheng Hsieh for expert statistical support, Ms Janice Gary and Ms Ruth Cho for administrative support, and the staff who retrieved and sent tissue blocks from the following military medical treatment facilities: National Naval Medical Center, Bethesda, MD; Naval Medical Center, San Diego, CA; Walter Reed Army Medical Center, Washington, DC; Naval Medical Center, Portsmouth, VA; Dwight D. Eisenhower Army Medical Center, Fort Gordon, GA; Tripler Army Medical Center, Honolulu, HI; Brooke Army Medical Center, Fort Sam Houston, TX; William Beaumont Army Medical Center, El Paso, TX; Madigan Army Medical Center, Fort Lewis, WA; 59th Medical Wing, Wilford Hall Medical Center, Lackland Air Force Base, TX; Wright-Patterson Medical Center, Wright-Patterson Air Force Base, OH; Womack Army Medical Center, Fort Bragg, NC; 81st Medical Group, Keesler Medical Center, Keesler Air Force Base, MS; 60th Medical Group, David Grant Medical Center, Travis Air Force Base, CA; Darnall Army Community Hospital, Fort Hood, TX; Naval Hospital Camp Pendleton, Camp Pendleton, CA; Ireland Army Community Hospital, Fort Knox, KY; 121st General Hospital, Seoul, Korea; Martin Army Community Hospital, Fort Benning, GA; Naval Hospital Camp Lejeune, Camp Lejeune, NC; Reynolds Army Community Hospital, Fort Sill, OK; Winn Army Community Hospital, Fort Stewart, GA; Naval Hospital Great Lakes, Great Lakes, IL; Munson Army Health Center, Fort Leavenworth, KS; Naval Hospital Charleston, North Charleston, SC; 375th Medical Group, Scott Air Force Base, IL; 97th Medical Group, Eglin Air Force Base, FL; Blanchfield Army Community Hospital, Fort Campbell, KY; Naval Hospital Yokosuka, Yokosuka, Japan; Naval Hospital Jacksonville, Jacksonville, FL; 10th Medical Group, US Air Force Academy Hospital, US Air Force Academy, CO; General Leonard Wood Army Community Hospital, Fort Leonard Wood, MO; 72nd Medical Group, Tinker Air Force Base, OK; McDonald Army Community Hospital, Fort Eustis, VA; 90th Medical Group, F. E. Warren Air Force Base, WY; Naval Hospital Bremerton, Bremerton, WA; AK; Naval Hospital Pensacola, Pensacola, FL; Evans Army Community Hospital, Fort Carson, CO; Naval Hospital Long Beach, Long Beach, CA; Dewitt Army Community Hospital, Fort Belvoir, VA; Naval Ambulatory Care Center Groton, Groton, CT; Bassett Army Community Hospital, Fort Wainwright, AK; 55th Medical Group, Ehrling Bergquist Hospital, Offutt Air Force Base, NE; 355th Medical Group, Davis-Monthan Air Force Base, AZ; Bayne-Jones Army Community Hospital, Fort Polk, LA; Patterson Army Health Clinic, Fort Monmouth, NJ; US Air Force Regional Hospital, March Air Force Base, CA; 82nd Medical Group, Sheppard Air Force Base, TX; 6th Medical Group, MacDill Air Force Base, FL; Royal Air Force Lakenheath Hospital, Lakenheath Air Force Base, England; 28th Medical Group, Ellsworth Air Force Base, SD; Wuerzburg Army Hospital, Wuerzburg, Germany; Naval Hospital, Guam; Raymond W. Bliss Army Health Center, Fort Huachuca, AZ; Kenner Army Health Clinic, Fort Lee, VA; Naval Ambulatory Care Center, Newport, RI; Naval Hospital, Naples, Italy; Branch Medical Clinic Naval Support Activity Mid-South, Millington, TN; Naval Hospital Lemoore, Lemoore, CA; Moncrief Army Community Hospital, Fort Jackson, SC; 99th Medical Group, Mike O'Callaghan Federal Hospital, Nellis Air Force Base, NV; and Kimbrough Ambulatory Care Center, Fort George G. Meade, MD.

This work was supported by the National Cancer Institute (grants PO1 CA69266 and P50 CA96888).

The views expressed are those of the authors and should not be construed to represent the positions of the Department of the Army or Department of Defense.

Authorship

Contribution: L.I.L. and N.E.M. designed the research, secured funding, contributed to the analysis of results, and wrote the paper; E.T.C. performed the statistical analysis, contributed to the analysis of the results, and wrote the paper; R.F.A. designed the research, secured funding, oversaw the laboratory assessment of tumor EBV status, contributed to the analysis of results, and wrote the paper; E.T.L. oversaw the laboratory assessment of serum biomarkers and contributed to the analysis of the results; M.V.R. designed the research and oversaw the selection and retrieval of serum samples; R.B.M., M.B., and E.G.W. reviewed tumor tissue to confirm diagnosis and to determine EBV positivity; and S.L.A. contributed to the selection of eligible cases.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Lynn I. Levin, Department of Epidemiology, Preventive Medicine Program, Walter Reed Army Institute of Research, 503 Robert Grant Ave, Silver Spring, MD 20910-7500, e-mail: lynn.levin@us.army.mil.

References

1. Evans AS, Gutensohn (Mueller) NM. A population-based case-control study of EBV and other viral antibodies among persons with Hodgkin's disease and their siblings. *Int J Cancer.* 1984;34: 149-157.
2. Mueller N, Evans A, Harris NL, et al. Hodgkin's disease and Epstein-Barr virus: altered antibody pattern before diagnosis. *N Engl J Med.* 1989; 320(11):689-695.
3. Lehtinen T, Lumio J, Dillner J, et al. Increased risk of malignant lymphoma indicated by elevated Epstein-Barr virus antibodies: a prospective study. *Cancer Causes Control.* 1993;4(3):187-193.
4. Lennette ET, Rymo L, Yadav M, et al. Disease-related differences in antibody patterns against EBV-encoded nuclear antigens EBNA 1, EBNA 2 and EBNA 6. *Eur J Cancer.* 1993;29A(11):1584-1589.
5. Henle W, Henle G, Andersson J, et al. Antibody responses to Epstein-Barr virus-determined nuclear antigen (EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection. *Proc Natl Acad Sci U S A.* 1987;84(2):570-574.
6. Miller G, Grogan E, Rowe D, et al. Selective lack of antibody to a component of EB nuclear antigen in patients with chronic active Epstein-Barr virus infection. *J Infect Dis.* 1987;156(1):26-35.
7. Youinou P, Buisson M, Berthelot JM, et al. Anti-Epstein-Barr virus-nuclear antigen-1, -2A and -2B antibodies in rheumatoid arthritis patients and their relatives. *Autoimmunity.* 1992;13(3):225-231.
8. Winkelspecht B, Grasser F, Pees HW, Mueller-Lantzsch N. Anti-EBNA1/anti-EBNA2 ratio decreases significantly in patients with progression of HIV infection. *Arch Virol.* 1996;141(5):857-864.
9. Winkelspecht B, Mueller-Lantzsch N, Kohler H. Serological evidence for reactivation of EBV infection due to uraemic immunodeficiency. *Nephrol Dial Transplant.* 1997;12(10):2099-2104.
10. Mueller NE, Lennette ET, Dupnik K, Birmann BM. Antibody titers against EBNA1 and EBNA2 in relation to Hodgkin lymphoma and history of infectious mononucleosis. *Int J Cancer.* 2012;130(12): 2886-2891.
11. Weiss LM, Movahed LA, Warnke RA, Sklar J. Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease. *N Engl J Med.* 1989;320(8):502-506.
12. Glaser SL, Lin RJ, Stewart SL, et al. Epstein-Barr virus-associated Hodgkin's disease: epidemiologic characteristics in international data. *Int J Cancer.* 1997;70(4):375-382.
13. Herbst H, Dallenbach F, Hummel M, et al. Epstein-Barr virus latent membrane protein expression in Hodgkin and Reed-Sternberg cells. *Proc Natl Acad Sci U S A.* 1991;88(11):4766-4770.
14. Niedobitek G, Kremmer E, Herbst H, et al. Immunohistochemical detection of the Epstein-Barr virus-encoded latent membrane protein 2A in Hodgkin's disease and infectious mononucleosis. *Blood.* 1997;90(4):1664-1672.
15. Pallesen G, Hamilton-Dutoit SJ, Rowe M, Young LS. Expression of Epstein-Barr virus latent gene products in tumour cells of Hodgkin's disease. *Lancet.* 1991;337(8737):320-322.
16. Mueller NE. Epstein-Barr virus and Hodgkin's disease: an epidemiological paradox. *Epstein-Barr Virus Rep.* 1997;4:1-2.
17. Gallagher A, Perry J, Shield L, Freeland J, MacKenzie J, Jarrett RF. Viruses and Hodgkin disease: no evidence of novel herpesviruses in non-EBV-associated lesions. *Int J Cancer.* 2002; 101(3):259-264.
18. Rubertone MV, Brundage JF. The Defense Medical Surveillance System and the Department of Defense Serum Repository: glimpses of the future of public health surveillance. *Am J Public Health.* 2002;92(12):1900-1904.
19. Rothman K, Greenland S. *Modern Epidemiology.* Philadelphia, PA: Lippincott-Raven; 1998.
20. Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. *WHO Classification of Tumours: Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues.* Lyon, France: International Agency for Research on Cancer Press; 2001.
21. Wu TC, Mann RB, Charache P, et al. Detection of EBV gene expression in Reed-Sternberg cells of Hodgkin's disease. *Int J Cancer.* 1990;46(5):801-804.
22. Ambinder RF, Mann RB. Epstein-Barr-encoded RNA in situ hybridization: diagnostic applications. *Hum Pathol.* 1994;25(6):602-605.
23. Gulley ML, Glaser SL, Craig FE, et al. Guidelines for interpreting EBER in situ hybridization and LMP1 immunohistochemical tests for detecting Epstein-Barr virus in Hodgkin lymphoma. *Am J Clin Pathol.* 2002;117(2):259-267.
24. Begg CB, Zhang ZF. Statistical analysis of molecular epidemiology studies employing case-series. *Cancer Epidemiol Biomarkers Prev.* 1994; 3(2):173-175.
25. Chang ET, Zheng T, Lennette ET, et al. Heterogeneity of risk factors and antibody profiles in Epstein-Barr virus genome-positive and -negative Hodgkin lymphoma. *J Infect Dis.* 2004;189(12): 2271-2281.
26. Enblad G, Sandvej K, Lennette E, et al. Lack of correlation between EBV serology and presence of EBV in the Hodgkin and Reed-Sternberg cells of patients with Hodgkin's disease. *Int J Cancer.* 1997;72(3):394-397.
27. Srinivas SK, Sample JT, Sixbey JW. Spontaneous loss of viral episomes accompanying Epstein-Barr virus reactivation in a Burkitt's lymphoma cell line. *J Infect Dis.* 1998;177(6):1705-1709.
28. Razzouk BI, Srinivas S, Sample CE, Singh V, Sixbey JW. Epstein-Barr virus DNA recombination and loss in sporadic Burkitt's lymphoma. *J Infect Dis.* 1996;173(3):529-535.
29. Sixbey JW. Epstein-Barr virus DNA loss from tumor cells and the geography of Burkitt's lymphoma. *Epstein-Barr Virus Rep.* 2000;7:37-40.
30. Meij P, Vervoort MB, Bloemena E, et al. Antibody responses to Epstein-Barr virus-encoded latent membrane protein-1 (LMP1) and expression of LMP1 in juvenile Hodgkin's disease. *J Med Virol.* 2002;68(3):370-377.
31. Ambinder RF. Gammaherpesviruses and "Hit-and-Run" oncogenesis. *Am J Pathol.* 2000; 156(1):1-3.
32. Hjalgrim H, Askling J, Rostgaard K, et al. Characteristics of Hodgkin's lymphoma after infectious mononucleosis. *N Engl J Med.* 2003;349(14): 1324-1332.
33. Diepstra A, Niens M, Vellenga E, et al. Association with HLA class I in Epstein-Barr-virus-positive and with HLA class III in Epstein-Barr-virus-negative Hodgkin's lymphoma. *Lancet.* 2005; 365(9478):2216-2224.
34. Niens M, Jarrett RF, Hepkema B, et al. HLA-A*02 is associated with a reduced risk and HLA-A*01 with an increased risk of developing EBV+ Hodgkin lymphoma. *Blood.* 2007;110(9):3310-3315.
35. Urayama KY, Jarrett RF, Hjalgrim H, et al. Genome-wide association study of classical Hodgkin lymphoma and Epstein-Barr virus status-defined subgroups. *J Natl Cancer Inst.* 2012;104(3):240-253.
36. Hohaus S, Santangelo R, Giachelia M, et al. The viral load of Epstein-Barr virus (EBV) DNA in peripheral blood predicts for biological and clinical characteristics in Hodgkin lymphoma. *Clin Cancer Res.* 2011;17(9):2885-2892.
37. Sauce D, Larsen M, Curnow SJ, et al. EBV-associated mononucleosis leads to long-term global deficit in T-cell responsiveness to IL-15. *Blood.* 2006;108(1):11-18.
38. Giron-Michel J, Menard F, Negrini S, Devocelle A, Azzarone B, Besson C. EBV-associated mononucleosis does not induce long-term global deficit in T-cell responsiveness to IL-15. *Blood.* 2009; 113(19):4541-4547.