The 30-bp Deletion Variant of Epstein-Barr Virus–Encoded Latent Membrane Protein-1 Prevails in Acute Infectious Mononucleosis

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To assess the frequency of malignancy-associated 30-bp deletion variants of the latent membrane protein 1 (LMP-1) in benign conditions, a comparative sequence analysis was done using samples from 20 American children with acute infectious mononucleosis and 16 Swiss children with chronic tonsillar hyperplasia. The 30-bp deletion variant (LMP-1-del) was present in 66% of patients (12/20 with infectious mononucleosis and 12/16 with tonsillar hyperplasia). Two additional patients had a 3-bp deletion and an inframe insertion of 18 nucleotides, respectively. All but 1 isolate had numerous nonsilent point mutations. These data identify a hypervariable region within the C-terminus of LMP-1, in a domain required for maximal stimulation of NF-κB activity. These data demonstrate that LMP-1-del variants are frequent in acute infectious mononucleosis and tonsillar hyperplasia and identical to those observed in Epstein-Barr virus–associated AIDS-related lymphoma.

Epstein-Barr virus (EBV) causes infectious mononucleosis (IM) and is consistently associated with nasopharyngeal carcinoma (NPC) and lymphomas, especially in the immunocompromised host. EBV infection is ubiquitous; the virus persists lifelong in latent form and can be detected in B lymphocytes and in oropharyngeal secretions [1].

Latent membrane protein-1 (LMP-1) is considered a virus oncogene due to its ability to transform rodent fibroblasts in vitro and to render them tumorigenic in nude mice. It transforms human epithelial cells and inhibits their differentiation, induces DNA synthesis, up-regulates *bcl-2* expression, and is engaged in cellular signalling processes, including NF-κB [2] and the TNF receptor family. LMP-1 is expressed in IM and in the tumor cells of most EBV-associated malignancies (reviewed in [1]).

A naturally occurring carboxy-terminal 30-bp deletion variant (LMP-1-del) is found in persons with AIDS-related lymphoma (72% [3–5]), NPC [6], clinically aggressive Hodgkin's disease, and atypical lymphoproliferations [4]. This variant oncoprotein with a distinct 30-bp deletion within the carboxy-terminal NF-κB activation domain of LMP-1 has been associated with enhanced transforming potential compared with the oncoprotein prototype in vitro [7]. To further assess in vivo the relevance of this variant, we analyzed the LMP-1 C-terminus in DNA samples from American children with acute IM and Swiss children with EBV-associated chronic tonsillar hyperplasia (TH).

Material and Methods

Samples. Twenty children with acute IM defined by positive heterophilic antibodies, peripheral blood lymphocytosis, and lymphadenopathy were identified at University of Massachusetts Medical Center between April 1988 and October 1992. Peripheral blood mononuclear cells (PBMC) were collected during the acute phase of the disease. Sixteen EBV-seropositive children (IgG against viral capsid antigen) had undergone tonsillectomy for hyperplastic tonsils at the University Children's Hospital in Zurich from December 1992 to April 1994. At this time, tonsil tissue was harvested and frozen until extraction of genomic DNA.

Polymerase chain reaction (PCR). LMP-1 genomes were identified by PCR. Three different primer sets, specific for the C-terminal domain, were used as previously described [4]. PCR conditions and amplification strategy have been described in detail [4]. Independent triplicate amplification was performed for each sample. In the samples with LMP-1-del, an additional PCR using one primer (5′-TCATAGTCATGATTCCGGCC-3′) located within the 30-bp deletion was performed, in order to detect a low copy number of additional wild type LMP-1.

DNA sequencing. Double-stranded PCR products coding for the LMP-1 C-terminus were obtained by the primer pair 5′-AGCGACTCTGCTGGAAATGAT-3′/5′-TGATTAGCTAAGGCATTCCCA-3′ (primer pair 9/11 from [4]) or CP3/MS7 (5′-TGCTCTCAAAACCTAGGCGCA-3/5′-TCATCATCTCCACCGGAACCA-3′, positions 168609–168589/168200–168220). These amplification products were purified and directly se-

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Informed consent was obtained from patients and their parents. All procedures were done in accordance with the approved institutional guidelines for the conduct of clinical research.

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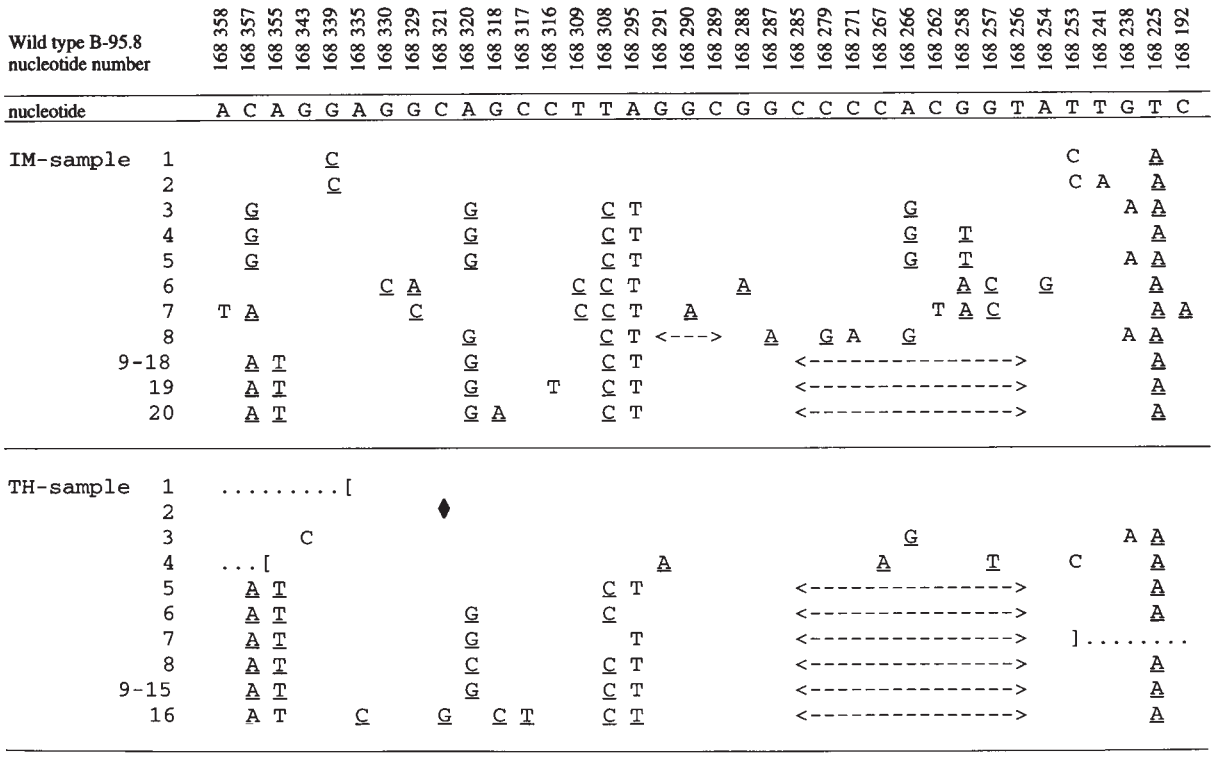

Figure 1. Mutational hot spots within carboxy-terminus of latent membrane protein-1 (LMP-1) in children with acute infectious mononucleosis (IM) and tonsillar hyperplasia (TH). Point mutations with following amino acid change are underlined; silent mutations are not underlined. Absence of letter indicates nucleotide identical to wild-type sequence. Dashed lines mark deletion of 30 bp and 3 bp, respectively. Dotted line plus bracket indicates sequence not read. ♦ stands for insertion of 18 nucleotides [5′-TAGTCTAGACTAGGTGAC-3′] between nt 168321 and 168322. IM, infectious mononucleosis; TH, tonsillar hyperplasia.

quenced with ^{35}S-labeled dATP using a Sequenase kit (Amersham Life Sciences, Arlington Heights, IL). Sequencing primers for LMP-1 were MS1 (5′-ACAATTGACGGAAGAGGTTGA-3′, positions 168358–168338) or primer 9 for the coding strand and MS7 or primer 11 for the noncoding strand. Sequencing from independent PCR products from 25% of samples showed identical results. In 36% of samples, both strands were sequenced.

Statistical analysis. The uncorrected χ^2 test was used to assess the association of LMP-1-del between samples from healthy carriers and from persons with AIDS-related lymphomas, IM, and TH.

Results

Sequencing results are summarized in figure 1. All deletions and most point mutations occurred within the 100 bp from positions 168324 to 168225, within the NF-κB activation domain of LMP-1. Mutations were identified in >75% of isolates at positions 168225, 168257, 168258, 168266, 168295, and 168308; 76% of isolates had a point mutation at position 168357 that was no longer in the NF-κB activation domain. Between position 168224 and the carboxy-terminal end (168160), only one single point mutation (IM-7) was identified; 60% of the IM samples and 75% of the TH samples showed a 30-bp deletion (positions 168285–168256). One IM-isolate (IM-8) had a 3-bp deletion (positions 168289–186291) and numerous point mutations. An insertion between position 168321 and 168322 containing 18 nucleotides (TAGTCTAGACTAGGTGAC) was identified in sample TH-2, which otherwise corresponded to the B95.8 sequence. Of interest, 13 bp within this insertion had complete homology to a measle virus nucleoprotein gene (TCTAGACTAGGTG), another 13 were homologous to the human herpesvirus-6 gene encoding the Rep protein (TAGACTAGGTGAC), and an additional 13- to 14-bp sequence to a coat protein gene of poliovirus Sabin 1 strain (TCACCTATTCTAGA). Only 1 isolate (TH-1) corresponded exactly with the prototype B95.8. Most nucleotide changes were characterized by different amino acid substitutions, defining them as mutational ''hot spots.'' Independent triplicate amplification of every sample consistently showed PCR products of identical size. In none of the patients were both LMP-1 variants found. A second sequencing of independent amplification products was performed in 9 cases (including the 7 cases with the highest number of point mutations) to further exclude sequence errors due to base misincorporation. This internal control revealed complete sequence homology with the initial results. Overall, four patterns of mutations were observed in patients with IM and TH: an insertion in 1 isolate, single point mutations and no deletion in 3 isolates, no 30-bp

Table 1. Frequency of latent membrane protein 1-del in healthy carriers and in persons with HIV-associated lymphoma, infectious mononucleosis, and tonsillar hyperplasia.

Group	LMP-1-del	(%)	Origin [reference]	*P**
A				
Healthy EBV carriers				
LCL from PBMC	10/34	29	Europe [8]	
LCL	7/24	29	Europe [9]	
Total	17/58	29		
B				
HIV-associated lymphoma				
Hodgkin's disease	11/11	100	Europe [3]	
B cell lymphoma	11/17	65	Europe [4]	
B cell lymphoma	11/18	61	North America [5]	
Total	33/46	72		A vs. B $< .01$
C				
Infectious mononucleosis	12/20	60	North America	
	6/12	50	Europe [9]	
	18/32	56		A vs. C $= .012$
D				
Tonsillar hyperplasia	12/16	75	Europe	
	10/17	59	Brazil [10]	
	22/33	67		A vs. D $< .01$

NOTE. LMP-1 del, 30-bp deletion variant of LMP-1; LCL, lymphoblastoid cell line; PBMC, peripheral blood mononuclear cells.

* Differences between groups B, C, and D are not significant ($P > .1$).

deletion but an accumulation of point mutations in 7 isolates, and the 30-bp deletion plus point mutations in 24 isolates.

The comparison of data from recent studies [3–5, 8–10] with our data (table 1) reveals a significant accumulation of LMP-1-del in IM and TH, as observed in AIDS-related lymphoma and in contrast with asymptomatic EBV carriers.

Discussion

The LMP-1 variant with a C-terminal 30-bp deletion and 6 clustered point mutations (LMP-1-del) has been detected predominantly in AIDS-related lymphoma and Asian NPC [3–6]. Experimental studies with LMP-1 variants from biopsies from persons with NPC (carrying LMP-1-del) indicated this variant to be more tumorigenic [11] and less immunogenic [12] than the LMP-1 prototype (B95.8) in mouse model systems. In particular, Li et al. [7] recently demonstrated that the deletion of these 30 bp in the LMP-1 prototype mediated enhanced oncogenic potential in vitro. In vivo, the sequence variation of LMP-1-del in a region critical for the protein's half-life (by prolongation of half-life) might lead to an accumulation of oncoprotein [2, 4], consistent with an enhanced transforming activity in the infected cell.

This sequence analysis of LMP-1 indicates the prevalence of LMP-1-del in 36 children with IM and HT. Although the total number of persons is epidemiologically limiting, the high frequency of LMP-1-del in IM and HT, both benign and self-limiting processes, is surprising and may raise doubts on the in vivo relevance of these in vitro tumor-promoting effects. However, the high frequency of LMP-1-del in persons with AIDS-related lymphoma, IM, and TH but not in asymptomatic carriers is consistent with the hypothesis that this evolving LMP-1 variant may arise only in the absence of T cell surveillance. Several findings are in support of this hypothesis.

First, our data identify LMP-1-del as a part of a hypervariable region different from a simple viral polymorphism. The presence of mutational hot spots, 3- and 30-bp deletions and an insertion, favors the generation of viral variants. Aware of the possibility that variants with and without deletion might be present in the same patient [4, 13, 14], we were not able to detect more than one variant by a very sensitive PCR approach in this collection. Viral inter- or intrastrain recombination during EBV replication has been shown in oral hairy leukoplakia and NPC [6, 14] and fits with the frequent detection of LMP-1-del in persons with hyperplastic tonsils and in HIV-infected patients, in whom enhanced viral replication occurs. Alternatively, the accumulation of LMP-1 variants in situations with ongoing germinal center activity, particularly as seen in persons with TH, IM, and AIDS-associated lymphoproliferations, is consistent with the generation of LMP-1 variants in germinal center reactions, where physiologically somatic hypermutations and isotype switching of immunoglobulin genes occur [15].

Second, the role of cellular immunity in controlling latent EBV infection is well established and, in particular, is maintained after primary infection by specific clones of cytotoxic T lymphocytes targeted against latent EBV proteins, including LMP-1 [1]. Moreover, cellular immunodeficiency results in

reactivation of oropharyngeal EBV replication, occurrence of variant virus strains, and expansion of the pool of EBV-infected lymphocytes. As seen in AIDS-related or posttransplant lymphoma [3–5], in which LMP-1-del is strongly expressed, the in vitro observed enhanced oncogenic potential may manifest in the T cell–deficient host.

Third, the identification of LMP-1-del in the healthy population (table 1) emphasizes the sustained infectivity and transmission, as well as the persistence of this variant in peripheral blood lymphocytes in healthy carriers. The accumulation of LMP-1-del in hyperplastic tonsils from Swiss and Brazilian children [10], in oral hairy leukoplakia [15], and in NPC [6] suggests a higher incidence of such LMP-1 variants in the oropharynx, where EBV might differ from its latent form in lymphocytes. However, in persons with acute IM, the frequency of LMP-1-del is significantly higher than in healthy EBV carriers if only North American and European persons and PBMC samples are compared. In contrast to the situation during latent EBV infection, during acute IM the high virus load in PBMC and the not yet functionally developed specific cellular immune response may contribute to this difference.

In summary, the high percentage of LMP-1 30-bp deletion variants in samples from persons with acute IM and TH indicates that their in vitro–enhanced oncogenicity may not be relevant in the healthy host in vivo. However, these variants may manifest their in vitro–reported oncogenic potential in settings characterized by lack of cellular immune surveillance, as demonstrated in persons with AIDS-related lymphoma.

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