Aberrant Expression of Immunoglobulin Heavy Chain Genes in Epstein-Barr Virus-Negative, Human Immunodeficiency Virus-Related Lymphoid Interstitial Pneumonia

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SUMMARY: The two-step polymerase chain reaction (PCR) and sequencing analysis was used to analyze the immunoglobulin heavy chain variable (Ig V_H) genes of open-chest biopsy or autopsy samples from five patients with Epstein-Barr virus-negative human immunodeficiency virus (HIV)-related lymphoid interstitial pneumonia (LIP), and the results were compared with those for Ig V_H genes from five HIV-negative LIP patients. The findings of this study are consistent with the different immunological situations of HIV-related and HIV-negative LIP. (a) The Ig V_H3 subgroup was underexpressed in three of five cases of HIV-related LIP. In contrast, none of the HIV-negative cases showed this abnormality. Because the Ig V_H3 subgroup encodes the largest portion of Ig V_H genes, a depletion of B cells expressing Ig V_H3 genes reflects a major alteration in the B-cell compartment. (b) All HIV-related LIP cases demonstrated two or three oligoclonal populations. HIV-negative cases showed minor monoclonal or polyclonal populations, but not oligoclonal ones. These oligoclonal populations suggest the coexistence of several occult clonal B-cell populations in HIV-related LIP. (c) Some oligoclonal clones in HIV-related LIP showed mutated framework regions not demonstrated in HIV-negative clones. This degree of variation exceeds the usual mutation rate for frameworks, suggesting a role for framework residues in antigen binding. (d) The frequency of D-D fusions of minor oligoclonal clones (HIV-related LIP) is higher than that of minor monoclonal clones (HIV-negative LIP). Such D-D fusions may enhance the probability of expression of antibodies capable of binding HIV glycoproteins. (*Lab Invest* 2000, 80:1891-1903).

Infection with human immunodeficiency virus (HIV) induces profound abnormalities in the host immune system, including a loss of CD4⁺ T cells, suppression of response to T-dependent antigens, and abnormal activation of B lymphocytes (Shirai et al, 1992). On the other hand, infection paradoxically involves a poor response to polysaccharides in vivo and pokeweed mitogen in vitro (Ambrosino et al, 1987; Terpstra et al, 1989). Many of these B-cell abnormalities occur early in infection, before substantial depletion of CD4⁺ T cells or clinically evident disease. These abnormalities are manifested even when purified B cells from HIV-positive individuals are tested with normal T cells, suggesting that functional deficits are intrinsic to the B cell (Miedema et al, 1989).

The immunoglobulin heavy chain locus on chromosome 14 contains an estimated 100 to 150 variable (Ig

V_H) genes, 30 diversity (D) genes, and 6 junctional (J_H) gene segments. During the maturation of a normal B cell, the unique combination of single germline V_H, D, and J_H gene fragments gives rise to a functional V_H-D-J_H unit (Stewart and Schwartz, 1994). Continued antigenic exposure selects for and stimulates the proliferation of antigen-reactive clones bearing Ig V_H exhibiting high affinity, which contain varying numbers of nucleotide substitutions (somatic hypermutation) with corresponding amino acid changes that alter the shape of the antigen-binding site and increase the fit for the antigen. Somatic hypermutation of Ig V_H genes is generally believed to occur in the germinal centers, and replacement mutations in complementarity-determining regions (CDRs) have been used as indicators of antigen selection (Schlomchik et al, 1987). The presence or absence of somatic hypermutation in B cells can pinpoint their developmental stage.

Children infected with HIV are at increased risk for lymphoid interstitial pneumonia (LIP). Although LIP is rare in adult HIV-infected patients, it was reported in 4 of 50 lung biopsy specimens (Noran et al, 1994; Travis et al, 1992). A detailed analysis of Ig V_H gene subgroup expression in patients infected with HIV-related LIP

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has not been reported. The structural analysis of the Ig V_H genes expressed in HIV-related LIP can provide insight into the mechanisms involved in B-cell abnormalities. It is not known whether the repertoire of Ig V_H genes expressed in HIV-related LIP is biased toward or against Ig V_H3 genes that encode antibodies with an innate binding activity for HIV glycoproteins. We previously reported the sequence of Ig V_H complementarity determining region 3 (CDR3) of low-grade bronchus-associated lymphoid tissue lymphoma as determined by polymerase chain reaction (PCR) and sequencing analysis on DNA obtained from appropriate regions by microdissection (Kurosu et al, 1997). Using this technique, we compared the Ig V_H region genes of biopsy samples from five patients with Epstein-Barr virus (EBV)-negative HIV-related LIP with the same genes from five HIV-negative LIP patients. We made some observations consistent with the different immunological situations of HIV-related and HIV-negative LIPs.

Results

Histopathologic Findings

All 10 LIP samples demonstrated dense and mixed interstitial infiltrates of lymphocytes, plasma cells, and histiocytes with prominent germinal centers. No cytologic atypia of the lymphoid infiltrates were seen. Immunohistochemical staining of the specimens revealed polyclonal B-cell populations with numerous T cells.

Two-Step PCR and Sequencing Analysis of the Ig V_H Regions of LIP

Because four cases were positive for the EBV genome by PCR analysis, they were excluded from our study. Another 10 cases of LIP, with no amplification of the EBV genome, showed a smear polyclonal band of Ig V_H gene (about 250 base pairs) on electrophoresis (Table 1, Fig. 1). The results of sequencing are presented as the incidence rates of the predominant sequences out of the total number of vector clones

Figure 1.

PCR analysis of the immunoglobulin heavy chain variable (Ig V_H) gene rearrangement. The numbers above the lanes correspond to the case numbers in Table 1. P, positive control; N, negative control.

analyzed (Tables 2 and 3). All five HIV-related LIP cases (Cases 1 through 5) showed minor oligoclonal sequences with frequencies ranging from 2 of 10 to 3 of 10 clones. The definition for oligoclonal population is to detect plural clones with high frequencies (more than 2 of 10 clones). In two HIV-negative LIP cases (Cases 6 and 7), all sequences were different. Another three HIV-negative cases (Cases 8 through 10) demonstrated minor monoclonal sequences with frequencies ranging from 2 of 10 to 3 of 10 clones. Other sequences obtained had different individual CDR3 sequences characteristic of contaminating normal reactive B cells.

The closest germline Ig V_H gene and its degree of similarity to each case are shown in Table 2. Two HIV-related (Cases 2 and 5) and all HIV-negative LIP subjects demonstrated the most frequently expressed Ig V_H3 family genes (60% to 90%). Another three HIV-positive cases (cases 1, 3, and 4) showed much lower frequencies (10% to 20%) of Ig V_H3 gene expression. All peripheral blood samples of HIV-

Table 1. Pathological and Molecular Details of Analyzed Samples

Case No.	Age/Sex	Specimens	Ig V _H rearrangement*	Pulmonary Symptoms	Complications
HIV-Related LIP					
1	2/M	autopsy	s	cough, dyspnea	HIV
2	3/F	autopsy	s	cough, dyspnea	HIV
3	41/M	OC	s	cough	HIV
4	35/F	OC	s	cough, chest pain	HIV
5	26/M	OC	s	dyspnea	HIV
HIV-Negative LIP					
6	2/M	OC	s	cough	None
7	60/F	OC	s	dyspnea, cough	rheumatoid arthritis
8	37/F	OC	s	cough	Sjögren Syndrome
9	67/F	OC	s	none	None
10	45/M	OC	s	cough	None

LIP, lymphoid interstitial pneumonia; OC, open-chest biopsy; * s, smear polyclonal band.

related LIP patients showed the most frequently expressed Ig V_H3 family genes (50% to 60%) (Table 4).

The distribution of somatic mutations among the minor monoclonal or minor oligoclonal Ig V_H genes is shown in Table 3. In HIV-related LIP cases, seven oligoclonal populations expressed Ig V_H genes derived from the Ig V_H3 family, whereas the other eight populations used members of the Ig V_H1 or Ig V_H4 families. HIV-negative LIP cases showed two Ig V_H3 (Clones 8-1, 2 and 10-1, 2) and one Ig V_H4 (Clone 9-1, 2, 3) minor monoclonal populations. For CDR, there were more replacement (R) mutations than expected in all cases, with significant ($p < 0.05$) clustering indicative of antigen detection in all cases.

Among a total of 50 sequenced Ig V_H rearrangements from the 5 HIV-related LIP cases, 41 rearrangements were potentially functional and 9 were nonfunctional (9 out-of-frame clones). The frequencies of out-of-frame clones in HIV-related and HIV-negative LIPs were 9 of 50 (18%) and 2 of 50 (4%), respectively. Comparison of the CDR3 with known J_H gene segments revealed that each minor oligoclonal clone, with some exceptions (Clones 1-8, 9), probably arose through D-D fusion with no major contribution from the D region (Table 5). This D-D fusion could be detected in only one of three minor monoclonal populations in HIV-negative LIP cases.

Discussion

This study presents the molecular analysis of Ig V_H genes used by B cells from five patients with HIV-related LIP compared with those from five patients with HIV-negative LIP. All 10 LIP cases were negative for EBV. In this study, some observations consistent with the different immunological situations between HIV-related and HIV-negative LIPs were made. (a) The Ig V_H3 subgroup was underexpressed in 3 of 5 cases of HIV-related LIP. In contrast, none of the HIV-negative or the two remaining HIV-related LIP cases showed this abnormality, leading to the most frequently expressed Ig V_H3 subgroup. (b) All HIV-related LIP cases demonstrated two or three oligoclonal populations with rates from 20% to 30%. HIV-negative LIP cases showed minor monoclonal or polyclonal populations, but no oligoclonal ones. (c) Some oligoclonal clones in HIV-related LIP showed mutated framework regions (FRs), a feature that was not found in HIV-negative LIP. (d) The frequency of D-D fusions of minor oligoclonal clones (HIV-related LIP) is higher than that of minor monoclonal clones (HIV-negative LIP).

Because the use of high fidelity DNA polymerases in PCR is essential for reducing the introduction of amplification errors in PCR products, we used the high-fidelity polymerase, Pfu. Unlike *Taq* DNA polymerase, Pfu possesses a 3' to 5' exonuclease proofreading activity that enables the polymerase-generated PCR fragments to have fewer errors than *Taq*-generated PCR inserts. The error rate of Pfu has been reported to be one mutation per 7.8×10^5 bp per cycle (Cline et al, 1996).

As mentioned in the "Results" section, the Ig V_H3 subgroup was underexpressed in 3 of 5 cases of HIV-related LIP. The Ig V_H3 subgroup has the largest number of functional Ig V_H genes in humans. Because Ig V_H3 genes ordinarily are used by approximately 45% to 60% of the blood B cells of normal adults (Cook and Tomlinson, 1995; Guigou et al, 1990), a depletion of B cells expressing Ig V_H3 genes reflects a major alteration in the B cell compartment. Prior studies noted that peripheral Ig V_H3 B cells are increased in early disease and depleted in later disease (David et al, 1995). It was recently reported that persons in the early stages of HIV infection show abnormal expression levels of Ig V_H3 genes and that persons with established acquired immunodeficiency syndrome show a significantly lower incidence of abnormalities in Ig V_H3 expression levels (Bessudo et al, 1998). We questioned whether the underexpression of the Ig V_H3 subgroup was a distinctive pattern in the LIP region or reflected a systemic lymphoid phenomenon. To address this issue, we analyzed the Ig V_H region genes of peripheral B-cells in HIV-negative LIP cases (Cases 2 to 5). We concluded that the Ig V_H3 subgroup was underexpressed in the focal LIP region, but not in peripheral B-cells of HIV-negative LIP cases. Our HIV-related LIP cases had normal CD4⁺ T cell counts (early stage HIV). Our findings of Ig V_H3 underexpressing HIV-related LIP cases are consistent with the prior studies.

B-cell superantigens may account for the abnormal levels of Ig V_H gene subgroups observed in patients with HIV. HIV gp120 binds to a conserved immunoglobulin motif unique to the immunoglobulin of the Ig V_H3 subgroup independently of the light chain isotype, and of D and J_H usage. Gp120 may function as a superantigen that induces an initial stimulation of B cells expressing Ig V_H3 genes followed by a depletion. A similar pattern exists for peripheral B cells bearing the D12 idiotype (Berberian et al, 1994). Recently, the superantigen residues of the gp120 engaged in binding to Ig V_H3 have been identified (Goodglick et al, 1995; Karray and Zouali, 1997). It has been reported that the repertoire of Ig V_H genes binding to gp120 is restricted to a limited number of Ig V_H3 genes (VH26, 3-30, 3-73, 3-15) with high homology (96% to 100%) to germline configuration and that gp120 binding is sensitive to somatic mutation (Berberian et al, 1993; Karray and Zouali, 1997). In Ig V_H3 underexpressed HIV-related LIP, we did not detect any clones belonging to those limited Ig V_H3 genes (VH26, 3-30, 3-73, 3-15). In contrast, one VH26 clone (clones 5-3, 4) was detected in another HIV-related LIP. This VH26 clone had some replacement mutations in the CDR2 region, but only one silent mutation in FR2 and FR3 regions. FR1 and FR3 are candidate subregions for gp120 binding, because they display strong sequence conservation among V_H3 family members. It was reported that four positions localized in FR1 and nine positions in FR3 might influence gp120 binding (Karray and Zouali, 1997). The VH26 clone (clones 5-3, 4) contained no replacement mutations at nine favorable sequences of FR3 for gp120 binding.

Table 2. V_H Genes Analysis of HIV-Related and Nonrelated LIPs

Case	Clones	Germline V _H (V _H family)	Similarity (%)	Replacement: Silent			D segment	J _H segment	Frame
				CDR	FRs	p [*]			
HIV-Related LIP									
1	1-1, 2, 3	DP-14 (I)	94.2	5.00	4.00	0.045 [*]	D2 & D21/10	JH1	In
	1-4, 5	4-39 (IV)	94.1	6.00	0.33	0.012 [*]	D4-C & DM1	JH3	In
	1-6, 7	1.9II (IV)	96.2	4.00	1.00	0.024 [*]	DXP'1 & DN4	JH4	Out
	1-8, 9	DP-35 (III)	94.2	5.00	4.00	0.038 [*]	DN4	JH4	In
	1-10	DP-73 (V)	96.7	1.00	0.00	0.233	DK1	JH6	In
2	2-1, 2, 3	3-33 (III)	93.7	6.00	4.00	0.015 [*]	DA1/4 & D2	JH6	In
	2-4, 5	hv3005 (III)	95.2	5.00	0.50	0.016 [*]	DK1 & DXP4	JH4	In
	2-6	cos 16 (III)	95.4	2.50	0.00	0.065	DN1	JH4	In
	2-7	DP-48 (III)	96.8	2.00	0.50	0.245	D4 & DLR5	JH5	Out
	2-8	H11 (III)	94.1	6.00	1.00	0.007 [*]	inv.DK5	JH6	In
	2-9	4.35 (IV)	95.2	1.50	0.33	0.163	DXP'1 & DK1	JH6	In
	2-10	DP-75 (I)	94.7	4.00	0.25	0.077	DK4	JH6	Out
	2-10	DP-75 (I)	94.7	4.00	0.25	0.077	DK4	JH6	Out
3	3-1, 2	DP-25 (I)	93.7	3.50	2.00	0.003 [*]	D2-C & DA1	JH1	In
	3-3, 4, 5	VI-2 (I)	90.5	7.00	4.00	0.035 [*]	D21/5 & D21/9	JH4	In
	3-6, 7	4.21 (IV)	95.7	4.00	0.50	0.039 [*]	DLR4 & D23/7	JH6	In
	3-8	DP-51 (III)	95.3	6.00	0.00	0.003 [*]	DXP'1	JH4	Out
	3-9	4.18 (IV)	95.2	2.00	0.20	0.297	DM1	JH5	In
	3-10	DP-75 (IV)	95.8	5.00	0.00	0.007 [*]	D2	JH6	Out
	3-10	DP-75 (IV)	95.8	5.00	0.00	0.007 [*]	D2	JH6	Out
4	4-1, 2	hv1263 (I)	94.7	5.00	0.33	0.026 [*]	D21/10 & DXP'1	JH4	In
	4-3, 4	DP-49 (III)	93.1	6.00	5.00	0.023 [*]	D21/9 & D5	JH6	In
	4-5, 6	DP-67 (IV)	95.7	4.00	0.50	0.039 [*]	DLR4 & D6	JH6	In
	4-7	4.16 (IV)	97.4	1.00	0.50	0.409	DA1/4	JH3	In
	4-8	DP-7 (I)	93.8	7.00	0.30	0.005 [*]	DM1 & DLR5	JH4	Out
	4-9	hv4005 (IV)	94.8	2.00	0.33	0.088	DXP'1	JH4	In
	4-10	VH32 (V)	95.3	2.50	0.00	0.016 [*]	D6	JH5	In
5	5-1, 2	1.9III (III)	94.7	5.00	1.00	0.026 [*]	D21/9 & DLR1	JH3	In
	5-3, 4	VH26 (III)	96.3	5.00	0.00	0.004 [*]	D2 & DXP5	JH4	In
	5-5, 6	cos 15 (III)	93.5	6.00	4.00	0.012 [*]	DM2 & DK5	JH6	In
	5-7	DP-49 (III)	96.3	1.00	0.50	0.264	D1-C & DXP4	JH3	In
	5-8	DP-81 (III)	93.7	6.00	4.00	0.012 [*]	DN1	JH4	In
	5-9	v3-48 (III)	95.8	3.00	0.33	0.134	DK1	JH6	Out
	5-10	4-61b (IV)	94.1	1.67	0.50	0.032 [*]	DA5	JH6	Out
HIV-Negative LIP									
6	6-1	DP-21 (I)	96.9	4.00	0.00	0.015 [*]	DLR5	JH4	In
	6-2	VH3-23 (III)	94.3	2.00	0.67	0.111	DN4	JH3	In
	6-3	DP-47 (III)	95.8	1.00	0.50	0.336	DN1	JH4	In
	6-4	DP-58 (III)	96.9	1.00	0.00	0.246	D21/9	JH4	In
	6-5	8-1B (III)	97.9	2.00	0.00	0.142	D22/12	JH4	In
	6-6	VH26 (III)	96.4	1.00	0.25	0.367	DXP'1	JH5	In
	6-7	YAC-5 (III)	95.2	6.00	1.00	0.003 [*]	DXP4	JH5	In
	6-8	DP-70 (IV)	95.8	1.00	0.33	0.285	DLR1	JH6	In
	6-9	v2-1 (IV)	100.0	0/0	0/0	0/0	DM5	JH6	In
	6-10	VH71-2 (IV)	96.8	2.00	0.50	0.233	DLR1-C	JH6	In
7	7-1	DP-46 (III)	95.3	4.00	1.00	0.066	DXP'1	JH1	In
	7-2	DP-50 (III)	95.8	2.00	0.00	0.046 [*]	DA1/4	JH4	In
	7-3	DP-32 (III)	95.3	6.00	0.00	0.003 [*]	DLR4	JH4	In
	7-4	DP-54 (III)	96.4	2.00	0.33	0.275	D21/10 & DLR3	JH4	In
	7-5	DP-49 (III)	94.8	1.33	0.50	0.088	DLR5	JH5	In
	7-6	DP-38 (III)	95.4	2.50	0.00	0.023 [*]	DM2-C	JH5	In
	7-7	hv3033 (III)	100.0	0/0	0/0	0/0	DXP4 & D21/9	JH5	Out
	7-8	DP-64 (IV)	95.8	3.00	1.00	0.134	D2-C	JH6	In
	7-9	4.11 (IV)	95.2	1.50	0.33	0.163	DN4	JH6	In
	7-10	DP-66 (IV)	95.8	2.00	0.25	0.285	DXP1 & DLR5	JH6	In

Table 2. (Continued)

Case	Clones	Germline V _H (V _H family)	Similarity (%)	Replacement: Silent			D segment	J _H segment	Frame
				CDR	FRs	p*			
8	8-1, 2	VH26 (III)	95.7	5.00	0.00	0.007*	DHQ52	JH4	In
	8-3	DP-77 (III)	98.4	1.00	0.00	0.384	D5	JH1	In
	8-4	DP-46 (III)	95.8	3.00	0.33	0.147	DXP'1	JH3	In
	8-5	VH9.1 (III)	97.9	1.00	0.00	0.406	inv.D6-C	JH4	In
	8-6	DP-77 (III)	93.8	1.00	0.20	0.236	DK1	JH4	In
	8-7	HHG19 (III)	94.8	2.00	0.33	0.088	DM2	JH4	In
	8-8	DP-54 (III)	95.3	1.50	0.33	0.176	D23/7	JH5	In
	8-9	4.18 (IV)	95.2	2.00	0.20	0.297	DLR5	JH6	In
	8-10	4.22 (IV)	95.8	5.00	0.00	0.007*	DLR1 & DXP'1	JH6	In
	9-1, 2, 3	4.11 (IV)	94.2	4.00	0.50	0.039*	DN1	JH4	In
9	9-4	DP-42 (III)	96.2	3.00	0.50	0.115	DN1	JH4	In
	9-5	VH26 (III)	95.3	1.50	0.33	0.176	D21/10	JH4	In
	9-6	18/2 (III)	93.7	4.00	0.17	0.133	DK1	JH4	In
	9-7	DP-49 (III)	100.0	0/0	0/0	0/0	DM1	JH4	In
	9-8	DP-29 (III)	94.9	2.00	0.33	0.099	DLR5	JH5	In
	9-9	DP-39 (III)	94.7	2.00	0.33	0.088	DM5	JH6	In
	9-10	DP-53 (III)	95.2	6.00	0.00	0.003*	DN1 & DLR5	JH6	In
	10-1, 2	DP-46 (III)	94.2	6.00	0.33	0.009*	DK5 & D3-C	JH1	In
	10-3	VH3-1 (I)	96.3	2.00	0.33	0.275	D21/7	JH4	In
	10-4	DP-59 (III)	95.8	3.00	0.33	0.147	DM2-C	JH4	In
10	10-5	DP-51 (III)	95.2	1.00	0.50	0.176	DXP'1	JH4	In
	10-6	DP-31 (III)	95.8	2.00	0.25	0.293	DN4	JH4	In
	10-7	DP-61 (III)	100.0	0/0	0/0	0/0	DA1	JH5	Out
	10-8	cos-6 (III)	95.7	0.67	0.50	0.285	DXP4	JH6	In
	10-9	H11 (III)	94.6	6.00	0.00	0.004*	D5	JH6	In
	10-10	DP-71 (IV)	95.7	4.00	0.50	0.046*	D2	JH6	In

CDR, complementarity-determining region; FRs, framework regions; J_H, junctional.

* *p* is the probability of obtaining the number of R mutations found in the CDR by chance.

Pellicci and colleagues described the presence of two or more low-intensity immunoglobulin gene rearrangement bands within reactive persistent generalized lymphadenopathy tissues in 4 of 11 HIV-infected persons, leading to the presence of oligoclonal B cell expansions (Pellicci et al, 1986). Considering that only 5 in 100,000 circulating B cells share the same Ig V_H CDR3 (Yamada et al, 1991), the rate of minor oligoclonal clones in cases 1 through 5 is unusually higher than in normal reactive lymphocytes. It was reported that the numbers of specific V_H-N-D-N-J_H junctions is considered to be unusually high if they represent two or more identical copies among the Ig V_H CDR3 PCR products after cloning; in lymphadenitis, all lymphocyte clones differ in their V_H-N-D-N-J_H junctions (Linke et al, 1995). In our study, the two or three oligoclonal populations derived from HIV-related LIP cases suggest the co-existence of several occult clonal B-cell populations that are not identifiable by morphologic or immunophenotypic analyses. In contrast, it is very interesting that HIV-negative LIP demonstrates minor monoclonal or polyclonal, but not oligoclonal, populations. Polyclonal lymphomas have been described at the molecular level, demonstrating that approximately 40% of AIDS-related lymphomas are polyclonal in genotype (Shiramizu et al, 1992). These HIV-related polyclonal lymphomas may repre-

sent an earlier stage in HIV-related lymphomagenesis before the emergence of a dominant malignant clone. Alterations of the T-helper 1 (Th1)/T-helper 2 (Th2) regulatory networks may be important in determining the progressive immunosuppression of local immunocompetence. Following HIV infection, Th1 response, characterized by the release of interferon- γ (IFN- γ) and interleukin-2 (IL-2) which promote a strong cell-mediated immune response, can switch to a Th2 profile with the concomitant release of IL-10, IL-6, and IL-4 (Clerici and Shearer, 1993). The oligoclonal B-cell activation that is observed in the lungs of HIV-positive patients with LIP may also reflect local Th1 imbalance. It is, at present, largely unknown whether such a shift occurs in the LIP lung in response to HIV. More definitive molecular analyses will be required to understand the immunologic process of HIV-related LIP.

Among LIP cases, we observed frequent somatic mutations of the rearranged Ig V_H genes in all minor oligoclonal (HIV-related) or minor monoclonal (HIV-negative) populations. A higher CDR replacement (R):silent (S) mutation ratio (> 2.9, calculated for somatic mutations occurring randomly in a gene encoding a protein whose structure need not be preserved) reflects the positive selective pressure of an antigen (affinity-maturation) on those gene products that come into close contact with an antigen (Jukes

Table 3. IgV_H Sequences of Minor Monoclonal or Oligoclonal Populations Derived from LIPs^a

Case	Clones	Germline V _H	Sequences
1	1-1, 2, 3	I (DP-14)	<-----FR2-----> <-----CDR2-----> TGG GTG CGA GGC CCT GGA CAA GGG CTT GAG TGG ATG GGA TGG ATC AGC GCT TAC AAT GGT AAC ACA AAC TAT GCA CAG AAG CTC CAG GGC -----C-----G-----t-----A-----C-----C-----
		I (DP-14)	-----FR3----- AGA GTC ACC ATG ACC ACA GAC ACA TCC ACG AGC ACA GCC TAC ATG GAG CTG AGG AGC CTG AGA TCT GAC GAC ACG GCC GTG TAT TAC TGT GCG AGA -----T-----A-----G-----C-----
	1-1, 2, 3	IV (4-39)	-----FR2-----> <-----CDR2-----> TGG ATC CGC CAG CCC CCA GGG AAG GGG CTG GAG TGG ATT GGG AGT ATC TAT TAT AGT GGG AGC ACC TAC TAC AAC CCG TCC CTC AAG AGT -----g-----a-----C-----T-----C-----a-----G-----C-----
		IV (4-39)	-----FR3-----> CGA GTC ACC ATA TCC GTA GAC ACG TCC AAG AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG ACC GCC GCA GAC ACG GCT GTG TAT TAC TGT GCG AGA -----G-----a-----
	1-4, 5	IV (1.9II)	-----FR2-----> <-----CDR2-----> TGG ATC CGG CAG CCC CCA GGG AAG GGA CTG GAG TGG ATT GGG TAC ATC TAT TAT AGT GGG AGC ACC TAC TAC AAC CCG TCC CTC AAG AGT -----a-----G-----C-----T-----c-----A-----
		IV (1.9II)	-----FR3-----> CGA GTC ACC ATG TCA GTA GAC ACG TCC AAG AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG ACC GCC GTG GAC ACG GCC GTG TAT TAC TGT GCG AGA -----G-----
	1-6, 7	III (DP-35)	-----FR2-----> <-----CDR2-----> TGG ATC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTT TCA TAC ATT AGT AGT AGT GGT AGT ACC ATA TAC TAC GCA GAC TCT GTG AAG GGC -----A-----c-----G-----A-----t-----A-----
		III (DP-35)	-----FR3-----> CGA TTC ACC ATC TCC AGG GAC AAC GCC AAG AAC TCA CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG GCC GTG TAT TAC TGT GCG AGA -----G-----A-----G-----
	2-1, 2, 3	III (3-33)	-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG GCA CTT ATA TGG TAT GAT GGA AGT AAT AAA TAC TAT GCA GAC TCC GTG AAG GGC -----c-----C-----G-----GT-----G-----C-----
		III (3-33)	-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA -----t-----C-----A-----
2	2-1, 2, 3	III (hv3005)	-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGC AAG GGG CTA GAG TGG GTG GCA GTT ATA TCA TAT GAT GGA AGT AAT AAA TAC TAC GCA GAC TCC GTG AAG GGC -----c-----g-----C-----A-----C-----T-----A-----
		III (hv3005)	-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA -----A-----
	2-4, 5	III (hv3005)	-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GGC CCC GGA CAA AGG CTT GAG TGG ATG GGA TGG ATC AAC GCT GGC AAT GGT AAC ACA AAA TAT TCA CAG AAG TTC CAG GGC -----A-----G-----c-----A-----GC-----T-----T-----t-----
		I (DP-25)	-----FR3-----> TGG GTC CGC CAG GGC CCC GGA CAA AGG CTT GAG TGG ATG GGA TGG ATC AAC GCT GGC AAT GGT AAC ACA AAA TAT TCA CAG AAG TTC CAG GGC -----A-----
	3-1, 2	I (DP-25)	-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GGC CCC GGA CAA AGG CTT GAG TGG ATG GGA TGG ATC AAC GCT GGC AAT GGT AAC ACA AAA TAT TCA CAG AAG TTC CAG GGC -----A-----G-----c-----A-----GC-----T-----T-----t-----

Table 3. (Continued)

Case	Clones	Germline V _H	Sequences
4	3-1, 2	I (DP-25)	<-----FR3-----> AGA GTC ACC ATT ACC AGG GAC ACA TCC GCG AGC ACA GCC TAC ATG GAG CTG AGC AGC CTG AGA TCT GAA GAC ACG GCT GTG TAT TAC TGT GCG AGA -----G-----C----->
		I (VI-2)	<-----FR2-----> TGG GTG CGA CAG GCC CCT GGA CAA GGG CTT GAG TGG ATG GGA -----C-a-----A-----G-----G-----T-----A-----C-----C-----T-----a----->
	3-3, 4, 5	I (VI-2)	<-----FR3-----> AGG GTC ACC ATG ACC AGG GAC ACG TCC ATC AGC ACA GCC TAC ATG GAG CTG AGC AGG CTG AGA TCT GAC GAC ACG GCC GTG TAT TAC TGT GCG AGA -----C-----G-----T-----A-----C-----C----->
		IV (4.21)	<-----FR2-----> TGG ATC CGC CAG CCC CCA GGG AAG GGG CTG GAG TGG ATT GGG -----C-----G-----T-----A-----C-----A-----G-----C-----G----->
	3-6, 7	IV (4.21)	<-----FR3-----> CGA GTC ACC ATA TCA GTA GAC ACG TCC AAG AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG ACC GCC GCG GAC ACG GCT GTG TAT TAC TGT GCG AGA -----A-----t----->
		I (hv1263)	<-----FR2-----> TGG GTG CGA CAG GCC CCT GGA CAA GGG CTT GAG TGG ATG GGA -----G-----G-----C-----T-C-G-----A-----C----->
	4-1, 2	I (hv1263)	<-----FR3-----> AGA GTC ACG ATT ACC GCG GAC AAA TCC ACG AGC ACA GCC TAC ATG GAG CTG AGC AGC CTG AGA TCT GAG GAC ACG GCC GTG TAT TAC TGT GCG AGA -----g-----C-----g----->
		III (DP-49)	<-----FR2-----> TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG GCA -----T-----a-----G-----T-----G-----A-----G-----T-----g-----G----->
	4-3, 4	III (DP-49)	<-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT GCG AAA -----C-----C-----A----->
		IV (DP-67)	<-----FR2-----> TGG ATC CGG CAG CCC CCA GGG AAG GGG CTG GAG TGG ATT GGG -----C-----C-----a-----G-----G-----t-----t-----A-----T----->
5	4-5, 6	IV (DP-67)	<-----FR3-----> CGA GTT ACC ATA TCA GTA GAC ACG TCC AAG AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG ACC GCC GCA GAC ACG GCC GTG TAT TAC TGT GCG AGA -----C----->
		III (1.9III)	<-----FR2-----> TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG GCA -----g-----g-----A-----t-----G-----T-----C-----g-----AG----->
	5-1, 2	III (1.9III)	<-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT GCG AAA -----G----->
	5-1, 2		

Table 3. (Continued)

Case	Clones	Germline V _H	Sequences
8	5-3, 4	III (VH 26)	<-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTC TCA GCT ATT AGT GGT AGT GGT AGC ACA TAC TAC GCA GAC TCC GTG AAG GGC -----C-----A-----t--t--G-----C----- <-----FR3-----> CGG TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG GCC GTA TAT TAC TGT GCG AAA -----a----- <-----FR2-----> <-----CDR2-----> TGG GTT CGC CGG GCT CCA GGG AAG GGT CTG GAG TGG GTA TCA GCT ATT GGT ACT GGT GGT GAT ACA TAC TAT GCA GAC TCC GTG ATG GGC -----A-----a-----T-----CG-----G-----a----- <-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAC GCC AAG AAG TCC TTG TAT CTT CAT ATG AAC AGC CTG ATA GCT GAG GAC ATG GCT GTG TAT TAT TGT GCA AGA -----G-----C-----
		III (COS 15)	<-----FR2-----> <-----CDR2-----> TGG GTT CGC CGG GCT CCA GGG AAG GGT CTG GAG TGG GTA TCA GCT ATT GGT ACT GGT GGT GAT ACA TAC TAT GCA GAC TCC GTG ATG GGC -----A-----a-----T-----CG-----G-----a----- <-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAC GCC AAG AAG TCC TTG TAT CTT CAT ATG AAC AGC CTG ATA GCT GAG GAC ATG GCT GTG TAT TAT TGT GCA AGA -----G-----C-----
	5-5, 6	III (VH 26)	<-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGA GGA CTG GAG TGG ATT GGG TAT ATC TAT TAC AGT GGG AGC ACC AAC TAC AAC CCC TCC CTC AAG AGT -----a-----G-----T-----G-----G-----T----- <-----FR3-----> CGG TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG TCC AAG AAC CAG TTC TCC CTG AGG CTG AGT TCT GTG ACC GCT GCG GAC ACG GCC GTA TAT TAC TGT GCG AGA -----g-----T----- <-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTG GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG AAG GGC -----a-----TC-----T-----g----- <-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA -----a-----C-----C-----
		III (DP-46)	<-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTG GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG AAG GGC -----a-----TC-----T-----g----- <-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA -----a-----C-----C-----
	10-1, 2	III (DP-46)	<-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTG GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG AAG GGC -----a-----TC-----T-----g----- <-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA -----a-----C-----C-----
9	5-3, 4	III (VH 26)	<-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTA TCA GCT ATT GGT ACT GGT GGT GAT ACA TAC TAT GCA GAC TCC GTG AAG GGC -----G-----T-----A-----G-----g-----G----- <-----FR3-----> CGG TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG GCC GTA TAT TAC TGT GCG AAA -----C-----
		III (VH 26)	<-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGA CTG GAG TGG ATT GGG TAT ATC TAT TAC AGT GGG AGC ACC AAC TAC AAC CCC TCC CTC AAG AGT -----a-----C-----c-----G-----G-----T----- <-----FR3-----> CGA GTC ACC ATA TCA GTA GAC ACG TCC AAG AAC CAG TTC TCC CTG AGG CTG AGT TCT GTG ACC GCT GCG GAC ACG GCC GTA TAT TAC TGT GCG AGA -----g-----T----- <-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTG GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG AAG GGC -----a-----TC-----T-----g----- <-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA -----a-----C-----C-----
	9-1, 2, 3	IV (VH 4.11)	<-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTG GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG AAG GGC -----a-----TC-----T-----g----- <-----FR3-----> CGA GTC ACC ATA TCA GTA GAC ACG TCC AAG AAC CAG TTC TCC CTG AGG CTG AGT TCT GTG ACC GCT GCG GAC ACG GCC GTA TAT TAC TGT GCG AGA -----g-----T----- <-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTG GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG AAG GGC -----a-----TC-----T-----g----- <-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA -----a-----C-----C-----
		III (DP-46)	<-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTG GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG AAG GGC -----a-----TC-----T-----g----- <-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA -----a-----C-----C-----
	10-1, 2	III (DP-46)	<-----FR2-----> <-----CDR2-----> TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTG GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG AAG GGC -----a-----TC-----T-----g----- <-----FR3-----> CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA -----a-----C-----C-----

^a Dashes indicate bases identical to the reference sequence, and only differences are shown using the uppercase letters for R mutations and lowercase letters for S mutations.

Table 4. V_H Genes Analysis of Peripheral Lymphocytes Derived from HIV-Related LIP

Case	Clones	Germline V _H (V _H family)	Similarity (%)	Replacement: Silent			D segment	J _H segment	Frame
				CDR	FRs	<i>p</i> *			
2	2P-1	DP-25 (I)	95.0	3.00	0.67	0.176	D3	JH4	In
	2P-2	V1-46 (I)	95.3	2.00	0.50	0.302	DN1	JH1	In
	2P-3	3-33 (III)	98.4	1.00	0.00	0.384	DXP5 & D1	JH4	In
	2P-4	DP-54 (III)	94.5	5.00	3.00	0.026*	DLR1 & D21/7	JH4	In
	2P-5	9-1 (III)	93.1	2.00	0.75	0.179	D21/10	JH4	In
	2P-6	DP-77 (III)	96.8	2.00	0.50	0.269	DA4	JH6	In
	2P-7	DP-49 (III)	94.5	5.00	3.00	0.027*	D23/7 & DK5-C	JH6	In
	2P-8	DP-70 (IV)	98.9	0.00	0.00	0.608	D4	JH3	In
	2P-9	hv4005 (IV)	94.4	2.50	2.00	0.026*	D6	JH6	In
	2P-10	DP-66 (IV)	95.6	3.00	0.33	0.147	DM2	JH6	In
3	3P-1	DP-50 (III)	95.6	1.00	0.20	0.336	DLR4	JH4	In
	3P-2	DP-83 (III)	97.3	0.50	0.00	0.410	DXP'1	JH4	In
	3P-3	VH26 (III)	95.8	5.00	2.00	0.014*	DM5 & DLR5	JH5	In
	3P-4	COS-16 (III)	95.2	1.50	0.33	0.201	DK4	JH6	In
	3P-5	V3-53 (III)	95.2	2.50	1.00	0.024*	D3-C	JH6	In
	3P-6	YAC-5 (III)	98.4	1.00	0.00	0.402	DA1/4	JH6	In
	3P-7	DP-72 (IV)	95.6	4.00	2.00	0.046*	D21/9 & DN1	JH4	Out
	3P-8	VH4.22 (IV)	98.4	1.00	0.00	0.384	DN4	JH4	In
	3P-9	V4-31 (IV)	94.4	4.00	1.50	0.088	DLR4	JH4	In
	3P-10	DP-68 (IV)	97.8	1.00	0.00	0.410	DXP4	JH6	In
4	4P-1	DP-15 (I)	96.2	3.00	0.50	0.115	D2	JH1	In
	4P-2	COS-5 (I)	98.4	1.00	0.00	0.384	DN1-C	JH4	In
	4P-3	DP-53 (III)	93.4	6.00	1.50	0.016*	DLR4 & DK1	JH4	In
	4P-4	DP-34 (III)	96.2	2.00	0.33	0.246	DXP'1	JH5	In
	4P-5	DP-81 (III)	94.5	2.00	1.00	0.088	D21/10	JH5	In
	4P-6	COS-15 (III)	92.9	6.00	0.20	0.023*	D4 & DM2	JH6	In
	4P-7	DP-59 (III)	95.1	3.00	0.25	0.176	D6-C	JH6	In
	4P-8	DP-63 (IV)	96.1	4.00	0.00	0.029*	DK4 & inv.D21/7	JH4	Out
	4P-9	V4-31 (IV)	95.0	2.00	0.20	0.302	DM2	JH6	In
	4P-10	DP-72 (IV)	97.2	1.00	0.00	0.410	DLR5	JH6	In
5	5P-1	hv1051 (I)	94.5	1.00	0.14	0.268	DA1/4	JH4	In
	5P-2	VH26 (III)	98.9	0.00	0.00	0.640	DN1	JH4	In
	5P-3	1.9III (III)	94.0	6.00	3.00	0.010*	D2 & DXP'1	JH4	In
	5P-4	V3-35 (III)	95.6	1.50	0.50	0.147	D22/12	JH4	In
	5P-5	DP-42 (III)	94.4	5.00	0.33	0.026	D23/7	JH4	In
	5P-6	DA-9 (III)	97.3	1.00	0.00	0.410	DK4	JH6	In
	5P-7	YAC-9 (III)	98.9	0.00	0.00	0.640	D6	JH6	In
	5P-8	DP-64 (IV)	95.0	3.00	0.25	0.176	DLR4	JH4	In
	5P-9	DP-67 (IV)	97.8	0.00	0.00	0.410	DM2-C	JH5	In
	5P-10	V71.4 (IV)	93.3	6.00	1.50	0.016*	D4 & inv.DM1	JH6	In

* *p* is the probability of obtaining the number of R mutations found in the CDR by chance.

and King, 1979). The accumulation of R mutations in CDR indicates a role for an antigen in the selection of lymphoma cell clones and that the cell of origin has been exposed to the hypermutation mechanism in the germinal center. For all minor oligoclonal or monoclonal populations, CDR showed more R mutations than expected, with significant ($p < 0.05$) clustering indicative of antigen selection.

Generally, somatic mutation is thought to occur randomly in Ig V_H genes but is largely seen in the CDRs because of antigen selection. However, 11 of 33 (33.3%) oligoclonal clones in HIV-related LIP showed an unusual number of nucleotide differences in the FRs, in particular in FR3. These framework mutations

that occurred with high frequencies could not be demonstrated in HIV-negative LIP. This degree of variation exceeds the usual mutation rate for frameworks, suggesting a role for framework residues in antigen binding. Higher FR R:S mutation ratios result in amino acid interchanges, suggesting an antigen-driven process. An unusual number of nucleotide differences occur in the FRs of Ig V_H genes in monoclonal cell lines derived from the peripheral blood cells of HIV-infected patients (Andris et al, 1991).

D-D recombination is believed to be an important mechanism for antigen-driven selection and is very rare ($< 1/33,000$) in pre-B cells. Thirty-one of 33 (93.9%) minor oligoclonal clones in HIV-related LIP

Table 5. CDR3 Sequences of PCR Products Obtained from Patients with LIP

Case	Clone	3' II end of V	N(P)-D-N region	5' end of J	CDR3 length (N-D-N length)
1	1-1, 2, 3	GCGAGA	gggttcATTGTAGTGGGAGTTATGgcccg D2 & D21/10	CAGCAC JH1	42 (30)
	1-4, 5	GCGAGA	acgttCAGCTGGTACTGGAACtAttgcatact D4-C & DM1	GATGTC JH3	45 (33)
	1-6, 7	GCGAGA	ccagtcctcATGGTTCGGCAGCTCGTcaagct DXP'1 & DN4	GACTAC JH4	44 (32)
	1-8, 9	GCGAGA	ggggaattgggTATAGCAGCTCGTcaaaacaaaccc DN4	TACTTTGACTAC JH4	54 (36)
2	2-1, 2, 3	GCGAGA	aattcagttactTAGTTACTGTAGTGGTGGTAGCccgggt DA1/4 & D2	GGTATGGACGTC JH6	57 (39)
	2-4, 5	GCGAGA	atttttgaggTATAGTGGATTTTGGAcccttg DK1 & DXP4	TAC JH4	42 (33)
3	3-1, 2	GCGAGA	ggggaatcggaTAGCAGCTACCggACAGTAACTccatgcca D2-C & DA1	CAGCAC JH1	54 (42)
	3-3, 4, 5	GCGAGA	cattccgatGATATTTTcACTATGATAGTAGTttca D21/5 & D21/9	ATGGACGTC JH6	51 (36)
	3-6, 7	GCTAGA	ccatgggaTAGTAGTAGTGGTTATTAccgatgggggggc DLR4 & D23/7	TACGGTATGGACGTC JH6	60 (39)
4	4-1, 2	GCGAGA	ccagagggggcccGATTACGTTTGGGGTTCGGGGAGcaat D21/10 & DXP'1	TTTGACTAC JH4	54 (39)
	4-3, 4	GCGAAA	aagactaatccgacGATAGTAGTGGACTGGTTATTATgcacc D21/9 & D5	GGTATGGACGTC JH6	60 (42)
	4-5, 6	GCGAGA	taggATTGTAGTAGTCGGGAGTTATTcgaac DLR4 & D6	TACTACGGTATGGACGTC JH6	57 (33)
5	5-1, 2	GCGAAA	caccccggaCTATGATAGTAACTGGTGTcccaggg D21/9 & DLR1	TTTGATGTC JH3	51 (36)
	5-3, 4	GCGAAA	ggaggttcttTGGTAGCTGCTTTGGACTGGTTcacaccc D2 & DXP5	TTTGACTAC JH4	54 (39)
	5-5, 6	GCAAGA	ggcacttgggAACCGGAACAGTGCTACGATccaaccc DM2 & DK5	GGTATGGACGTC JH6	57 (39)
8	8-1, 2	GCGAAA	tggCTGGGGAcc DHQ52	TAC JH4	21 (12)
9	9-1, 2, 3	GCGAGA	gtaagggcttCAGCTGGTACgacctcggtcgac DN1	TACTTTGACTAC JH4	51 (33)
10	10-1, 2	GCGAGA	ccagaggTAGTGTCTCACCACCAat DK5 & D3-C	CAC JH1	33 (24)

were encoded by 2 or 3 different D segments. In contrast, 2 of 7 (28.6%) minor monoclonal populations in HIV-negative LIP showed D-D fusion, and only about 10% of the Ig V_H genes expressed by normal adult B cells are reported to have CDR3 with evidence of D-D fusion (Yamada et al, 1991). The D-D fusions of HIV-related LIP may reflect an enhanced propensity of B cells to undergo D-D fusion. It is interesting that each of the characterized anti-HIV antibodies from patients infected with HIV has a heavy chain CRD3 that also probably results from D-D fusion (Andris et al, 1991; Marasco et al, 1992; Moran et al, 1993). Such D-D fusions may enhance the probability of the expression of antibodies capable of binding HIV glycoproteins. Considering that purified splenic B cells can be stimulated to induce recombination activating gene (RAG) expression by treatment with IL-4 along with either anti-CD40 or lipopolysaccharide (Hikida et al, 1996) and that RAG-1 and RAG-2 expression in mature splenic B cells can induce V_H-(D)-J_H gene recom-

bination (Papavasiliou et al, 1997), local Th1 imbalance of HIV-related LIP may cause receptor editing that leads to greater D-D fusions in the HIV clones.

In summary, we report observations consistent with the different immunological situations among EBV-negative, HIV-related, and HIV-negative LIPs.

Materials and Methods

Patients

For this study, 14 cases were collected from our own files and from those of other hospitals for the years 1982 to 1999 (Table 1). The study was performed on tissue samples obtained by open-chest lung biopsy or autopsy from 14 patients with a histopathologic diagnosis of LIP. Of these 14 cases, four were positive for EBV by PCR analysis and were excluded from the study. The remaining 10 samples were acceptable for our study. Five cases were HIV-positive patients with

normal absolute CD4⁺ T cell numbers classified as group II or III according to the Centers for Disease Control (CDC) classification (Centers for Disease Control, 1986). The remaining five samples were from HIV-negative cases. Samples of heparinized blood were obtained from patients (cases 2 to 5) with HIV-related LIP. Lymphocytes were prepared by Ficoll-Hypaque (Pharmacia, Tokyo, Japan) gradient centrifugation.

Histopathologic Studies

Open-chest biopsy or autopsy specimens were fixed in 10% formalin and embedded in paraffin wax. The sections were subjected to hematoxylin and eosin (H&E) and immunohistochemical stainings. Immunohistochemical studies were performed on the following markers: leukocyte-common antigen (LCA; Dakopatts, Glostrup, Denmark), CD20 (L26, Dakopatts), CD45RA (MB1; Euro-Diagnostica, Malmö, Sweden), CD43 (MT1, Euro-Diagnostica), CD45RO (UCHL1, Dakopatts), and anti-kappa and lambda light chains (Dakopatts).

Microdissection

To increase the concentration of lymphocyte DNA relative to total tissue DNA, surgically resected materials were microdissected in the following way. An H&E section was visualized under a $\times 5$ objective and the nonlymphoid tissue was carefully scraped off leaving a zone as small as 25 mm² rich in the infiltrate of interest. Four serial sections, 5- μ m thick, were laid on top of the first H&E section and the corresponding zones were selected; the rest was scraped off. Microdissected areas from four sections were then scraped into one tube.

DNA Extraction and PCR

DNA was isolated from the microdissected materials and peripheral lymphocytes using TAKARA DEXPAT (Takara Corporation, Kyoto, Japan) and COLLECTAGENE (Takara Corporation).

The two-step semi-nested PCR method was performed in a GeneAmp PCR 9600 system (Perkin Elmer-Cetus, Norwalk, Connecticut). For the amplification of Ig V_H genes, the primer sequences were as follows: 5'-TGG-[A/G]TCCG[A/C]CAG[G/C]C[T/C][T/C]C[A/G/T/C]GG-3' (termed FR2) for the second framework portion of the Ig V_H region, and 5'-TGAGGAGACGGTG-ACC-3' (termed LJH) and 5'-GTGACCAGGGT[A/G/C/T]CCTTGGCCCCAG-3' (termed VLJH) for the J_H region (Ramasamy et al, 1992; Trainor et al, 1991). Each PCR experiment included a sample without DNA template as a negative control and a sample with DNA extracted from a Burkitt's lymphoma lymph node (as a positive control) whose Ig V_H gene rearrangement was detected by Southern blotting. For amplification, the high-fidelity polymerase, Pfu, was used. One PCR cycle consisted of denaturation for 45 seconds at 94° C, annealing for 45 seconds at 60° C, and extension for 45 seconds at 72° C. The first step consisted of 30 cycles with primers FR2 and LJH and 5 μ l of template DNA; the second step

consisted of 20 cycles with FR2 and VLJH, with 10 μ l of a 1 to 1000 dilution of the first step PCR product as a template.

Our procedure for PCR amplification of the EBV genome is given in detail in Oda et al (1993). For the amplification of the EBV genome, one set of primers specific for the *Bam*HI W region was synthesized: 5'-CACTTTAGAGCTCTGGAGGA-3' (termed EBV1) and 5'-TAAA-GATAGCAGCAGCGCAG-3' (termed EBV2). These primers give rise to a 153 base-pair amplified product. Each PCR experiment included a negative control and a sample with DNA extracted from a RAJI cell (as a positive control). For amplification, the high-fidelity polymerase, Pfu, was used. The samples were subjected to 30 amplification cycles (45 seconds at 94° C, 45 seconds at 54° C, and 45 seconds at 72° C).

The amplified DNA was precipitated from the PCR product (40 μ l) and dissolved in TE buffer (5 μ l). The mixture was electrophoresed in a 2% agarose gel and stained with ethidium bromide to visualize the DNA under short-wavelength UV light.

Sequence Analysis

The other portion of the Ig V_H gene PCR product was ligated to the PCR-BluntII-TOPO vector, and the ligation mixture was transformed into One Shot™-competent cells using a Zero Blunt™ TOPO PCR Cloning Kit (Invitrogen Corporation, Carlsbad, California). Ligated clones were chosen at random and phage DNA was purified. The inserts in the pCR-BluntII-TOPO vector were sequenced by the Dye Primer method using a Taq Dye Primer Cycle Sequencing Core Kit (Applied Biosystems, Norwalk, Connecticut). The Ig V_H gene sequences were aligned to European Molecular Biology Laboratory (EMBL)/Gen Bank databases.

Analysis of Mutations

The probability (*p*) that an R mutation will localize to CDR was calculated using the formula $CDR_f \times CDR_{rel}$, where CDR_f is the replacement frequency inherent to the CDR sequence, and CDR_{rel} is the relative size of the CDR. We calculated the probabilities that an excess or scarcity of R mutations arose by chance using the binomial distribution model $p = (n!/k! [n-k]!) q^k (1-q)^{n-k}$, where *n* is the total number of R mutations and *k* is the number of observed R mutations in the CDR (Bahler and Levy, 1992; Chang and Casali, 1994).

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