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Serum IgA to Epstein-Barr virus Early Antigen-Diffuse identifies Hodgkin's Lymphoma

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Abstract

Hodgkin's lymphoma is associated with immune dysregulation. Immune impairment often results in aberrant immune responses and lytic reactivation of ubiquitous Herpesviruses such as Epstein-Barr virus (EBV) in mucosal tissues. Accordingly, the specificity of IgA to EBV early-lytic antigens, which are important for reactivation, was evaluated to determine Hodgkin's lymphoma-specific sero-reactive patterns. Sera from 42 previously described patients with Hodgkin's lymphoma were compared to sera from 17 patients with infectious mononucleosis (IM), another EBV-related condition that often presents in a similar manner; and to sera from 15 healthy EBV-seropositive subjects. Flow cytometry analysis demonstrated that like IM sera, most Hodgkin's lymphoma sera contained IgA that labeled cells expressing EBV early-lytic antigens whereas healthy EBV-seropositive sera did not. Further evaluation to distinguish Hodgkin's lymphoma from IM showed that IgA in most Hodgkin's lymphoma, irrespective of the presence of EBV in primary tumors, detected only modified forms of EBV lytic Early Antigen-Diffuse (EA-D) while IM sera detected the un-modified form as well, further supporting the presence of immune dysregulation in Hodgkin's lymphoma patients. This IgA pattern distinguished Hodgkin's

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lymphoma from IM sera with a sensitivity of 92.9%, specificity 100%, positive predictive value 100%, and negative predictive value 85%. Our findings lay the groundwork for additional scientific and clinical investigation, particularly into the potential for developing Hodgkin's lymphoma -associated diagnostic and prognostic biomarkers.

Keywords

Hodgkin's lymphoma; Epstein-Barr virus; immune dysregulation; biomarker; lytic antigens

Introduction

Epstein-Barr virus (EBV), a human Herpesvirus, persists in B lymphocytes of most healthy humans. Up to 30% of Hodgkin's lymphoma in the western hemisphere is associated with EBV [Ambinder, 2007]. However, irrespective of the presence of EBV in tumors, Hodgkin's lymphoma is associated with immune dysregulation [Landgren et al., 2006]. Such loss of immune control is associated with repeated reactivation of Herpesviruses and production of infectious virus particles [Fields et al., 2007]. Immune dysregulation in Hodgkin's lymphoma may also drive unique serum IgA profiles to reactivated Herpesviruses such as EBV compared to infectious mononucleosis (IM). IM results from first-time infection with EBV and can clinically resemble Hodgkin's lymphoma.

In an earlier study, a flow cytometry-based assay was used to demonstrate transient expression of serum IgA specific for EBV early-lytic antigens in patients with IM but not in healthy EBV-seropositive individuals [Bhaduri-McIntosh et al., 2007]. Others have demonstrated serum IgA directed against lytic EBV antigens in Hodgkin's lymphoma [Chang et al., 2004; Mueller et al., 1989] and nasopharyngeal carcinoma [Li et al., 2010]. This indication of EBV lytic reactivation during Hodgkin's lymphoma, likely in the oropharyngeal mucosal compartment, suggested that sera from Hodgkin's lymphoma patients may contain IgA antibodies to early-lytic antigens. Early-lytic antigens are essential for viral DNA replication, which is central to productive reactivation. The present study demonstrates that sera from a set of Hodgkin's lymphoma patients, irrespective of detectable EBV in tumors, contain IgA that recognizes EBV lytic antigens. Like patients with IM, sera from Hodgkin's lymphoma patients also contain IgA to early-lytic antigens including Early Antigen-Diffuse (EA-D) but unlike IM, IgA in Hodgkin's lymphoma sera almost exclusively detect modified forms of EA-D, distinguishing Hodgkin's lymphoma from IM sera.

Methods

Serum samples

Serum samples from 42 age- and gender-matched patients with Hodgkin's lymphoma [Chang et al., 2004], 17 IM [Bhaduri-McIntosh et al., 2007; Bhaduri-McIntosh and Miller, 2006] (including two additional patients in the present study recruited from the Clinical Virology Laboratory at Yale-New Haven Hospital), and 15 healthy EBV-positive controls [Bhaduri-McIntosh et al., 2007; Bhaduri-McIntosh and Miller, 2006] were obtained after written informed consent via IRB-approved protocols at Yale and Harvard Universities. All

Hodgkin's lymphoma patients had serologic evidence of past infection with EBV determined in clinical laboratories. All IM patients were adolescents and their sera were collected within 48 hours of serologic confirmation.

Detection of serum IgA specific for EBV lytic antigens by flow cytometry

Induction of lytic replication and detection of EBV lytic antigens by flow cytometry were performed as described previously [Bhaduri-McIntosh et al., 2007; Bhaduri-McIntosh and Miller, 2006]. Briefly, HH514-16 cells (EBV-positive Burkitt lymphoma cell line) were induced to express all kinetic classes of EBV lytic proteins by treatment with sodium butyrate (NaB). Alternatively, cells were induced to express only EBV early lytic proteins by treatment with NaB and phosphonoacetic acid (PAA). Following permeabilization of target cells and incubation with study sera, serum IgA with specificity to EBV lytic antigens was detected with a validated biotinconjugated anti-human IgA antibody (BD Pharmingen, San Jose, California) followed by an Avidin-Cyochrome secondary antibody (BD Pharmingen, San Jose, California). To gate on IgA-bound lytically infected cells, labeled cells were compared to similarly-treated cells incubated with reference healthy EBV-seronegative sera (or without human serum) followed by the biotin-Avidin sandwich.

Detection of serum IgA specific for EBV lytic antigens by immunoblotting

HKB5/5 cells were transfected with constructs expressing EBNA1 (*BKRF1*), ZEBRA (*BZLF1*), LR2 (*BLRF2*), sVCA (*BFRF3*), LMP1, RTA (*BRLF1*), or EA-D (*BMRF1*). Total extracts from HKB5/5 cells transfected with a construct expressing an EBV gene of interest or empty vector pFLAG-cytomegalovirus 2 (CMV-FLAG) were resolved using SDS-PAGE. The blots were probed with serum samples from Hodgkin's lymphoma or IM patients followed by detection of bound IgA antibodies with horseradish peroxidase-conjugated anti-human IgA (Jackson ImmunoResearch Laboratories, West Grove, PA) antibody using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

Statistics

Statistical analyses were performed using unpaired t test.

Results

Hodgkin's lymphoma sera contain IgA to lytic proteins of EBV

Serum IgA to EBV lytic antigens is transiently detectable in primary EBV infection during which robust viral replication occurs [Bhaduri-McIntosh et al., 2007]. Immune dysregulation, as is observed in Hodgkin's lymphoma patients, is also associated with active viral replication [Fields et al., 2007]. Since viral replication likely occurs in the oropharyngeal compartment, the presence of IgA to lytic proteins of EBV, expressed during viral reactivation, was measured in Hodgkin's lymphoma sera. Using a validated flow cytometry based IgA assay [Bhaduri-McIntosh et al., 2007] in which lytically infected cells are enumerated following their detection by IgA antibodies, 42 sera from Hodgkin's lymphoma patients [Chang et al., 2004], half with EBV in tumors, were tested (Table 1). Irrespective of EBV detection in tumors, all Hodgkin's lymphoma patients had serological

evidence of past infection with EBV. No EBV-negative Hodgkin's lymphoma patients were included in this study as they represent a minority of Hodgkin's lymphoma patients.

Expression of EBV lytic genes in HH514-16 cells, a EBV-positive Burkitt lymphoma cell line, was induced with NaB. Detection by IgA of greater than 1% cells over background detection using a reference EBV-seronegative serum was considered positive. Serum IgA from 90.5% of EBV-positive Hodgkin's lymphoma and 100% of EBV-negative Hodgkin's lymphoma patients recognized 1.09% to 52.8% of NaB-treated cells, whereas none of the healthy sera recognized target cells (Figure 1 and Table 2). Expression of only early-lytic antigens was induced by treatment with NaB and PAA. PAA blocks EBV DNA replication and thus expression of late-lytic viral genes. IgA from 71.4% of EBV-positive Hodgkin's lymphoma and 90.5% of EBV-negative Hodgkin's lymphoma patients recognized 1.45% to 48.5% of NaB+PAA-treated cells while only 20% of healthy sera recognized small fractions of target cells (Figure 1 and Table 2). The average percent total- and early-lytic antigen-expressing cells detected by Hodgkin's lymphoma sera, irrespective of EBV status in tumors, was significantly higher than those detected by healthy sera. Detection of lytic antigens by IgA in Hodgkin's lymphoma sera was similar over three age tertiles (data not shown). Thus, similar to IM patients [Bhaduri-McIntosh et al., 2007], presence of serum IgA to total- and early-lytic antigens distinguished Hodgkin's lymphoma from healthy sera. Percent lytic cells detected by IgA in Hodgkin's lymphoma was in a range similar to that detected by IgA in IM [Bhaduri-McIntosh et al., 2007].

IgA in Hodgkin's lymphoma sera uniformly detect EBV early lytic antigen EA-D

To determine the specific EBV antigens detected by Hodgkin's lymphoma sera, six Hodgkin's lymphoma samples were screened for reactivity to recombinant EBV proteins by Western blot. Serum IgA in three EBV-positive and three EBV-negative Hodgkin's lymphoma patients reacted heterogeneously with EBNA1 (*BKRF1*), ZEBRA (*BZLF1*), LR2 (*BLRF2*), and sVCA (*BFRF3*) (Table 3). LMP1 and RTA (*BRLF1*) were not detected, consistent with the known poor antigenicity of these two proteins. In contrast, all six sera, irrespective of tumor EBV status, contained IgA to EA-D (*BMRF1*). Subsequent Western blot analysis of all forty-two Hodgkin's lymphoma sera demonstrated uniform detection of EA-D by serum IgA (data not shown). Thus, regardless of the presence of EBV in tumor tissue, IgA in Hodgkin's lymphoma sera detected EBV EA-D.

Serum IgA to modified forms of EA-D distinguishes Hodgkin's lymphoma from IM

Since the flow cytometry-based assay detected IgA to lytic antigens in both Hodgkin's lymphoma and IM sera and was therefore unable to distinguish between Hodgkin's lymphoma and IM, the possibility of differential detection of specific early lytic antigens by IgA in Hodgkin's lymphoma and IM sera was investigated. Given that phosphorylation results in detection of three forms of EA-D by immunoblot [Tsurumi, 1993], here referred to as bands A, B, and C (Fig.2A-E), we asked if Hodgkin's lymphoma and IM sera recognized distinct forms of EA-D. IgA in all Hodgkin's lymphoma sera recognized band B, only 3 recognized band C, while recognition of band A was heterogeneous (Fig.2A-C, F, G). Most IM sera recognized bands B and C (Fig.2A, D, H). With a positive test for Hodgkin's lymphoma defined as presence of band B but absence of band C, and a negative test defined

as any other pattern, the sensitivity of the test for distinguishing Hodgkin's lymphoma from IM was 92.9% and specificity was 100% (Table 4). The positive predictive value was 100% and negative predictive value was 85%. Thus, IgA reactivity, directed preferentially against modified forms of a viral lytic protein EA-D, marked Hodgkin's lymphoma sera but not IM sera.

Discussion

Using a combination of highly sensitive assays the data presented here demonstrate that most Hodgkin's lymphoma patients, when examined within a year of diagnosis, demonstrate serum IgA to EBV lytic antigens, including antigens belonging to the class of early lytic proteins. Increased titers of anti-EBV antibodies have been associated with malignant and non-malignant inflammatory diseases such as combination of highly sensitive assays the data presented here demonstrate that most Hodgkin's lymphoma [Ambinder, 2007; Brousset et al., 1993; Chang et al., 2004; Mueller et al., 1989; Pallesen et al., 1991; Tselis and Jenson, 2006], nasopharyngeal carcinoma [Billaud et al., 1989; Li et al., 2010; Tselis and Jenson, 2006], IM [Bhaduri-McIntosh et al., 2007; Tselis and Jenson, 2006], systemic lupus erythematosus [Mohammad et al., 2007], and multiple sclerosis [Munger et al., 2011] when compared with healthy controls. The presence of IgA antibodies in Hodgkin's lymphoma sera reactive to primarily modified forms of EA-D is the first demonstration of an immunoreactive pattern able to distinguish between two EBV-related diseases. This characteristic, which is independent of the presence of EBV in tumors of nearly all Hodgkin's lymphoma sera but not IM sera, lends further support to the existence of immune dysregulation during Hodgkin's lymphoma [Chang et al., 2004; Lamprecht et al., 2008; Marshall et al., 2004]. Although patients with NPC frequently have IgA to EA-D, whether such antibodies are directed to modified forms of EA-D is not known [Tabuchi et al., 2011].

Consistent with earlier studies [Weitzman et al., 1977], the average concentration of total serum IgA for all samples was not statistically different between study groups and all were within published reference ranges (data not shown). Therefore, the different patterns of IgA reactivity in Hodgkin's lymphoma and IM sera represent alterations of EBV-directed immunoreactivity rather than a concentration effect. Increased titers of IgG specific for VCA and EBNA in pre-morbid sera have been shown to increase the likelihood for subsequent development of Hodgkin's lymphoma. This association was strongest in pre-morbid sera collected at least three years prior to diagnosis [Mueller et al., 1989]. In a more recent study using the Hodgkin's lymphoma sera examined here, higher titers of IgG and IgA specific for VCA were more strongly associated with EBV-positive Hodgkin's lymphoma, as was a lower ratio of IgG specific for EBNA1 compared with EBNA2 [Chang et al., 2004]. The unique EA-D-specific IgA pattern common to both EBV-positive and EBV-negative Hodgkin's lymphoma patients presented in this study is likely due to differences in the sensitivities of the tests used here compared to the earlier study. Notably, DFA, which is not a particularly sensitive assay, was used to detect seroreactivity to EBV antigens in the previous study. The robust performance characteristics of the Hodgkin's lymphoma IgA Western blots, irrespective of whether tumors were EBV-positive or -negative, make this an attractive candidate for development into a predictive or prognostic biomarker for Hodgkin's lymphoma. Whether this pattern of IgA reactivity to EA-D precedes development of

Hodgkin's lymphoma, persists with disease progression, disappears with remission, and whether the pattern is a feature of other EBV-related diseases, all merit investigation.

Loss of band C recognition in Hodgkin's lymphoma may represent selection of B cell clones restricted to phosphorylated EA-D or predominant expression of phosphorylated forms of EA-D. EA-D can be phosphorylated by both cellular and viral kinases [Tsurumi, 1993; Yang et al., 2008]. Hodgkin's lymphoma is known to be associated with increased activity of cellular kinases [Tselis and Jenson, 2006]; these alterations of kinase activity may result in rapid phosphorylation of all available EA-D molecules. EA-D has been previously shown to activate the gastrin promoter, a mechanism by which EBV may potentiate the development of gastric cancer [Holley-Guthrie et al., 2005]. Alterations in patterns of EA-D expression may similarly influence pathogenesis of Hodgkin's lymphoma.

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Butyrate-treated (total lytic-expressing) cells

Butyrate+PAA-treated (early-lytic expressing) cells

Figure 1. IgA antibodies in sera from Hodgkin's lymphoma patients detect butyrate- and butyrate+PAA-treated HH514-16 cells

Representative FACS dotplot analyses of butyrate-treated (A, B, and C) and butyrate+PAA-treated (D, E, and F) cells recognized by serum IgA in a patient with EBV⁺ - Hodgkin's lymphoma (A and D), a patient with EBV⁻ - Hodgkin's lymphoma (B and E), and a healthy EBV-seropositive individual (C and F). Numbers in the upper right hand quadrants indicate percentage of cells recognized by IgA antibodies in serum samples. G and H, Comparison of the fraction of butyrate-treated (G) or butyrate+PAA-treated (H) HH514-16 cells recognized by IgA antibodies present in sera from patients with EBV⁺ - Hodgkin's lymphoma, EBV⁻ - Hodgkin's lymphoma, and healthy EBV-seropositive individuals. Horizontal bars represent mean values.

F				G				H			
EBV+ lymphoma serum	EA-D Band			EBV- lymphoma serum	EA-D Band			IM Serum	EA-D Band		
	A	B	C		A	B	C		A	B	C
1	+	+	-	22	-	+	-	1	+	+	+
2	-	+	-	23	+	+	-	2	+	+	+
3	-	+	-	24	+	+	-	3	-	+	+
4	-	+	-	25	-	+	-	4	-	-	-
5	-	+	-	26	+	+	-	5	+	+	+
6	+	+	+	27	+	+	-	6	+	+	+
7	+	+	-	28	-	+	-	7	+	+	+
8	-	+	-	29	-	+	-	8	-	-	-
9	+	+	-	30	+	+	-	9	+	+	+
10	+	+	-	31	-	+	-	10	+	+	+
11	-	+	-	32	+	+	-	11	+	+	+
12	+	+	-	33	+	+	-	12	+	+	+
13	-	+	-	34	-	+	-	13	+	+	+
14	+	+	+	35	+	+	-	14	+	+	+
15	-	+	-	36	-	+	-	15	+	+	+
16	+	+	-	37	+	+	-	16	+	+	+
17	+	+	-	38	+	+	-	17	+	+	+
18	+	+	-	39	-	+	-				
19	+	+	-	40	+	+	-				
20	+	+	-	41	+	+	+				
21	+	+	-	42	+	+	-				

Figure 2. Detection of a specific pattern of EA-D by IgA antibodies in sera distinguishes Hodgkin's lymphoma from IM

(A-E) Immunoblot detection of EA-D by serum IgA. Total extracts from EBV-negative HKB5/5 cells transfected with a construct expressing EA-D or empty vector pFLAG-cytomegalovirus 2 (CMV-FLAG) were subjected to IgA immunoblotting using Hodgkin's lymphoma or IM sera. Letters A, B, and C at approximately 50kDa indicate hyper-phosphorylated (A), hypo-phosphorylated (B), and un-phosphorylated (C) forms of EA-D. Blots representative of different patterns are shown. A summary of detection of bands A, B, and C of EA-D by IgA antibodies present in sera of patients with EBV⁺ - Hodgkin's lymphoma, EBV⁻ - Hodgkin's lymphoma, and IM is shown in F-H. Sera that scored positive by this test are in shaded boxes.

Table 1

Characteristics of Hodgkin's lymphoma patients

EBV Status of Hodgkin's lymphoma		Positive	Negative
		21 (50%)	21 (50%)
Age (yrs)			
Mean		47.6 ± 16.6	48.1 ± 17
Median		44	45
Males		14 (66.7%)	14 (66.7%)
Females		7 (33.3%)	7 (33.3%)
Race			
White		15 (71.4%)	15 (71.4%)
Black		1 (4.8%)	0.0%
Unknown		5 (23.8%)	6 (28.6%)
Interval between diagnosis and blood draw (days)			
Mean		291.3 ± 148.4	397.9 ± 295.8
Median		319	379
Tumor Classification			
Mixed Cellularity		38.1%	19.0%
Nodular Sclerosis		33.3%	52.4%
Lymphocyte-rich classical HD		9.5%	0.0%
Lymphocyte-depleted		0.0%	4.8%
Interfollicular variant		4.8%	4.8%
Unclassifiable		14.3%	19.0%
Disease stage			
Stage I/II		12 (57.1%)	15 (71.4%)
Stage III/IV		7 (33.3%)	4 (19%)
Not staged		2 (9.5%)	2 (9.5%)
IgG to EBV antigens (median titer by DFA)			
Viral Capsid Antigen		2560	1280
EBNA		320	320
Patients with antibodies to EA-D (by DFA)			
IgG		8 (38.1%)	2 (9.5%)

Table 2

Fraction of total lytic (butyrate-treated) cells and early lytic (butyrate + PAA-treated) cells recognized by IgA antibodies in sera from patients with EBV⁺- Hodgkin's lymphoma, EBV⁻ - Hodgkin's lymphoma, and healthy EBV-seropositive individuals

Butyrate-treated cells						Butyrate+PAA-treated cells					
EBV ⁺ -lymphoma serum	Percent cells recognized	EBV ⁻ -lymphoma serum	Percent cells recognized	Healthy EBV-seropos serum	Percent cells recognized	EBV ⁺ -lymphoma serum	Percent cells recognized	EBV ⁻ -lymphoma serum	Percent cells recognized	Healthy EBV-seropos serum	Percent cells recognized
1	41.57	22	32.43	1	20.6	1	20.6	22	35.78	1	0.15
2	29.79	23	1.82	2	18.6	2	18.6	23	0.86	2	0.7
3	42.64	24	26.65	3	0	3	0	24	12.7	3	0.2
4	28.13	25	2.77	4	17.6	4	17.6	25	8.93	4	0.5
5	28.76	26	6.42	5	38.64	5	38.64	26	16.25	5	0.8
6	52.83	27	2.4	6	48.51	6	48.51	27	0.83	6	1.9
7	4.64	28	32.36	7	0	7	0	28	23.04	7	0.12
8	34.8	29	35.14	8	28.94	8	28.94	29	39.95	8	0.4
9	12.78	30	26.24	9	23.79	9	23.79	30	20.2	9	0.05
10	21.07	31	2.52	10	26.58	10	26.58	31	0.24	10	0.2
11	44.02	32	4.3	11	47.99	11	47.99	32	0.4	11	2
12	41.83	33	1.25	12	38.24	12	38.24	33	0.75	12	0.02
13	2.28	34	2.62	13	0.01	13	0.01	34	2.3	13	0.04
14	37.97	35	9.32	14	42.28	14	42.28	35	6.65	14	1.2
15	34.51	36	18.03	15	0.08	15	0.08	36	15.47	15	0.1
16	22.56	37	47.7		12.19	16	12.19	37	45.33		
17	5.26	38	1.17		0.36	17	0.36	38	1.99		
18	0.12	39	23.91		1.45	18	1.45	39	19.27		
19	1.09	40	11.35		0.93	19	0.93	40	2.76		
20	0.48	41	36.51		0.08	20	0.08	41	34.72		
21	9.39	42	4.27		12.84	21	12.84	42	2.02		
Mean	23.64	Mean	15.67	Mean	18.08	Mean	18.08	Mean	13.83	Mean	0.56

Note: Data are percentages. PAA, phosphonoacetic acid

Table 3

Recognition by immunoblot analyses of EBV latent (EBNA1 and LMP1), early lytic (RTA, ZEBRA, and EA-D), and late lytic (LR2 and sVCA) antigens by IgA antibodies in sera from patients with Hodgkin's lymphoma.

Lymphoma Serum #	Latent		Lytic				
	EBNA1	LMP1	RTA	ZEBRA	EA-D	LR2	sVCA
4 (EBV ⁺)	+	-	-	-	+	-	+
8 (EBV ⁺)	-	-	-	+	+	+	+
11 (EBV ⁺)	-	-	-	+	+	+	+
22 (EBV ⁻)	+	-	-	-	+	-	+
28 (EBV ⁻)	+	-	-	+	+	-	-
29 (EBV ⁻)	+	-	-	+	+	-	+

Table 4

Performance characteristics of the diagnostic EA-D banding pattern for identifying Hodgkin's lymphoma (presence of band B but absence of band C)

	Patients with lymphoma	Patients without lymphoma	Total
Positive Test	39	0	39
Negative Test	3	17	20
Total	42	17	