RAPID COMMUNICATION

Deletion Variants Within the NF-κB Activation Domain of the LMP1 Oncogene Prevail in Acquired Immunodeficiency Syndrome-Related Large Cell Lymphomas and Human Immunodeficiency Virus-Negative Atypical Lymphoproliferations

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This sequencing study of 17 acquired immunodeficiency syndrome-related lymphomas (9 primary brain, 8 systemic) and 8 human immunodeficiency virus-negative atypical lymphoproliferations expressing large amounts of the latent membrane protein 1 (LMP1) of Epstein-Barr virus was performed to characterize the carboxy terminal NF-κB activation domain of LMP1 at the molecular level in an immunocompromised host. In-frame deletions within the NF-κB activation domain were identified in all but 2 primary brain lymphomas, 4 systemic lymphomas, and 4 atypical lymphoproliferations, ie, in 60% of cases. In addition, non silent point mutations (range 1 to 5, mean 3.3) were detected in all cases. Although all changes occurred within the first 100 nucleotides of the carboxy terminal NF-κB activation domain—a critical sequence for the protein half-life—not a single point mutation was found in the remaining 62 nucleotides, necessary for malignant transformation. Such a clustering of nonrandom sequence variations, associated with a high oncoprotein expression in immunocompromised hosts, suggests that this part of the LMP1 oncogene behaves as a hypervariable region with natural selection of growth-promoting variants through prolongation of the protein half-life.

LMP1, AN INTEGRAL membrane protein, encoded by the BNLF1 gene of Epstein-Barr virus (EBV),[1] is expressed in the multinucleated Reed-Sternberg (RS) cells of EBV-associated Hodgkin's disease (HD), and in the immunoblasts of most acquired immunodeficiency syndrome (AIDS)-related large cell lymphomas and EBV-associated, human immunodeficiency virus (HIV)-negative, premalignant and malignant lymphoproliferative disorders.[2-5] A naturally occurring LMP-1 deletion variant (LMP1-del), characterized by mutational hot spots and a distinct 30-bp deletion within the carboxy terminal region, is identified in the same conditions.[6]

LMP1 is considered to be a viral oncogene because of its capacity to transform rodent fibroblasts in vitro and to render them tumorigenic in nude mice.[7] It transforms human keratinocytes,[8] inhibits human epithelial cell differentiation,[9] induces DNA synthesis,[10] and upregulation of bcl-2 expression.[11] In transgenic mice, LMP1 induces hyperplastic dermatitis and abnormal keratin expression.[12]

Recent studies indicate that LMP1 may stimulate intercellular and intracellular signal transduction pathways.[13] In particular, the membrane-spanning segments together with the carboxy terminal 55 amino acids of this oncoprotein are required for maximal stimulation of NF-κB, a transcription factor controlling the expression of genes involved in cell activation and growth control.[14,15] Stimulation of NF-κB activity appears to occur through phosphorylation and degradation of the inhibitory molecule IκBα, followed by translocation of free NF-κB to the nucleus.[16]

We recently reported in this journal the identification of clustered point mutations and deletions within that region of the oncogene in clinically aggressive cases of HIV-negative HD with high LMP1 expression.[17,18] High LMP1 expression associated with bcl-2 oncoprotein expression in AIDS-related primary brain lymphomas (HIV-PBL) was just reported in the same forum.[19] These observations prompted us to test whether clustering of nonrandom point mutations within the NF-κB activation domain of the LMP1 gene was a common finding in immunocompromised hosts. Overall, 17 AIDS-related large cell lymphomas (including 6 brain lymphomas and 7 systemic large cell lymphomas [HIV-LCL] from ref 19) and 8 HIV$^-$, LMP1-expressing atypical lymphoproliferations have been analyzed. Our findings show that a particular variant sequence of the NF-κB activation domain of the LMP1 gene is predominant in EBV-related lymphoproliferative disorders in immunocompromised hosts.

MATERIALS AND METHODS

Tissue samples, histology, and immunohistology. A survey of the origin of the 25 cases analyzed is presented in Table 1. Diagnosis of lymphoma or lymphoproliferative disorder was performed on 4-μm tissue sections stained with hematoxilin-eosin. LMP1 was de-

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Table 1. Clinical and Histologic Data of the 25 LMP-1 Oncoprotein Positive Cases

Cases	Characteristics
1-9	HIV-associated primary large cell brain lymphomas. Immunoblastic morphology, abundant LMP-1$^+$ tumor cells.
10-17	HIV-associated large cell lymphomas without secondary brain involvement. Anaplastic large cell or immunoblastic morphology. Abundant LMP-1$^+$ tumor cells.
18-25	HIV$^-$ lymphoproliferative disorders
18	Chronic lymphoproliferative syndrome in a child. Few LMP-1$^+$ immunoblasts. JH and TCRβ in germline configuration.
19	Biclonal B-cell lymphoma after renal transplant in an EBV$^-$ host.
20	Angioimmunoblastic lymphadenopathy (AILD). JH and TCRβ in germline configuration.
21	T-cell lymphoma of AILD-type. TCRγ rearrangement.
22	T-cell lymphoma of AILD-type. TCRβ rearrangement.
23	AILD. LMP-1$^+$ B-immunoblasts. JH in germline configuration. Oligoclonal rearrangement of TCRβ.
24	Chronic lymphoproliferative syndrome in a child from an HIV$^+$ mother. Oligoclonal rearrangement of JH and TCRβ.
25	Hodgkin's disease. Splenic relapse with abundant LMP-1$^+$ tumor cells.

tected on deparaffinized 4-μm sections or fresh-frozen sections by incubation with monoclonal antibody cocktail CS1-4 (DAKO, Glostrup, Denmark) followed by identification of the CS1-4/LMP1 immune-complex with standard alkaline phosphatase-antialkaline phosphatase (APAAP) methods as previously described.[20,21] The immunohistologic findings of cases 1, 2, 4-7, 10, and 12-17 have been reported previously.[19] All these cases showed numerous LMP1-expressing tumor cells.

In situ hybridization. In situ hybridization (ISH) for detection of intracellular EBV EBER1 mRNA in histologic sections was performed with single-stranded digoxigenin-labeled RNA probes, complementary and anticomplementary (negative control) to EBER1 mRNA transcripts as described previously.[20,21]

Polymerase chain reaction (PCR). In all samples the LMP1 genome was identified by PCR. Three different primer sets, specific for the carboxy terminal domain of the LMP1 gene, were used. Detailed methods describing PCR conditions, amplification strategy, and primer sequences have recently been published.[22]

DNA sequencing. All sequencing data are original and have not been published previously. Sequencing data of case 18 will also be published in a detailed clinical case report. Double-stranded PCR products obtained with the primer pair 5′-AGCGACTCTGCTGGAAATGAT-3′/5′-TGATTAGCTAAGGCATTCCCA-3′ (primer pair 9/11 from ref 22) coding for regions adjoining the deletions were purified with a Geneclean II kit (BIO 101, La Jolla, CA) and directly sequenced with ^{35}S dATP using a Sequenase kit (Amersham Life Sciences Inc, Arlington Heights, IL). Sequencing primers were MS1 (5′-ACAATTGACGGAAGAGGTTGA-3′; nucleotide positions 168 358-168 338)[23] for the coding strand, and MS7 (5′-TCATCATCTCCACCGGAACCA-3′; positions 168 200-168 220) and primer 11 (5′-TGATTAGCTAAGGCATTCCCA-3′; positions 168 075-168 095) for the noncoding strand as reported.[22] From our previous experience[6,17,18] primer 11 yielded unambiguous sequencing results in all cases; therefore, this primer was used in all cases of this study. As an internal control, all brain lymphomas and three further cases were also sequenced with primer MS7. Primer MS1, including the mutational hot spot at positions 168 355-168 357, was additionally used in case 23 (eight point mutations, but none of them within the sequence needed for annealing of MS1).

Controls. DNA from 52 EBV$^+$ nonmalignant conditions (17 cases of florid infectious mononucleosis, 17 lymphoblastoid cell lines mainly from cancer patients, 11 reactive tissue lesions associated with EBV, 6 samples of peripheral blood mononuclear cells [PBMC] associated with inflammation, and PBMC from the EBV$^+$ healthy kidney donor of case 19) has been assessed for the presence of carboxy terminal LMP1 deletions. In the case of the EBV-associated B-cell lymphoma of patient 19 the tumor occurred in a host without serologic evidence of EBV infection before the renal transplant, indicating that the origin of EBV in this patient was of donor origin.

Statistical analysis. The χ^2 test with Yates correction was used to assess the association between LMP1 deletions and malignant lymphoproliferation.

RESULTS

EBV genomes were detected by ISH, and high LMP1 expression was observed within the immunoblasts of all cases. A comparative view of the sequencing data is given in Fig 1. Seven of 9 HIV-PBLs and half of the HIV-LCLs and HIV$^-$ LPDs showed LMP1 deletions. All deletions and most point mutations were located within the first hundred base pairs of the carboxy terminal NF-κB activation domain extending from nucleotide 168 324 to 168 225. Not a single point mutation occurred within the remaining 62 nucleotides from 168 224 to 168 163, identical with the 3′ end of the LMP1 coding region. Nonsilent point mutations were identified in 100% of cases at position 168 225, in 88% at positions 168 308 and 168 320, and in 84% at positions 168 266 (mutation or part of the deletion) and 168 357 (no more in the NF-κB activation domain). Therefore, by far the most frequent sequence variation was characterized by non silent point mutations at positions 168 357, 168 320, 168 308, 168 266, and 168 225. The corresponding amino acid changes within the NF-κB activation domain are summarized in Fig 2. A high rate of mutation is seen within amino acid positions 332 to 338 (Fig 3), which are identical to the first six amino acids of the carboxy terminal region of the NF-κB activation domain. Interestingly, the 69-bp deletion originates also in this region. Overall, the same mutation pattern was observed in HIV-PBL, HIV-LCL, and HIV$^-$ LPD.

In case 19 the posttransplant B-cell lymphoma occurred in a pretransplant EBV$^-$ host, indicating the donor kidney as a source of EBV infection. PCR-driven amplification of DNA extracted from donor PBMC using primers for the critical carboxy terminal region of the LMP1 gene showed two bands migrating at 316 and 286 bp, respectively. Sequencing confirmed the presence of the deletion variant identified in the B-cell lymphoma, but showed also presence of wild-type LMP1, not identifiable in the tumor. In the 52 nonmalignant reactive conditions, carboxy terminal LMP1 deletions were identified in 10 samples (19%). The presence of carboxy terminal LMP1 deletions was significantly associated with malignant lymphoproliferations compared with

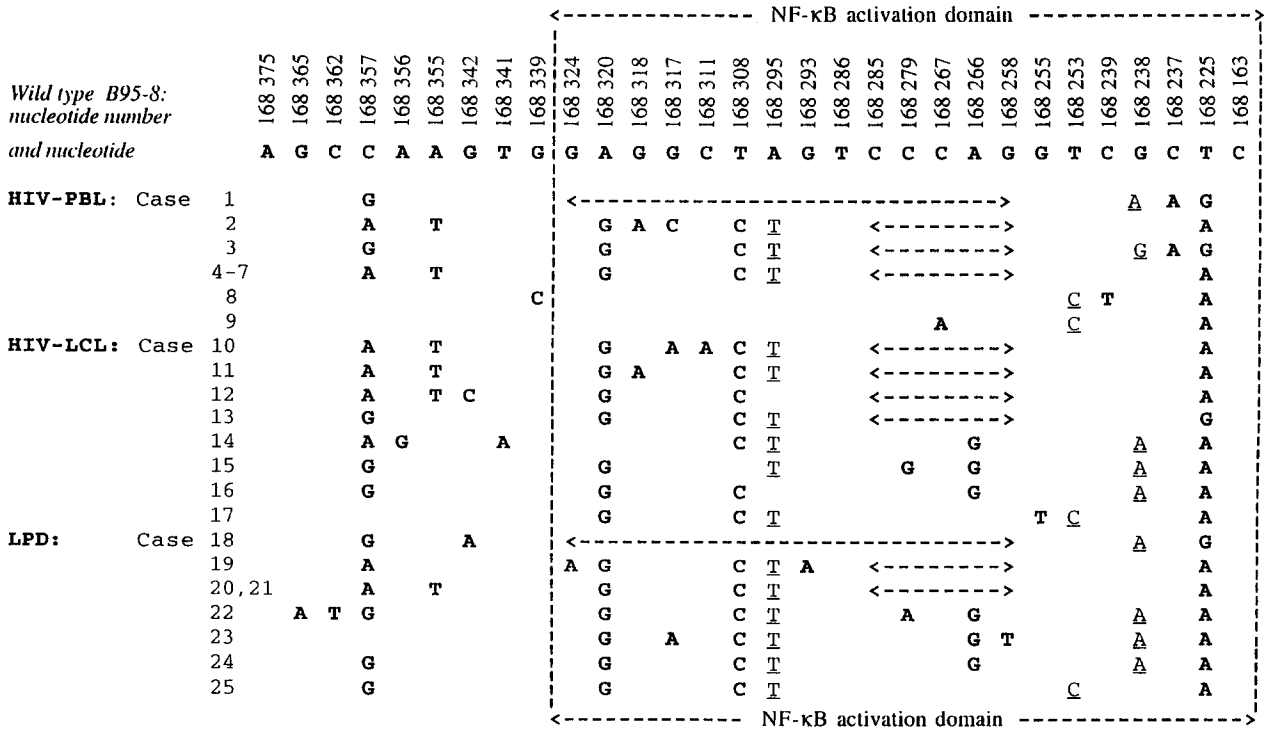

Fig 1. Mutational hot spots within the NF-κB activation domain of the LMP1 Oncogene in HIV-associated lymphomas and atypical lymphoproliferations. Point mutations inducing amino acid changes are in boldface type, silent mutations are underlined. Absence of letter indicates nucleotide identical to the wild-type sequence. Dotted lines indicate deletions of 69 and 30 bp, respectively.

overall reactive conditions ($P < .001$). Interestingly, in reactive conditions most LMP1 deletions (6 of 17) occurred in the florid infectious mononucleosis cases.

DISCUSSION

The results of this study indicate that the carboxy terminal NF-κB activation domain of the LMP1 gene expressed in AIDS-related primary brain lymphomas and disseminated lymphomas is characterized by the presence of a high number of nonrandom point mutations including distinct 30- and 69-bp deletions. This mutational pattern was previously identified in aggressive (relapsing) HD[6,17,18] atypical B- or T-cell lymphoproliferative disorders,[24-26] posttransplant lymphomas,[27] and HIV-associated oral hairy leukoplakia.[28] Our original observation of strong LMP1 oncoprotein expression in such cases[17] is now confirmed in a larger series because 13 of the AIDS-related lymphomas (6 primary brain, 7 systemic) molecularly analyzed in the present report have been shown to express LMP1 oncoprotein at high levels when assessed by immunostaining.[19] The identification of numerous tumor cells with a strong staining signal for LMP1 appears to us indicative of oncoprotein expression at high levels because abundant LMP1 mRNA transcripts have been identified in similar conditions.[29]

Interestingly, this mutational pattern is found in a setting of profound T-cell anergy, ie, central nervous system lymphoma in AIDS patients, and of diminished T-cell–mediated immunity, ie, angioimmunoblastic lymphadenopathy (AILD) and HD. The chief difference lies in the number of LMP1-expressing cells, which is very high in HIV-PBL and HIV-LCL, but small to intermediate in AILD and HD. However, when transformation of AILD into B-immunoblastic lymphoma occurs, abundant LMP1-expressing tumor cells are observed.[26] This pattern is best explained by the hypothesis that the mutations within the carboxy terminal domain of LMP1 confer a growth advantage to lymphoid cells, still partially counterbalanced in AILD and HD by the host's immune response. A further decrease in cell-mediated immunity may then lead to an uncontrolled immunoblastic proliferation resulting in B-immunoblastic lymphoma or lymphocyte-depleted HD. Indeed, the outgrowth of the donor-derived LMP1 deletion variant but not of the donor-derived LMP1 wild type in the B-immunoblastic lymphoma of the initially EBV^- host after renal transplantation (case 19) is consistent with a growth advantage of the LMP1 deletion variant over the wild type in the immunosuppressed host.

Several recent findings are in favor of a natural selection process of LMP1 deletion variants. First, all deletions and most amino acid substitutions are located within the carboxy terminal LMP1 domain required for maximal NF-κB–mediated transcription.[14,15] Deletions and mutations are restricted to the first 33 amino acids in this region, whereas not a single substitution is observed within the remaining 22 amino acids, a region that is necessary for transformation of Rat-1 fibro-

Wild type B95-8: amino acid number and designation		332 Gly	333 Asp	334 Gln	335 Gly	337 Pro	338 Leu	343 Gly	346 His	348 His	352 His	355 Gly	356 Asp	361 Thr	362 Leu	366 Ser	386 Ala
HIV-PBL:	Case 1		<----------	----------	----------	----------	----------	----------	----------	----------	----------	---------->			Met	Ala	
	2			Arg	Thr		Ser		<----------	----------	----------	---------->				Thr	
	3			Arg			Ser		<----------	----------	----------	---------->			Met	Ala	
	4-7			Arg			Ser		<----------	----------	----------	---------->				Thr	
	8													Met		Thr	
	9										Asn					Thr	
HIV-LCL:	Case 10			Arg	Asp	His	Ser		<----------	----------	----------	---------->				Thr	
	11			Arg	Ser		Ser		<----------	----------	----------	---------->				Thr	
	12			Arg			Ser		<----------	----------	----------	---------->				Thr	
	13			Arg			Ser		<----------	----------	----------	---------->				Ala	
	14						Ser				Arg					Thr	
	15			Arg						Asp	Arg					Thr	
	16			Arg			Ser				Arg					Thr	
	17			Arg			Ser						Tyr			Thr	
LPD:	Case 18		<----------	----------	----------	----------	----------	----------	----------	----------	----------	---------->				Ala	
	19		Asn	Arg			Ser	Asp	<----------	----------	----------	---------->				Thr	
	20,21			Arg			Ser		<----------	----------	----------	---------->				Thr	
	22			Arg			Ser			Asn	Arg					Thr	
	23			Arg	Asp		Ser				Arg	Cys				Thr	
	24			Arg			Ser				Arg					Thr	
	25			Arg			Ser									Thr	

Fig 2. Mutational hot spots within the NF-κB activation domain (amino acids 332-386) of the LMP1 oncogene in HIV-associated lymphomas and atypical lymphoproliferations. Absence of amino acid designation indicates amino acid identical to the wild-type sequence. Dotted lines indicate deletions of 23 and 10 amino acids, respectively.

blasts.[30] Such naturally occurring deletion variants (the most frequent variant as present in cases 4 through 7 was tested) maintain their transforming capacity when expressed in Rat-1 fibroblasts.[31] Furthermore, they are preferentially expressed in multinucleated RS cells with the same frequency as the wild-type LMP1 when transfected into the EBV$^-$ HD cell line L-428.[32] These data suggest that such LMP1-del variants maintain an oncogenic potential identical to the wild type. Second, such deletion variants may escape immunologically mediated elimination. It has been shown that the LMP1 deletion variant NPC CAO (same point mutations and deletion within the carboxy terminal NF-κB activation domain as observed in cases 4 through 7) is nonimmunogenic in a murine carcinoma model system, in contrast to the wild-type homologue B95-8.[33] Third, the immunomodulatory properties of the LMP1-del variants may differ from the wild type because of a longer protein half-life. Mutations between amino acids 322 and 364, which encompass a critical sequence for the protein half-life,[30,34] might lead to accumulation of LMP1. Considering the large amount of oncoprotein identified within the tumor cells of this series, it is reasonable to assume that a growth advantage of LMP1-del might result

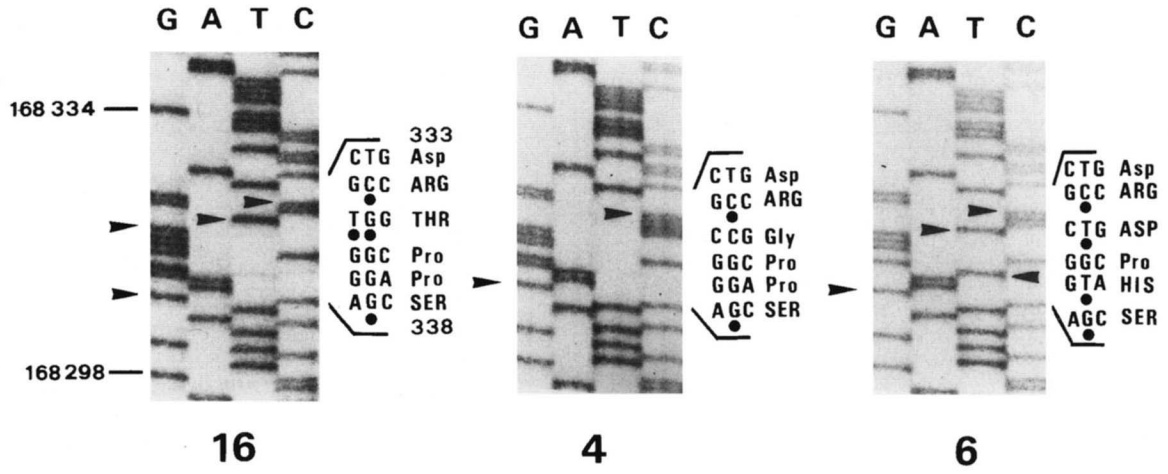

Fig 3. Mutational hot spots within the NF-κB activating domain of the LMP1 oncoprotein of AIDS-related lymphomas. All tumors have large cell (immunoblastic) morphology and strongly express LMP1. Mutations are clustered between amino acids 333 and 338. The most frequent variant is shown in the center (cases 4 through 7 from Fig 2). Number 16 on the left corresponds to case 2 of Fig 2. Number 6 on the right corresponds to case 10 from Fig 2.

from prolongation of the oncoprotein half-life. This basic mechanism which favors virus growth would also explain that identical carboxy terminal LMP1-del variants are observed in both strain A and B of EBV,[25,35] and that LMP1-del variants are found in 30% to 35% of patients presenting with florid infectious mononucleosis[24,27] but in only 10% of EBV$^+$ controls without clinical history of this disorder.

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