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EBNA1 Sequences in Argentinean Pediatric Acute and Latent Epstein–Barr Virus Infection Reflect Circulation of Novel South American Variants

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Epstein–Barr virus (EBV) is related to the development of lymphomas and is also the etiological agent for infectious mononucleosis (IM). Sequence variation of the EBNA1 gene, consistently expressed in all EBV-positive cells, has been widely studied. Based on the amino acid at codon 487 five major EBNA1 variants have been described, two closely related prototypic variants (P-ala and P-thr) and three variant sequences (V-leu, V-val, and V-pro). Sub-variants were then further classified based on mutations other than the originally described. While several studies proposed associations with tumors and/or anatomical compartments, others argued in favor of a geographical distribution of these variants. In the present study, EBNA1 variants in 11 pediatric patients with IM and 19 pediatric EBV lymphomas from Argentina were compared as representatives of benign and malignant infection in children, respectively. A 3-month follow-up study of EBNA1 variants in peripheral blood cells and in oral secretions of patients with IM was performed. A new V-ala variant which includes five V-ala sub-variants and three new V-leu sub-variants was described. These data favor the geographical association hypothesis since no evidence for a preferential compartment distribution of EBNA1 variants and sub-variants was found. This is the first study to characterize EBNA1 variants in pediatric patients with infection mononucleosis worldwide. ***J. Med. Virol. 82:1730–1738, 2010.***

KEY WORDS: EBNA1 sequence variations; infectious mononucleosis; pediatric patients; anatomical compartments; pediatric lymphoma

INTRODUCTION

Epstein–Barr virus (EBV) is an oncogenic virus in the *Lymphocryptovirus* genus which is a member of the *Gammaherpesvirinae* subfamily from the large *Herpesviridae* family. EBV is one of the most successful viruses, infecting over 90% of human adult population and establishing lifelong persistence in circulating B memory cells with minimal or no viral gene expression. Lytic replication, spontaneously activated in only a small percentage of latently infected B cells, is essential for virus propagation among individuals. Infective particles are transmitted through saliva and replicative infection is established within resting B cells in the oropharynx [Cohen, 2000]. EBV infection at an early age is usually mild or asymptomatic; but when the infection occurs during adolescence or early adult life frequently causes an acute, self-limited, infectious mononucleosis (IM). In developing countries, like Argentina, seroconversion usually occurs during early childhood, soon after the disappearance of maternal antibodies, and is almost universal by the age of 6 years [Chabay et al., 1999; Chan et al., 2001 and references herein]. Pediatric EBV infection can occasionally develop in an IM, characterized by pharyngitis, lymphadenopathy, headache, fever,

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hepatosplenomegaly, and malaise [Thorley-Lawson and Gross, 2004; Vetsika and Callan, 2004].

EBV infection is also associated with the development of lymphoid and epithelial cell malignancies such as Burkitt's lymphoma (BL) and Hodgkin's lymphoma (HL) and nasopharyngeal carcinoma (NPC). Given that the list of EBV-related malignancies continues to increase, the World Health Organization classified EBV as a carcinogenic agent in 1997 [Delecluse et al., 2007]. Infection in EBV-associated tumors is latent and viral antigens expressed in these malignancies are limited to a small set of genes, which define different latency programs specific to each tumor type.

Even though EBV infects most of the global population, only a small proportion of infected individuals develop EBV-associated malignancies. Both viral and host factors may play a role in determining the clinical outcome by modulation of viral gene expression in infected cells and the ability of the host's immune system to recognize cells that express growth-promoting proteins and its aptitude to eliminate them [Klein et al., 2007].

Only EBV-encoded nuclear antigen 1 (EBNA1), a 641 amino acid protein, is essential for the persistence and replication of the viral genome in latently infected cells through sequence specific binding to its replication origin, OriP [Young and Rickinson, 2004]. EBNA1 physically interacts with DNA through its core domain (amino acids 461–504) in its carboxy-terminal region (C-ter) [Ambinder et al., 1991]. As the only EBV protein consistently expressed in all types of latency programs, EBNA1 may play a critical role in the onset, progression, and maintenance of these malignancies. Tumor-promoting activity was further supported by Wilson et al. [1996] who described how two independent lines of transgenic mice expressing EBNA1 succumbed to B-cell lymphoma. On the other hand, Humme et al. [2003] proved that EBNA1 is not essential to establish tumor growth in mice but significantly promotes the efficiency of this process. Furthermore, EBNA1 was also shown to have anti-apoptotic properties in BL causing inhibition of p53-dependant apoptosis. However, the oncogenic role of EBNA1 to date is still not fully understood [Kennedy et al., 2003].

Sequence variations in the C-ter region of EBNA1 were first described by Snudden et al. [1995] in cells derived from NPC. Soon after, sequence analyses enabled the classification of EBNA1 variants into five main groups, two prototype sequences (P-ala, present in B95.8 cell line, and P-thr) and three variant sequences (V-leu, V-val, and V-pro) named according to the amino acid (aa) present at codon 487 [Bhatia et al., 1996]. These variants were then further classified into sub-variants according to mutations other than codon 487. A possible tumor association was proposed by Bhatia et al. [1996] since they described V-leu variants in BL samples from America and Africa but failed to detect it in healthy carrier's samples from those same geographic regions. Gutierrez et al. [1997] reinforced this theory when reported that P-ala and P-thr were rarely identified in malignant samples but were usually detected in peripheral blood lymphocytes (PBLs) and oral secretions (OS) from European and North American healthy carriers. V-val showed preferential compartment distribution since it could be detected in OS from adult healthy donors but not in PBLs [Gutierrez et al., 1997]. Later, several studies suggested the possibility for a geographical association of EBNA1 variants when malignant and control samples from other world regions where studied. Chen et al. [1998] described V-val variant in gastric carcinoma and in EBV-positive reactive follicular hyperplasia from Japan while P-ala, P-thr, and V-leu were described in North America. A study by Chang et al. [1999] compared variants present in HL with those present in reactive lymphoid tissues from Brazil and USA. They described P-thr, P-ala, and V-leu in these two conditions from both regions, and similarly, P-ala was rarely found in malignancy. In contrast, MacKenzie et al. [1999] studied BL and HL from UK and Brazil and found different variants. Further evidence for a geographic distribution of EBNA1 variants was provided by Habeshaw et al. [1999] and Sandvej et al. [2000] who studied malignant and normal samples from different regions and concluded that different geographic areas have different spectra of EBNA1 variants within their host population and no differences are observed between tumors and the healthy population of the same area. Therefore, V-val variants appear to be predominant in China, V-leu in America and Africa, while P-thr and P-ala in European Caucasians [Chen et al., 1998, 1999; Chang et al., 1999; Habeshaw et al., 1999; MacKenzie et al., 1999; Fassone et al., 2000; Greiner et al., 2000; Sandvej et al., 2000; Wang et al., 2002, 2010]. At the same time, some further evidence for the tumor-associated variant hypothesis was also provided by Wang et al. [2003] who identified V-val in NPC, but when studying NPC with matched PBLs, V-val was found in tumor tissue but mixed populations of V-val and P-ala were observed in PBLs. On the other hand, only P-ala was detected in PBLs from normal donors from that region. Further work by Zhang et al. [2004] and Do et al. [2008] compared EBNA1 variants in NPC and matching PBLs and throat washings (TW). They concluded that V-val is the predominant variant in Asia but also stated that it occurs preferentially in NPC since it was found as the unique variant in NPC samples while their matched PBLs and TW, as well as PBL and TW from healthy individuals presented V-val, P-ala, P-thr, or a mixture of V-val and P-ala. Additionally, Do et al. [2008] found that a V-val isolate from Asia had increased transcriptional activity when compared with the prototype P-ala variant. In line with previous findings, Wang et al. [2010] recently described V-val in the majority of the Chinese population studied. Some of these contributions were recently reviewed by Chang et al. [2009].

Most of these studies were conducted in North American, European, and Asiatic patients, but little work has been done in South American patients. This prevents definitive conclusions regarding geographic and disease patterns to be made. In addition, none of

them were conducted in pediatric EBV acute infection. The objective of this work is to characterize EBNA1 gene variants circulating in this geographic region in the different anatomical compartments from pediatric patients with EBV benign acute infection and its convalescence as well as in malignant EBV-associated lymphomas.

METHODS

Patients and Samples

This study was conducted on 30 pediatric patients, 11 with IM and a median age of 4 years (range, 2–9 years), 55% male; and 19 EBV-positive lymphomas (13 HL and 6 non-Hodgkin lymphoma (NHL)), with a median age of 7 years (range, 3–15 years), 74% male. Hospital's ethic committee reviewed and approved this study which is in accordance with the human experimentation guidelines of our institution. A written informed consent was obtained from all patients' parents.

Lymph node biopsies were collected at the time of lymphoma diagnosis before therapy. EBV presence was assessed on formalin-fixed, paraffin-embedded tissue sections by in situ hybridization (ISH) for EBERs according to the manufacturer's instructions (NCL-EBV-K, Novocastra Laboratories Ltd, New Castle, UK). Those cases with positive nuclear staining in tumor cells without staining in infiltrating lymphocytes were included.

A peripheral blood (6 ml) and OS samples were obtained from patients with presumptive acute IM at the time of diagnosis (D0), at 1 month (D30) and at 3 months from diagnosis (D90). Follow-up samples were not available for all patients due to patients lost to follow-up (Table I). IM was identified on clinical grounds

TABLE I. Viral Type and EBNA1 Variants Distribution

Patient	Sample	Disease	EBV type	EBNA1 variant		
				D0	D30	D90
P1*	PBLs	IM	1	P-thr″	P-thr″	P-thr″/P-ala
	Throat swab		1	P-thr″	P-thr″	P-thr″
P2	PBLs	IM	1	V-leu Ag	NA	NA
	Throat swab		1	V-leu Ag	NA	NA
P3	PBLs	IM	1	P-thr′	NA	NA
	Throat swab		1	P-thr′	NA	NA
P4	PBLs	IM	1	P-thr′	P-thr′	P-thr′
	Throat swab		1	P-thr′	P-thr′	P-thr′
P5	PBLs	IM	1	V-leu Ag	V-leu Ag	V-leu Ag
	Throat swab		1	V-leu Ag	V-leu Ag	V-leu Ag
P6*	PBLs	IM	1	V-leu Ag	V-leu Ag	P-thr′
	Throat swab		1	V-leu Ag/P-thr′	P-thr′	P-thr′
P7	PBLs	IM	2	V-leu	V-leu	V-leu
	Throat swab		2	V-leu	V-leu	V-leu
P8	PBLs	IM	1	V-leu Ag	V-leu Ag	V-leu Ag
	Buccal swab		1	V-leu Ag	V-leu Ag	V-leu Ag
P9*	PBLs	IM	1	P-ala/V-ala-i	NA	NA
	Buccal swab		1	V-ala-i	NA	NA
P10	PBLs	IM	1	P-thr″	P-thr″	P-thr″
	Throat swab		1	P-thr″	P-thr″	P-thr″
P11	PBLs	IM	1	P-thr′	P-thr′	P-thr′
	Throat swab		1	P-thr′	P-thr′	P-thr′
T1	LNB	NHL	2	V-leu Ag		
T2	LNB	NHL	2	P-thr′		
T3	LNB	NHL	1	V-ala-ii		
T4	LNB	HL	1	V-ala-iii		
T5	LNB	HL	2	V-leu-vii		
T6	LNB	HL	2	V-leu		
T7	LNB	HL	1	P-thr′		
T8	LNB	HL	1	V-ala-iv		
T9	LNB	HL	2	V-leu Ag-i		
T10	LNB	HL	1	V-leu Ag-ii		
T11	LNB	HL	1	P-thr′		
T12	LNB	HL	1	V-leu Ag		
T13	LNB	HL	1	V-leu Ag		
T14	LNB	HL	1	V-leu Ag		
T15	LNB	NHL	1	V-ala-ii		
T16	LNB	NHL	2	V-ala-v		
T17	LNB	NHL	1	V-leu-viii		
T18	LNB	HL	1	P-ala′		
T19	LNB	HL	1	P-thr′		

PBLs, peripheral blood lymphocytes; IM, infectious mononucleosis; NA, not available; *, co-infection; LNB, lymph node biopsy; NHL, non-Hodgkin lymphoma; HL, Hodgkin's lymphoma; D0, diagnosis day; D30, 30 days from diagnosis; D90, 90 days from diagnosis; V-leu Ag is the sequence present in Ag 876 cell line. GenBank accession numbers for the sequences reported in this work are GU475384–GU475456.

and confirmed by indirect immunofluorescent assay (IFA), and those patients with IgM, with or without IgG antibodies against virus capsid antigen (VCA) at (D0) were included in the study. As a differential diagnosis for other mononucleosis-like conditions, IgM anti-*Cytomegalovirus* and anti-*Toxoplasma gondii* were assessed by ELISA. All patients were negative for both of them. Average time between onset of IM symptoms and diagnosis (D0) was 8 days (range, 4–12 days).

DNA Extraction

EDTA-PBLs were separated from whole blood (6 ml) with Ficoll-Paque plus (GE Healthcare, Uppsala, Sweden). Genomic DNA was extracted from PBLs, OS samples, and lymph node biopsies using QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) following manufacturer's instructions.

EBV Typing

EBV type was determined from 300 ng of genomic DNA from each sample by PCR with specific primers for EBNA3C (5′-agaaggggagcgtgtgttgt-3′ (99939–99958 of EBV genome, GenBank accession number V01555) and 5′-ggctcgtttttgacgtcggc-3′ (100072–100091)) which yield an amplification product of 153 bp for EBV1 and a product of 246 bp for EBV2, as previously described [Sample et al., 1990]. PCR products were separated by electrophoresis in 2% agarose gel stained with ethidium bromide and visualized with UV light.

EBNA1 Carboxy-Terminal Region Analysis

EBNA1 C-ter region was amplified by nested PCR from each sample. Primers used in the first round were 5′-agatggtgagcctgacgtg-3′ (109218–109236) and 5′-gctgaggttttgaaggatgc-3′ (109663–109682). PCR was performed in 50 μl using 200 ng of genomic DNA, 1 mM $MgSO_4$, 1× amplification buffer, 1× enhancer solution, 0.2 mM dNTPs, 0.6 μM of each primer, and 0.375 U Platinum pfx DNA polymerase, with proof-reading activity (Invitrogen, Carlsbad, CA). One microliters of this product was re-amplified with primers 5′-cccgcagatgacccaggaga-3′ (109261–109280) and 5′-tttggaatggcccctggaccc-3′ (109570–109590). PCR conditions consisted of 5 min at 95°C, 35 cycles of 45 sec at 94°C, 45 sec at 60°C, 45 sec at 68°C followed by a 7-min extension at 68°C. Both steps were performed under the same conditions. PCR products were separated by electrophoresis in 2% agarose gel stained with ethidium bromide and purified with QIAEXII gel extraction kit (Qiagen GmbH) according to manufacturer's instructions. These purified PCR products were directly sequenced using Big Dye Terminator v3.1 kit (Applied Biosystems, Foster City, CA) in an automated Genetic Analyzer 3130xl (Applied Biosystems). At least two independent sequencing reactions were performed with the inner primers to confirm each sequence.

Sequence Analysis

Sequences were aligned and analyzed with Bioedit V7.0.1 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were compared with B95.8 reference sequence (GenBank V01555) and with other isolates from GenBank database. The most appropriate model of evolution for this 330 bp segment was inferred using Modeltest v.3.7 [Posada and Crandall, 1998], and the phylogenetic tree was calculated with the previously defined evolutionary model using PAUP* v.4.0.b10. [Swofford, 2003].

Statistical Analysis

Statistical analysis was performed using GraphPad InStat software, version 3.05 (GraphPad, San Diego, CA). For the univariate analysis, Fisher's exact test or chi-square test was used to assess the association between categorical variables. All tests were two-sided, and a P-value of <0.05 was considered statistically significant.

RESULTS

EBNA1 Variations in IM Patients

Direct sequencing (sense and antisense) of EBNA1 amplification products was performed to avoid potential selection against variants with poorer transforming capabilities when establishing lymphoblastoid cell lines. According to the terminology introduced by Bhatia et al. [1996], EBNA1 variants were classified into three major groups related to the amino acid present at position 487 (Table I). These variants were then further classified into sub-variants according to extra mutations other than codon 487. Five previously described sub-variants were found, namely P-ala, P-thr′, P-thr″, V-leu, and V-leu Ag (sequence present in Ag876 cell line) (Tables I and II). Finally, in patient P9 a new sub-variant harboring an alanine at position 487 which also contained other 11 nucleotide substitutions was found (Table II). These variations represent eight aa changes and justify its classification as a new sub-variant. Following the nomenclature of MacKenzie et al. [1999] we named this finding as a new V-ala sub-variant, V-ala-i. It was demonstrated through phylogenetic that this isolate segregated from other prototype P-ala sub-variants and clustered in a novel genetic lineage (Fig. 1).

Regarding compartmentalization, six patients (P4, P5, P7, P8, P10, and P11) presented each, at D0, the same sub-variant in their PBLs and in their OS. Furthermore, these sub-variants were sustained as the sole one in both anatomical sites during the 3 months of follow-up (Table I). Co-infection with two EBNA1 sub-variants was evidenced by the presence of multiple peaks in the chromatogram of certain samples from three patients. In PBLs of P1 and P9, P-ala sub-variant was detected in the presence of P-thr″ or V-ala-i, respectively. Co-infection in P1 was only evidenced at D90. P9 showed co-existence of EBNA1 variants in PBLs at D0 but, unfortunately, no follow-up samples were available. Finally, in P6 at D0 V-leu Ag and P-thr′ were detected in OS while only V-leu Ag was detected in PBLs. At D30, only P-thr′ could be detected in OS

TABLE II. EBNA-1 Variants in IM and Lymphoma Patients

		Codons																						
Patients	Variant	468	471	475	476	483	484	486	**487**	492	499	500	502	508	511	514	517	520	524	525	528	529	532	533
Infectious mononucleosis																								
B95.8; P1*; P9*	P-ala	CAT	CAA	ACC	CCG	GAA	GGT	AGA	**GCT**	AGT	GAC	GAA	ACT	TTC	GGA	AAG	CTT	CTA	ACT	GCC	ATT	CCA	CGT	CTT
		His	Gln	Thr	Pro	Glu	Gly	Arg	**Ala**	Ser	Asp	Glu	Thr	Phe	Gly	Lys	Leu	Leu	Thr	Ala	Ile	Pro	Arg	Leu
P9*	V-ala-i		GAA		ACG			AAA			GAG		GAT		GGA			CTC	ATT		GTT			ATT
			Glu		Thr			Lys			Glu		Asp		Gly			Leu	Ile		Val			Ile
P3; P4; P11; P6*	P-thr′				CAG				**ACT**	TGT	GAT							CTC	ATT					
					Gln				**Thr**	Cys	Asp							Leu	Ile					
P1*; P10	P-thr″		CAT		CAG				**ACT**	TGT	GAT							CTC	ATT					
			His		Gln				**Thr**	Cys	Asp							Leu	Ile					
P7	V-leu	CGT	GAA		CAG				**CTT**	TGT	GAG	GAT	AAT					CTC	ATT	GGC				
		Arg	Glu		Gln				**Leu**	Cys	Glu	Asp	Asn					Leu	Ile	Gly				
P2; P5; P6*; P8	V-leu Ag		GAA	AGC	CAG				**CTT**	TGT	GAG	GAT	AAT					CTC	ATT	GGC				
			Glu	Ser	Gln				**Leu**	Cys	Glu	Asp	Asn					Leu	Ile	Gly				
Lymphomas																								
T18	P-ala′										GAA								GTT					
											Glu								Val					
T3; T15	V-ala-ii					GAC					GAG		AAT				CTA	CTC	ATT		GTT			
						Asp					Glu		Asn				Leu	Leu	Ile		Val			
T4	V-ala-iii				CAG	GAC					GAG		AAT				CTA	CTC	ATT		GTT	CAA		
					Gln	Asp					Glu		Asn				Leu	Leu	Ile		Val	Gln		
T8	V-ala-iv					GAC	AGT				GAG		AAT	TTA				CTC						
						Asp	Ser				Glu		Asn	Leu				Leu						
T16	V-ala-v		GAA		CAG			AAA			GAG		AAT				TTA	CTC	ATT		GTT	CAA		
			Glu		Gln			Lys			Glu		Asn				Leu	Leu	Ile		Val	Gln		
T2; T7; T11; T19	P-thr′				CAG				**ACT**	TGT	GAT							CTC	ATT					
					Gln				**Thr**	Cys	Asp							Leu	Ile					
T6	V-leu	CGT	GAA		CAG				**CTT**	TGT	GAG	GAT	AAT					CTC	ATT	GGC				
		Arg	Glu		Gln				**Leu**	Cys	Glu	Asp	Asn					Leu	Ile	Gly				
T1; T12; T13; T14	V-leu Ag		GAA	AGC	CAG				**CTT**	TGT	GAG	GAT	AAT					CTC	ATT	GGC				
			Glu	Ser	Gln				**Leu**	Cys	Glu	Asp	Asn					Leu	Ile	Gly				
T9	V-leu Ag-i		GAA	AGC	CAG				**CTT**	TGT	GAG	GAT	AAT					CTC	ATT	GGC			CTT	
			Glu	Ser	Gln				**Leu**	Cys	Glu	Asp	Asn					Leu	Ile	Gly			Leu	
T10	V-leu Ag-ii		GAA	AGC	CAG				**CTT**	TGT	GAG	GAT	AAT			AGG		CTC	ATT	GGC				
			Glu	Ser	Gln				**Leu**	Cys	Glu	Asp	Asn			Arg		Leu	Ile	Gly				
T5	V-leu-vii				CAG				**CTT**	TGT	GAG	GAC	AAT					CTC	ATT					
					Gln				**Leu**	Cys	Glu	Asp	Asn					Leu	Ile					
T17	V-leu-viii		GAA					AAA	**CTT**		GAG		AAT					CTC	ATT		GTT	TCA		ATT
			Glu					Lys	**Leu**		Glu		Asn					Leu	Ile		Val	Ser		Ile

Codon 487 is highlighted in bold. *Co-infection.P-ala, P-thr′, and V-leu were described by Bhatia et al. [1996]. P-thr″ and P-ala′ were described by Fassone et al. [2000]. V-leu Ag is the sequence present in Ag876 cell, described by Dolan et al. [2006].

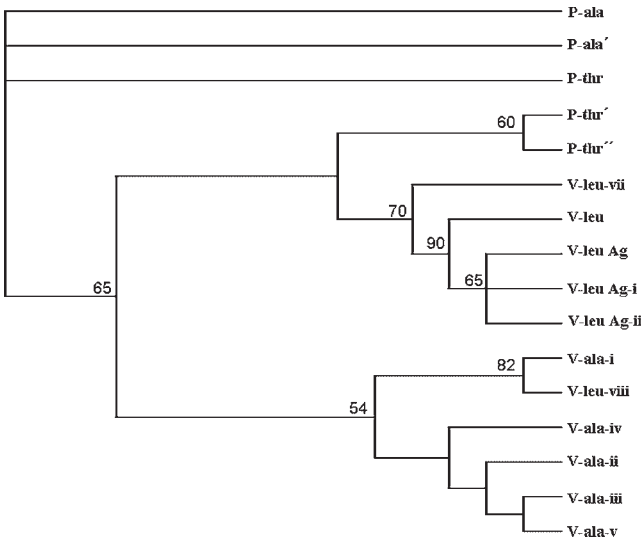

Fig. 1. Phylogenetic tree obtained from the analysis of the amplification fragments of the C-ter region of EBNA1 gene. The values obtained after 100 boostrap resamplings are indicated.

and V-leu Ag still remained as the single sub-variant in PBLs. By D90, P-thr′ was the only detectable sub-variant in both compartments (Table I).

EBNA1 Variations in EBV-Associated Lymphomas

A total of 19 pediatric EBV lymphomas were included in this study. DNA was successfully isolated and amplified from every sample. Direct sequencing analysis revealed five previously described sub-variants (P-ala′, P-thr′, V-leu, V-leu Ag) and one termed as isolate 3383 by MacKenzie et al. [1999], that we renamed V-leu Ag-i to uniformly name the sub-variants and seven new sub-variants (Table I). Sequence variations present in lymphomas T9 and T10 differed from V-leu Ag in only one nucleotide position each and clustered altogether in the phylogenetic tree (Fig. 1). The sequence present in T9 was V-leu Ag-i and the one from T10 was named V-leu Ag-ii (Table II). As described above for EBNA1 sequence found in P9, sequences present in T3, T4, T8, T15, and T16 displayed an alanine residue at codon 487 but also accumulated enough variations to segregate independently from the prototype P-ala sub-variant as demonstrated through phylogenetic analysis (Fig. 1). Sub-variant termed V-ala-ii was present in two biopsies, T3 and T15. Consistently, sub-variants harbored by T4 and T8 were termed V-ala-iii and V-ala-iv, respectively (Tables I and II). EBNA1 sub-variant present in T16, termed V-ala-v, is similar to the isolate 3478 described by MacKenzie et al. [1999] between codons 487 and 525 but, since the analyzed fragment is longer, extra variations from prototype P-ala before codon 487 and after 525 were found. Finally, sub-variants present in T5 y T17, with a leucine at position 487, were termed V-leu-vii and V-leu-viii, respectively (Tables I and II). Each lymphoma sample presented only one EBNA1 sub-variant. Unfortunately, no matching PBLs or OS were available for these cases.

EBV Typing

The majority of IM patients, 10/11 (91%), were infected with EBV1 (Table I). In all of these cases the same viral strain was detected in PBLs and OS, and no co-infection with both strains was evidenced through follow-up. Similarly, lymphoma biopsies were preferentially infected with EBV1, 13/19 (68%), and no co-infection was observed.

EBNA1 Variants Correlation

When EBNA1 sequences present in IM and in lymphomas were combined, no significant association was found between EBV type and EBNA1 variants ($P = 0.2459$) or between malignancy and EBNA1 variant ($P = 0.4141$). No association was found between any particular variant and anatomical compartment distribution ($P = 0.2904$). In a previous study with these IM patients, viral load levels were monitored in plasma during acute infection and follow-up [Lorenzetti et al., 2009]. Viral load levels showed no remarkable changes through-up the study and no EBNA1 variant associated with an increase in viral load.

DISCUSSION

Since mutations in the C-ter region of EBNA1 protein were first described, several studies have addressed sequence analysis of this gene in numerous EBV-associated malignancies where disease and compartment-associated polymorphisms were initially proposed [Bhatia et al., 1996; Gutierrez et al., 1997, 1998; Bhatia and Magrath, 1999]. Later on, the hypothesis of geographically associated polymorphisms was introduced [Chen et al., 1998; Habeshaw et al., 1999; MacKenzie et al., 1999], but still definitive conclusions regarding geographical and/or disease associations are lacking. Controversial findings were provided by different groups [Sandvej et al., 2000; Wang et al., 2003; Zhang et al., 2004; Mai et al., 2007; Do et al., 2008]. In this work, EBNA1 sub-variants present in PBLs and OS from pediatric patients with IM as well as sub-variants from EBV-pediatric lymphomas as representatives of benign and malignant EBV infection, respectively, were characterized.

Initially, V-leu sub-variants were described as tumor associated as they were found in 17/36 BL biopsies from Africa and North America but not in healthy EBV carriers' PBLs from those same regions [Bhatia et al., 1996]. Tumor association was then reinforced by Gutierrez et al. [1997] who detected this variant in 11/28 BL samples from Africa, North, and South America but not in PBLs and OS from healthy donors of that same regions. However, V-leu was also proposed to be geographically restricted to Africa and America, but not tumor associated. At first it was described in 4/17 gastric carcinomas but also in 4/11 lymphoid reactive tissues

from the USA but not from Japan [Chen et al., 1998]. Later, this same variant was detected by Habeshaw et al. [1999] in 37/55 BL biopsies and in 18/32 PBLs of healthy donors from Africa while only in 1/32 healthy donors from Europe and in no sample from Asia. MacKenzie et al. [1999] also found V-leu preferentially in 12/20 lymphomas from Brazil rather than from the United Kingdom (1/14), and Chang et al. [1999] reported this variant as one of the most common either in HL or reactive tissues from Brazil and the USA. Additionally, no V-leu variants were detected in any kind of sample from Danish or Chinese patients by Sandvej et al. [2000].

In this study we detected V-leu sub-variants both in 4/11 pediatric IM patients and in 9/19 lymphoma samples, supporting the idea that V-leu sub-variants preferentially circulate in this region and are not related to lymphoma development. On the other hand, V-leu sub-variants found in IM patients were detected in both anatomical compartments, PBL and OS. To the best of our knowledge, this is the first study to report the presence of V-leu sub-variants in OS.

In most cases, the sequences found correspond to previously described V-leu sub-variants from North and South America or differ from them in one (V-leu Ag-i and V-leu Ag-ii) or few (V-leu-vii) nucleotide positions (Table II) [Bhatia et al., 1996; Chen et al., 1998; Triantos et al., 1998; Chang et al., 1999; MacKenzie et al., 1999]. Only V-leu-viii, described in T17, differs significantly from previously described V-leu sub-variants. In fact, besides a leucine at codon 487, exclusively found in V-leu sub-variants, it harbors substitutions at codons 486, 528, and 533 which make it similar to V-ala-i sub-variant and also contains substitutions at codons 471, 499, 502, 520, and 524 which are common to both. Additionally, V-leu-viii displays codons 475, 492, and 500, which are typical of P-ala sub-variants (Table II). This complex pattern of mutations could explain the fact that even though this is a V-leu sub-variant, it segregates together with V-ala sub-variants in the phylogenetic tree (Fig. 1).

Interestingly, we detected P-ala sub-variants, only in co-infection, in 2/11 IM (P1 and P9) and as the sole variant in only 1/19 lymphoma sample (T18). Previous reports preferentially detected this sub-variant in PBLs and OS from European healthy donors and always as a minor variant in EBV-related lymphomas [Gutierrez et al., 1997; Habeshaw et al., 1999; Sandvej et al., 2000]. Instead, we detected and characterized by phylogenetic analysis five new V-ala sub-variants in five lymphoma samples (T3, T4, T8, T15, and T16) and in both, OS and PBLs samples of one IM case (P9). The occurrence of V-ala sub-variant, which accumulated enough substitutions to be considered separately from P-ala was first described by MacKenzie et al. [1999] in three lymphoma cases from Brazil. This finding adds more evidence for geographical distribution of these sub-variants which appear to circulate preferentially in South America, as they were not detected elsewhere.

P-thr sub-variants were detected in 6/11 IM and in 4/19 lymphomas. This not surprising, given that these EBNA1 sub-variants have been previously described in OS and PBLs from healthy donors in all geographic regions and also in different lymphomas such as BL, HL, NPC, and primary effusion lymphoma [Bhatia et al., 1996; Gutierrez et al., 1997; Chen et al., 1998; MacKenzie et al., 1999; Fassone et al., 2000].

On the other hand, we have not observed any V-val sub-variant which is almost exclusively described in Asia as the major one circulating, either in NPC cases as in OS and PBLs of healthy EBV carriers [Chen et al., 1998; Sandvej et al., 2000; Wang et al., 2002, 2003, 2010; Zhang et al., 2004; Do et al., 2008].

On this prospective, EBNA1 variants distribution in two different anatomical compartments during acute infection and the 3 months of follow-up were studied. In most cases (9/11) acute infection was caused by only one viral variant, which we detected in both compartments, although we cannot exclude the possibility that distinct viral variants with the same pattern of substitutions in the EBNA1 gene are responsible for these patients illness. The same variant exhibited at D0 was sustained in both compartments during the follow-up period in 8/11 cases. One patient (P1) displayed signs of co-infection in the PBLs compartment only at D90. This observation could be interpreted as a re-infection with a different variant over the convalescence period and may explain why previous reports performed in adult patients show up to 62% co-infection with different EBNA1 variants or sub-variants [Bhatia et al., 1996; Gutierrez et al., 1997; Sandvej et al., 2000; Wang et al., 2003, 2010; Zhang et al., 2004; Do et al., 2008]. The remaining two patients (P6 and P9) showed signs of co-infection since D0. P6 presented a dynamic distribution of viral variants between both anatomical compartments during the studied period. Finally, the same variant prevailed in both compartments indicating no preferential anatomical compartment distribution or cell tropism. Unfortunately, no follow-up samples were available for patient P9.

As expected, each of the lymphoma biopsies analyzed contained only one EBNA1 sub-variant. This could be due to the clonal expansion of the original infective virus, although co-infection with multiple sub-variants has been described in nasal lymphomas and other types of tumors [Gutierrez et al., 1998; Sandvej et al., 2000; Wang et al., 2003, 2010].

EBV type 1 was detected in most of IM cases 10/11 (91%) and in 13/19 (68%) patients with lymphoma. These results are in straight agreement with previous reports which describe 75.9% of adult healthy EBV carriers and up to 60% of pediatric patients with EBV lymphomas in Argentina infected with EBV1 [Chabay et al., 2004; Correa et al., 2004]. Consequently, EBV1 is the predominating viral strain circulating in Argentina. Interestingly, no sign of co-infection with both viral types was evidenced, in contrast to 6.5% of co-infection reported by Correa et al. [2004] in adult healthy carriers. This difference can be explained by the small number of

samples analyzed in this work, or alternatively, that pediatric patients are still not subjected to subsequent re-infections as adults.

Concerning viral type and EBNA1 variants, controversial results have been reported. While some studies failed to find a significant association [Bhatia et al., 1996; Chang et al., 1999; MacKenzie et al., 1999], others did. Wang et al. [2010] reported an association between EBV2 and a P-thr sub-variant, namely, P-thr-v in Northern China. Habeshaw et al. [1999] also described a significant association between EBV2 and V-leu sub-variants in Africa and P-ala in Europe. In contrast to this, we have not observed any statistical association between EBV type and EBNA1 variants from Argentina. However, P-ala and P-thr variants showed a trend towards preferentially occurring in EBV1 (7/8 P-ala and 9/10 P-thr cases were EBV1) while V-leu was more homogeneously distributed between both viral types (9/14 cases were EBV1). A larger study, including more pediatric patients from this region is needed to draw any conclusion.

Regarding the combined analysis of EBNA1 and BZLF1 variants, no pattern of association between the two gene sequences was found. This is in accordance with Chang et al. [2009] who described that the few studies which have evaluated diversity in more than a single gene suggested an incomplete linkage across different gene regions. On the other hand, much more genetic variation was observed in the C-ter region of EBNA1 compared with BZLF1 indicating that both genes are subjected to different selective pressure. In contrast to BZLF1 promoter region, EBNA1 is an immunogenic latency antigen and consequently expected to be under immunologic selective pressure. This would explain the greater genetic diversity, in the form of many variants, observed in EBNA1.

This is the first study to describe EBNA1 sequence variations in pediatric patients in a developing country where EBV has a particular epidemiology. We characterized EBNA1 sequence variations in patients with IM and EBV lymphomas and described five new V-ala and four new V-leu sub-variants. We not only provided further evidence in favor of a geographical distribution of EBNA1 variations but also proved that there is no preferential distribution of viral variants between anatomical compartments during the course of pediatric infectious mononucleosis. Furthermore, the EBNA1 sequences provided enlarge the existing data from South America, which until now was sparse and mainly restricted to Brazil and Peru.

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