Frequent T and B Cell Oligoclones in Histologically and Immunophenotypically Characterized Angioimmunoblastic Lymphadenopathy

John Lewis Smith,* Elizabeth Hodges,*
Christine Tara Quin,* Keith Paul McCarthy,[‡] and
Dennis Howard Wright[†]

From the Molecular Immunology Group of the Wessex
Immunology Service* and the Department of Pathology,[†]
Southampton University Hospitals National Health Service Trust,
Southampton; and the Department of Pathology,[‡] Cheltenham
General Hospital, Cheltenham, United Kingdom

The identification of clonal rearrangements of T cell receptor (TCR) genes is central to the diagnosis of T cell lymphomas. However, in angioimmunoblastic lymphadenopathy (AILD), first described as a non-neoplastic proliferation associated with immunodeficiency, the heterogeneity of TCR and IgH gene rearrangements suggest that some cases may harbor multiple lymphoid clones. In this study we have isolated DNA from archival paraffin biopsy material from 22 cases of AILD identified on the basis of classical histological and immunohistochemical features with the aim of establishing the occurrence of clones and oligoclonal products, the frequency of TCR and immunoglobulin heavy chain (IgH) variable (v) gene use, and the relationship of these findings to the presence of Epstein-Barr virus. DNA extracted from the biopsies was amplified using the polymerase chain reaction (PCR) and sequenced to detect functional and non-functional gene rearrangements. Epstein-Barr virus-encoded short RNA species (EBERs) were detected using *in situ* hybridization combined with immunohistochemistry to identify the phenotype of the Epstein-Barr virus-infected cells. Fifty-seven clonal products were found in 20/22 patients: TCR γ clonal products were identified in 16/22, TCR β clonal products in 16/22 and IgH clonal products in 6/22 cases. Oligoclonal PCR products were seen for TCR in 3/22 and for IgH in 3/22 cases. In one biopsy PCR products from all reactions were polyclonal. Sequence analysis revealed functional TCR γ , TCR β , and IgH sequences in 6/12, 9/11, and 8/8 cases, respectively. Functional TCR and/or IgH oligoclonal products were detected in 6/20 (30%) cases. In addition, nonfunctional TCR and IgH sequences were found in 11 cases. EBERs were identified in 18/20 cases varying from occasional to 25 to 30% nuclei staining and were associated with both T and B cells, although the majority were of indetermi-

nate phenotype. The presence of EBERs was not associated with all clonal IgH gene rearrangements but was associated with B cell oligoclonal products. Patterns of gene recombinations indicated that the majority of TCR γ recombinations used GV1 and GJ1S3/2S3 genes. Six out of eleven cases used TCR BV4S1 or BV2S1 genes associated with various BJ and BD1/2 genes. No common IgH gene usage was identified, but 8 clones had varying degrees of replacement and silent mutations (0.6–10.1%), consistent with B cell clones having undergone somatic mutation in the germinal center, and 3 clones harbored unmutated V genes, consistent with naive B cells. Our data do not support the concept of AILD as a clearly defined peripheral T cell lymphoma (PTCL). Rather, they suggest that AILD as defined by histology and immunohistochemistry is either a heterogeneous entity or represents a lymphoproliferation associated with immunodeficiency in which clonal T cell or B cell proliferation may occur. (*Am J Pathol* 2000, 156:661–669)

Early reports of AILD stressed the nonneoplastic nature of this disease,^{1,2} noting that neither the cytology nor the pattern of infiltration seen in biopsies or autopsies was that of a neoplasm. The disease was thought to be due to abnormal immune regulation, the majority of patients dying of infections, often with opportunistic organisms.² An association with lymphoma was, however, noted by Lukes and Tindie,³ who reported the development of immunoblastic lymphoma in 3 of their 32 patients. Subsequently, there has been controversy over the separation of AILD from AILD with immunoblastic lymphoma (IBL) based on histological features such as atypical clear cells, which has contributed to the variation in the reported incidence of lymphoma in AILD from 0 to 35%.^{4–6} In addition, both groups show clonality or lack of clonality.^{4,7} Frizzera et al⁸ has proposed that three AILD-related disorders can be recognized as AILD, AILD-like dysplasia, and AILD-like peripheral T cell lymphoma (PTCL) based on a combination of morphology, phenotype, clonal-

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Address reprint requests to Dr. J. L. Smith, Molecular Immunology Group, Wessex Immunology Service, Southampton University Hospitals NHS Trust, Southampton SO16 6YD, UK. E-mail: smithj6@hotmail.com.

Table 1. Clinical Details of AILD Cases

Case no.	Age	Sex	Lymphadenopathy	Splenomegaly	Skin rash	Fever	Hemolytic anemia	Cytopenia
1	67	F	+	+	+	+		
2	72	M	+			+		
3	62	F	+			+		
4	70	F	+	+	+			
5	61	M	+		+	+		
6	58	F	+			+		
7	60	M	+			+		
8	45	F	+	+				
9	48	M	+				+	
10	66	M	+		+			
11	57	F	+			+		+
12	61	M	+	+				
13	75	F	+		+			
14	62	M	+			+		
15	71	F	+	+	+			
16	62	M	+					
17	68	M	+	+				
18	63	M	+					
19	52	M	+	+				+
20	50	M	+		+	+		
21	60	F	+					
22	59	M	+		+	+		

+ Clinical symptoms of patients. Blanks represent the absence of the symptoms in the patients.

ity, and karyotype. However, since karyotypic abnormalities⁹ and clonal rearrangements of TCR and/or immunoglobulin (Ig) genes may occur in most cases, irrespective of morphology, this proposal is difficult to sustain.

Clonal rearrangements of TCR genes are a useful additional diagnostic index of T cell lymphoma but the interpretation of clonality in AILD is complicated by the heterogeneity of TCR and IgH gene rearrangements.⁷⁻¹⁶ In addition, clonal rearrangements have been shown to regress or appear during the course of the disease, suggesting that these cases may harbor multiple clones¹¹ similar to those found in cases with acquired immunosuppression.^{13,16} This conclusion is supported by cytogenetic studies that reveal a characteristic chromosome pattern consistent with the frequent presence of karyotypically unrelated abnormal clones, which may also appear and regress in a high proportion of AILD cases.^{9,17} Given the heterogeneous nature of AILD it is reasonable to propose that a relationship between reactive and clonal T and B cell lymphoproliferations exist in this entity.

Clonal studies in AILD have depended on the identification of rearranged alleles by Southern blot analyses or detection of clonal products after polymerase chain reaction (PCR) amplification. The detection of gene rearrangements do not necessarily imply that they are functional indicators of lymphocyte lineage, as nonfunctional rearrangements may occur in both T and B cells during differentiation. Cross-lineage IgH rearrangements also occur in T cell lymphoma and leukemia,¹⁸ with a higher incidence occurring in lymphoblastic T cell malignancies (10–15%) compared to mature T lymphocyte neoplasms (5%).¹⁹ This problem can be overcome by sequence analyses of PCR products to reveal the functional or nonfunctional nature of a specific TCR or IgH gene rearrangements in these cases.

We have undertaken a retrospective molecular study of T and B cell populations in 22 archival cases of AILD using PCR amplification of DNA isolated from formalin-fixed biopsy tissue to detect clonal TCR β , TCR γ , and IgH gene rearrangements for the purpose of establishing i) occurrence of clonal/oligoclonal TCR and IgH rearrangements among the group; ii) the presence of functional rearrangements as established by sequence analysis to investigate T cell lineages associated with each case, evidence for preferential V gene family use by these cell populations, and presence of B cell clones; and iii) the role, if any, of EB virus in the etiology of these lymphoproliferations, especially in those cases where functional IgH rearrangements have been identified.

Materials and Methods

Patients

Twenty cases diagnosed as AILD in the histopathology files of Southampton University Hospitals NHS Trust (SUHT) between 1990 and 1995 were entered into the study. Two additional cases were referred by one of us (K. McC.). The patients ranged in age from 45 to 75 years and the male:female ratio was 13:9. Clinical details of patients in the study are given in Table 1. Biopsies were derived from a variety of lymph node sites and fixed in 10% formalin. All biopsies were reviewed and the histological diagnosis confirmed by immunohistochemistry. One patient (case 17) had a splenic biopsy 3 months postpresentation.

In addition, 18 formalin-fixed, paraffin-embedded tissue biopsies diagnosed as reactive were collected from the SUHT files and included as polyclonal controls for the study. These biopsies included 13 lymph nodes, 2

spleens, and 3 tonsils. In addition, DNA isolated from blood collected from four healthy normal donors was also included as a control in all PCR assays.

Histology and Immunohistochemistry

Paraffin sections were cut at 5 μ m and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Giemsa, and reticulin using the Gordon and Sweet method. Immunohistochemistry was performed using the streptavidin-biotin peroxidase complex technique on 5- μ m sections mounted on silane-coated slides. The antibodies used included CD3, BF1(TCR β), and 1F8(CD21) on sections pre-treated with pronase, CD8, UCHL1(CD45RO), and CD79a on sections pre-treated in a microwave with citrate buffer, CD4 on sections pre-treated in a pressure cooker in EDTA, and L26(CD20) on sections with no pretreatment.

Epstein-Barr Virus (EBV)

Formalin-fixed, paraffin-embedded sections were prepared from all biopsies and used for *in situ* hybridization (EBV polyprobe kit, Novacastra Laboratories, UK) to detect EBV-encoded short RNA species (EBER). EBV-positive controls consisting of a known EBV-positive case and negative controls were assayed in the same run. Double staining for CD3, CD45RO, CD20, and EBER was performed on 5- μ m sections using the streptavidin-biotin complex technique. The peroxidase label was identified using 3-amino-9-ethylcarbazole (AEC), giving a red reaction product. *In situ* hybridization for EBERs was then performed using the Novacastra EBV probe kit following the manufacturer's protocol.

Genotypic Studies

All PCR methods were first established using positive controls including 3 T cell lines and 17 clonal T cell neoplasms previously characterized by Southern blot analysis. For Southern blot analysis, high molecular weight DNA was extracted from the biopsies by routine methods, digested with 3/4 restriction enzymes, Southern blotted, and probed with ³²P-labeled TCR and Ig probes as previously described.²⁰

PCR Analysis of TCR γ , TCR β , and IgH Gene Rearrangements

DNA was isolated from paraffin-embedded biopsy material as described previously.²¹ TCR γ PCR analysis was adapted from McCarthy et al²² and used primers VG11 (detects VGI genes, VG101 (detects VGIII and VGIV genes), VG9 (detects VGII gene), JG12 (detects JG1.3 and JG2.3 genes) and JP12 (detects JG1.1 and JG2.1 genes). The primers were used in the following combinations: VG11, VG101, VG9, JG12 and VG11, VG101, JP12. TCR β PCR analysis was performed using VBJB2 and DB1JB2 primer combinations.²⁴ TCR γ and - β PCR samples were subjected to 30 cycles of PCR (93°C for 1

minute, 52°C (TCR β) or 55°C (TCR γ) for 1 minute, 73°C for 1 minute). PCR products were electrophoresed through a 10% polyacrylamide gel. Sizes of the TCR PCR products ranged from 75 to 110 bp for TCR γ products and from 55 to 100 bp for TCR β products. Clonal bands are defined as one or two narrow sharp intense bands visible on polyacrylamide gels after electrophoresis, whereas polyclonal PCR products appear as a smear within the appropriate size range. Oligoclonal populations are defined by three or more distinct bands. These definitions also apply to IgH FR2 PCR analyses.

Fluorescent TCR β PCR amplification for gene scanning analysis was included in the study due to the reported increased detection rate of TCR β in T cell leukemia/lymphoma.²⁵ PCR amplification was performed using a seminested approach using VBcon, JBI(2), and JBII(2) primers in the first round of amplification, and VBcon (5' fluorescently labeled), JBI(1) and JBII(1) for the second round of amplification.²⁵ Two percent of the volume of the first-round PCR products was used for the second round of amplification. Samples were subjected to 40 (first round)/30 (second round) PCR cycles (92°C for 1 minute, 50°C (first round)/56°C (second round) for 40 seconds, 72°C for 30 seconds). For analysis on the ABI automated gene sequencer, 1 μ l of PCR product was mixed with 3 μ l formamide and 0.5 μ l of an internal size standard (Genescan-2500 Rox; Applied Biosystems, Warrington, UK) and denatured for 3 minutes at 90°C before being separated on a high resolution polyacrylamide gel and analyzed using GENESCAN 672 software. Products from clonal cell populations produce one or two sharp peaks of fluorescence corresponding to the PCR-amplified clonal rearranged allele(s), whereas DNA extracted from normal polyclonal peripheral blood produce a fluorescence spectrum composed of polyclonal PCR fragments of different sizes in a normal Gaussian distribution. Oligoclonal scan profiles are observed as three or more distinct peaks above a normal polyclonal background.

The TCR PCR methods in this study were established using DNA from clonal T cell tumors, including 4 lymphocytic leukemias, 7 lymphoblastic lymphomas, 2 unclassified peripheral T cell lymphomas, 2 enteropathy-associated T cell lymphomas, and 1 T cell angiocentric lymphoma, previously characterized by Southern blot analysis as clonal for TCR γ and TCR β loci. In these experiments, TCR γ rearrangements were detected by PCR in 16/17 (94%) cases using VJ and VJP primers. TCR β PCR with primers for VJ and DJ detected rearrangements in 8/17 (47%) and 10/17 (59%) cases, respectively; using both primer combinations the success rate was 71%. In comparison, TCR β gene scanning was successful in 13/16 (81%) of cases. IgH Southern blot and PCR analyses were completed in all cases and IgH clonal gene rearrangements were identified in 2 cases by both techniques. Three T cell lines (Molt4, Jurkat, and HPBALL) were also shown to be clonal by TCR γ PCR analysis and gene scanning; two of the lines were shown to be clonal with McCarthy's VJ primers but no clonal product was found with Molt4 due to lack of specificity of the consensus VB primer with BV2S1.²⁴ Sequence analysis of Molt4 and Jurkat confirmed published sequenc-

es.^{26,27} In a study of frozen and paraffin material of the same biopsy from 30 patients, concordant results were obtained in all but one case. In that case, the DNA was too degraded from the paraffin biopsy to produce unambiguous PCR results.

IgH FR2 PCR analysis was performed using a semi-nested PCR amplification method.²⁸ The first-round PCR consisted of 30 cycles of amplification of 250 ng template DNA using primers FR2 and LJH followed by 20 cycles of amplification using 0.5 μ l from the first round PCR using primers FR2 and VLJH. Cycling conditions for both rounds consisted of 93°C for 45 seconds, 50°C for 45 seconds, and 72°C for 110 seconds. PCR products were electrophoresed through a 5% polyacrylamide gel. Sizes of the IgH FR2 PCR products ranged from 240 to 280 bp. Our detection rate was 88% for demonstrating B cell clonality in a series of 40 immunohistologically characterized B cell neoplasms, including 24 B cell leukemias (22 B cell chronic lymphocytic leukemia, 1 B cell prolymphocytic leukemia, 1 hairy cell leukemia) and 16 B cell non-Hodgkin's lymphoma (data not shown).

Sensitivity

DNA from known positive clonal controls were serially diluted (comprising 100, 50, 40, 30, 20, 10, 5, 2, and 1% of tumor DNA) with DNA from reactive polyclonal controls. After appropriate PCR amplification, clonal products could be detected at a level of 2% clonal DNA on a polyclonal background smear/electrophoregram for all PCR protocols.

Cloning and Sequencing of PCR Products

Amplified TCR γ , TCR β , and IgH FR2 PCR products were gel electrophoresed and subsequently purified using GeneClean or MERmaid kits (Anachem, Luton Bedfordshire, UK) before transformation into JM109-competent cells (Promega, Madison, WI). We preferentially sequenced clonal TCR β products from TCR β scanning, because VB gene usage can be assigned from these products. Clones were randomly selected for each case. In some instances, the final extension time of the PCR cycling was extended to 1 hour to increase the cloning efficiency of PCR products.²⁹ Single-stranded DNA was prepared and sequenced by the dideoxy chain termination method using sequenase (Amersham International PLC, Little Chalfont, Buckinghamshire, UK) and M13 primers. Sequence data analysis was performed using GenBank, Vbase,³⁰ and current databases with MacVector 4.1.4 sequence analysis software for determination of V, D, J gene usage and N region addition, as well as stop codons and frame shifts which result in nonfunctional sequences. Clonal populations were defined where two or more sequences with identical CDR3 sequences were obtained from two independent PCR reactions.

Results

Histology and Immunohistochemistry

The lymph nodes in all cases of AILD showed loss of normal lymph node architecture with no reactive follicles and occasional burned-out germinal centers. In most biopsies the lymphoid infiltrate extended through the capsule of the node, often into the perinodal fat. Despite this, the subcapsular sinus was usually easily visible and often appeared to gape. All biopsies showed a proliferation of arborizing high endothelial venules well delineated by silver staining for reticulin and PAS staining that highlighted amorphous perivascular material. The lymphoid cells ranged from small lymphocytes and plasma cells through medium and large lymphoid cells. Clusters of clear cells of variable size and prominence were seen in all cases. Morphologically atypical cells accompanied by atypical mitoses were seen in only one case (case 19). These occurred as single cells with large, often irregular, nuclei and included multinucleated forms.

Immunohistochemistry showed the lymphoid infiltrate to comprise predominately CD3+, CD45RO+ T cells, mostly of the CD4+ subclass, expressing TCR β . The majority of these cells were small to medium-sized lymphocytes with occasional blast cells. The clusters of clear cells were always of the T cell phenotype. A diffuse infiltrate of small CD8+ lymphocytes was present in all biopsies. These were usually a minority population constituting 5 to 20% of all T cells. However, in 2 cases (cases 3 and 12) they constituted 50 to 60% of the T cells.

The B cell population identified by antibodies to CD20 and CD79a was always greatly reduced, consisting of clusters of small lymphocytes that often appeared to have been pushed to the periphery of the lymph node. Occasional B blasts, often situated at the edge of aggregates of small B cells, were present in all cases. Small aggregates of B blasts were seen in 2 cases (case 9 and 17). The atypical cells seen in case 19 were CD20-negative and some expressed membrane CD3; however, the close proximity of small T cells made it difficult to determine the phenotype of many of the atypical cells. Antibody to CD21 showed an expanded network of dendritic reticulum cells either focally or throughout the node in all cases.

The histology of the follow-up splenectomy on case 17 showed a follicle center cell lymphoma, grade I.

Genotypic Findings

Control Tissue

In 18 biopsies with reactive histology and 4 peripheral bloods, polyclonal products were found in all reactions. TCR β sequence analysis of 34 sequences from 2 peripheral bloods and 2 reactive tonsils revealed different sequences within the same case and between the four cases.

TCR β PCR products from 8 clonal T cell cases (2 lymphocytic leukemias, 4 lymphoblastic lymphomas, and 2 PTCL), used for verification of PCR methods (see Meth-

Table 2. EBV and Genotype of AILD Cases

Case no.	EBV	Genotype					IgH FR2/JH
		TCR				G	
		VJ	VJ	DJ	VJ Scan		
1	+	C ²	P	C	O ³	O ³	
2	S	C ²	P	P	P	P	
3	—	C	P	P	P	P	
4	+	C	C	P	P	P	
5	n.t.	C	C	P	P	P	
6	—	C*	P	P	P	C	
7	+	C*	C	P	C	P	
8	+	O ³	C	C	C ²	P	
9	+	C	P	C	P	P	
10	S	C ²	P	P	C	O ³	
11	—	C	P	P	C ²	C	
12	+	C ²	C ²	C ²	P	P	
13	—	C, C* ²	P	P	C	C	
14	n.t.	P	C	P	P	P	
15	+	C	P	P	C ²	O ³	
16	+	C	P	P	C	P	
17	+	P	P	P	C ²	P	
18	S	C	P	P	C ²	C ²	
19	—	P	P	P	C ²	C	
20	+	C	P	P	P	C	
21	+	P	P	P	O ³	P	
22	+	P	P	P	P	P	

EBV staining: +, positive; —, negative; n.t., not tested; S, occasional labeled nuclei identified.

Genotype: C, clonal; P, polyclonal; O, oligoclonal.

*PCR product identified by VGJGP primer combination.

Superscripts indicate number of bands identified by gel electrophoresis/fluorescent electrophoregrams, if > 1.

ods), were sequenced. In each case a clonal sequence was found, corresponding in size to the clonal peak on TCR β scan analysis.

AILD Study Group

Twenty-five TCR γ clonal bands were found in 17 cases (cases 1–13, 15, 16, 18, and 20) including 3 oligoclonal bands in case 8 (Table 2). With the primer pair VGJG, 19 clonal bands were detected in 15 cases, with 2 or more clonal bands being detected in cases 1, 2, 8, 10, and 12. In cases 6, 7, and 13, clonal products were identified with the VGJGP primer pair combination.

TCR β clonal bands, assessed by electrophoresis for VJ and DJ primers, were found in 6 and 4 cases, respectively (Table 2). In case 12, two clonal bands were detected by VJ as well by DJ primers. In contrast, TCR β clonal bands defined by scanning of fluorescent products after PCR amplification were found in 10/22 cases; 1 clonal and 2 clonal peaks were identified in 4 cases (cases 7, 10, 13, 16) and 6 cases (cases 8, 11, 15, 17–19), respectively. In addition, oligoclonal peaks were observed in cases 1 and 21. Representative scans are shown in Figure 1.

IgH clonal products were found in 6 cases after DNA amplification with FR2 primers. In 5 cases (cases 6, 11, 13, 19, 20), one clonal band was detected, whereas in the remaining case (case 18), two clonal bands were

Figure 1. Electrophoretic profiles of fluorescent TCR β products from one positive control, one polyclonal control and six cases. Relative fluorescence intensities (ordinate) are plotted as a function of PCR fragment size (abscissa). Cases are numbered according to Table 2.

detected. In an additional 3 cases (cases 1, 10, 15) oligoclonal bands were observed. In total, IgH clonal and oligoclonal bands were found in 9/22 cases (Table 2, Figure 2).

In case 17 with a follow-up splenectomy 3 years later, DNA analysis revealed a clonal IgH FR2 rearrangement not present in the original biopsy. Furthermore, the orig-

Figure 2. Polyacrylamide gels showing IgH FR2/JH PCR products. **Lanes M:** pBR322 HaeIII molecular weight marker (basepairs indicated on right side). **Lane 1:** positive clonal control; **lane 2:** polyclonal control; **lane 3:** negative control; **lane 4:** case 6; **lane 5:** case 10; **lane 6:** case 11; **lane 7:** case 13; **lane 8:** case 15; **lane 9:** case 18; **lane 10:** case 19; **lane 11:** case 20.

Table 3. Sequence Analysis

Case	Locus		No. of identical clones/total clones	CDR3 sequence	Functional (F)/non-functional (NF)	Gene Usage		
	TCR	Ig				V	D	J
1	G		4/10	SGVYYCATWDENIRNSLAVEQHL	NF	GVI	—	GJ1S3/J2S3
			6/10	SGVYYCATWDRIIRNSLAVEQHL	NF	GVI	—	GJ1S3/J2S3
2	G		5/12	SGVYYCATWDRRIIRNSLAVEQHL	NF	GVI	—	GJ1S3/J2S3
			3/12	SGVYYCATWDRLAIRNSLAVEQHL	NF	GVI	—	GJ1S3/J2S3
3	G		3/5	SGVYYCATWDIQDKKLFSGTTL	F	GVI	—	GJ1S3/J2S3
4	G		5/7	SGVYYCATWDGPYYKKLFSGT	F	GVI	—	GJ1S3/J2S3
5	G		6/12	SGVYYCATWDGL*KKYKLFGT	NF	GVI	—	GJ1S3/J2S3
6	G		3/3	VYYCATWDLPLVGSRYLLKGLSS	NF	GVI	—	GJ1S1
		FR2	4/6	TAVYYCARVSLWFGKEGYMDVWGQGT	F	VH3-21	DH3-10	JH6
7	G		6/8	SGVYYCATWDACVMDQDVCKRD*AH	NF	GVI	—	GJ2S1
	B		2/14	YFCASSGTSYSGYNEQFFGPG	F	BV13S3	BD2	BJ2S1
			2/14	YLCASIATSTSAQAPG*G	NF	BV17S1	?	BJ2S3
8	G		3/15	SGVYYCATWEGEVFGSGTTL	F	GVI	—	GJ1S3/J2S3
			2/15	SGVYYCATWDIL*ETLWQWNNT	NF	GVI	—	GJ1S3/J2S3
			4/15	SGVYYCARTLSYSCRRDGS GTTL	F	GVI	—	GJ1S3/J2S3
	B		4/4	KAVTLWGLAGGCSSYEYFGPGTRLTV	NF	—	BD2	BJ2S7
9	G		11/26	SGVYYCATWDGPYKLFSGS	F	GVI	—	GJ1S3/J2S3
			10/26	SGVYYCATWDDL*ETLWQWNNT	NF	GVI	—	GJ1S3/J2S3
10	G		4/5	SGVYYCATWDVLWHS LAVEQHL	NF	GVI	—	GJ1S3/J2S3
	B		4/9	SSIYLCRQA*REH*SFLWT	NF	BV4S1	BD1	BJ1S1
			4/9	YICSAEAWPYEQYFGAGTRLG	F	BV2S1	?	BJ2S7
		FR2	6/18	TAVYYCARDPPGIAVAGTSVWGQG	F	VH3-21	DH6-19	JH6b
			7/18	TALYYCKDLNGKYSRRVFDHWGQGT	F	VH3-23	DH6-13	JH5
11	G		6/8	SGVYYCATWDEYKLFSGSGTTL	F	GVI	—	GJ1S3/J2S3
	B		3/9	YICSARDWTCENYGPFG	F	BV2S1	BD1	BJ1S2
		FR2	3/9	TAVYYCAREDLVATNWGAW	NF	VH4-61	DH5-12	JH4
			6/9	TAVYTCASMYYYGSENDYHFDYWGQGT	F	VH3-23	DH3-10	JH4
12	G		5/9	SGVYYCATWDGLESSSDWIKT	F	GVI	—	GJ2S1
13	B		4/4	SSIYLCRQALKGTLKLS	NF	BV4S1	BD1	BJ1S1
		FR2	6/7	TAVYYCATNCSSTSCYTGRDYWGQG	F	HHG4(VH3)	DH2-2	JH4
14	B		4/5	YLCASSQDQGYSEYQYFGPGTRLTV	F	#	BD1	BJ2S7
15	B		2/11	YLCASSRTSAPGQYFG	F	BV17S1	BD2	BJ2S4
			6/11	YLCASSQGAHEQFFGGPGTRLTVT	F	BV5S1	BD1	BJ2S1
		FR2	6/17	TAVYYCAKDRFDVGRDLDDSSAMDYWGQG	F	VH3-30	DH3-22	JH4
			3/17	TAVYYCARAGFGYSYGRNFDYWG DG [†]	NF	VH3-21	DH5-5	JH4
			10/17	TAVYYCARGYCSGSGYCPDPWGQGT	F	VH3-33	DH2-15	JH4
16	B		3/3	SSFYICSAEARPYEQYFGPG	F	BV2S1	BD1/2	BJ2S7
17	B		4/6	YLCASSWDRPSGANVLTG	F	BV6S7	BD1	BJ2S6
18	B		5/7	SSIYLCSEAGRGVREAFFGQG	F	BV4S1	BD2	BJ1S1
		FR2	3/6	TAVYYCARDNDWNYFHTPLNYYYYYGM DVWGQG	F	VH3-33	DH3-22	JH6b
			3/6	TAMYHCAGGSKVKIFDWGQG	F	VH3-07	DH3-9	JH4
19	B		8/12	YICSARESTDPKNEQFFAPG	F	BV2S1	BD2	BJ2S1
		FR2	2/9	TAVYYCARLLQYDILTGSYVDYWGQG	F	VH4-61	DH3-9	JH4
20		FR2	5/8	TAVYYCARGLRYFDWLLSYLDYWGQG	F	VH4-30.4	DH3-9	JH4

All distinct PCR clonal products were cloned and sequenced. All sequences were aligned to the nearest germline gene. TCRG, TCR gamma; TCRB, TCR beta; F, functional sequence; NF, nonfunctional.

* stop codon.

unable to identify VB gene usage from McCarthy VB primer.

? unable to assign BD gene.

[†] stop codon identified further upstream. Sequences have been submitted to the EMBL database under accession numbers AJ223510-AJ223530.

inal TCR gene rearrangement was also present. The spleen biopsy histology was consistent with follicle center cell lymphoma, grade I.

Sequence Analysis

Sequences were obtained from 44 clonal/oligoclonal products from 20 cases; the deduced amino acid sequences are summarized in Table 3. TCR γ and TCR β products were sequenced from 12 and 11 cases, respectively, and IgH products were sequenced from 8 cases. Multiple sequences per locus were identified in 9 cases including TCR γ in cases 1, 2, 8, and 9, TCR β in cases 7, 10, and 15, and IgH in cases 10, 11, 15, and 18. Twenty-

eight functional in-frame sequences were identified including 7 in-frame TCR γ sequences (cases 3, 4, 8, 9, 11, 12), 10 in-frame TCR β sequences (cases 7, 10, 11, 14–19) and 11 in-frame IgH sequences (cases 6, 10, 11, 13, 15, 18–20). Both functional TCR and IgH sequences were found in cases 11 and 18 (Table 3). Nonfunctional TCR γ and TCR β sequences were found in 8 cases (cases 1, 2, and 5–10) and 5 cases (cases 7, 8, 10, 13, 15) respectively. Nonfunctional IgH sequences were found in 2 cases (cases 11, 15; Table 4).

TCR γ and β gene usage is tabulated in Table 3. The majority of TCR γ gene recombinations used GVI and GJ1S3/J2S3 genes (including 6 functional and 8 non-functional sequences); in 2 cases GVI was rearranged to

J2S1 gene and in the remaining case GVI was rearranged to GJ1S1. In cases with functional TCR β rearrangements TCRBV2S1 genes were used in 4 cases, whereas in the remaining cases BV4S1, BV5S1, BV6S7, BV13S3, and BV17S1 were used. TCRBV4S1 and BV17S1 were used in 3 cases with nonfunctional sequences. A variety of TCRB-J gene segments and both BD genes were used, although in case 16 the D segment could not be unambiguously assigned, and in cases 7 and 10 no D gene segment could be assigned (Table 3). A unexpected BD2 rearrangement to TCR BJ1S1 gene was also identified in a sequence from case 18 (Table 3).

The majority of cases with IgH rearrangements used VH3 and VH4 genes and a wide range of D segment genes with a predominance of the JH4 genes (Table 3). In these cases the incidence of somatic mutations was assessed by comparing the VH sequences with the closest germ line sequences in VBase.³⁰ Three clones from 3 cases harbored unmutated V genes, whereas in 8 clones from 8 cases there were varying degrees of replacement and silent mutations ranging from 0.6 to 10.1%. Case 11 had significant clustering of replacement amino acids in CDR2, indicative of antigen selection based on the method of Chang and Casali³¹ (data not shown).

EBV

In situ hybridization for EBERs showed positive cells in 15 of 20 cases (Table 3). In two of these, only occasional labeled nuclei were identified. In 11 cases, 1 to 2% of nuclei were labeled; these were of intermediate to large size. In case 12, 25 to 30% of the nuclei were positive. In all positive cases, double staining identified some EBER-positive cells expressing CD20. Occasional EBER-positive cells expressing CD3 and/or CD45RO were also seen. However, the majority of cells expressing EBERs appeared to be of indeterminate phenotype. Case 12 had 50 to 60% CD8+ T cells. The follicle center cell lymphoma in case 17 was EBV-negative.

Discussion

Although AILD was first described as a nonneoplastic proliferation associated with immunodeficiency, it is clear that the authors of the REAL classification³² regard this entity "as a T cell lymphoma because most cases show clonal rearrangements of T cell receptor genes." Nevertheless, the situation is more complex, and in a recent review, Frizzera collated the findings of nine studies comprising specimens from 107 patients with AILD on which DNA analysis had been performed by Southern blot analysis.⁸ Clonality was detected in specimens from 78 of 107 patients (73%) at presentation and clonal rearrangements of the TCR β gene were found in 68 of 84 specimens (81%). Ig genes were rearranged in 6 of 84 specimens (7%) and with TCR genes in 10 of 84 specimens (12%) giving a total of 16/84 specimens (19%) with Ig rearrangements. Although these data imply heterogeneity of cell lineages among clonal populations in AILD with a higher incidence of IgH clonal rearrangements than

that observed in other mature T cell neoplasms,¹⁹ they do not allow unequivocal lineage assignment in individual cases, as it is well documented that cross lineage rearrangements occur in lymphoma and leukemia.¹⁸⁻¹⁹

In this study we have investigated cell lineages in 22 cases of AILD by PCR amplification of DNA isolated from paraffin-embedded biopsy specimens and sequence analysis of PCR products. PCR amplification, unlike Southern blot analyses, can be applied to poor quality DNA for amplification of products <300 bp but suffers from the disadvantage of limited primer homology annealing to all V gene sequences, which can lead to false negative and discordant results using different primer sets for a chosen chromosome locus. For this purpose the methods used in this study were first characterized by comparison with Southern blot analysis and by analysis of immunohistologically characterized tumors, with success rates exceeding 80% for TCR γ , TCR β (gene scanning), and IgH primers, comparable with other published studies using PCR.^{23,24} A combination of TCR β protocols using two different VJ primer sets to identify full recombinations and one DJ primer set to identify partial gene recombinations was used to identify the majority of T cell rearrangements.

TCR β and/or TCR γ clonal bands defined as one/two bands on gel electrophoresis or as TCR β clonal peaks on scans, were detected in 20/22 patients, an incidence of 91%. TCR γ clonal bands were observed 17 patients (77%). TCR β gene scanning revealed clonal peaks in 10 cases and oligoclonal peaks in a further two cases, a total of 60% of cases and was more successful than McCarthy's VJ primers,²⁴ that detected clonal bands in 6 cases. Incomplete DJ rearrangement were detected in 4 cases. Using all of the combinations of primers and techniques, TCR β clonal rearrangements were detected in 16/22 cases; if oligoclonal rearrangements are included, the incidence is 18/22 (82%), compatible with Frizzera et al.⁸ However, results with the IgH FR2 primer combination revealed a higher incidence of Ig clonal rearrangements than that previously observed.⁸ IgH rearrangements were identified in 9/22 cases, an incidence of 41%, including 3 cases (14%) with oligoclonal bands.

Forty-four PCR products from 20 cases were sequenced for lineage assignment including TCR γ , TCR β , and IgH products from 12, 11, and 8 cases, respectively. Functional and nonfunctional sequences were determined by alignment to germline sequences to indicate in-frame rearrangements, frame shifts or the acquisition of stop codons. Functional sequences were found in 17 cases and more than one functional sequence was found in 6 cases. Nonfunctional sequences were associated with other functional TCR or IgH rearrangements in 8 cases. Nonfunctional rearrangements occur during T and B cell maturation in association with a functional allele and the failure in 3 cases to find a functional sequence probably reflects the limitation of PCR analysis to detect all possible rearrangements.³³

TCR γ sequences were found in 13 cases, where the majority of recombinations used GVI and GJ1S3/J2S3 genes reflecting common GV and GJ gene usage in T cell lymphomas.³⁴ It is interesting to note that TCR γ was

functionally rearranged in 6 cases and was the only functional gene in 5 cases, all predominantly infiltrated with T cells expressing CD3 and TCR β . Whether this finding represents TCRGD clones in a predominant population of TCRAB positive cells is difficult to assess as antibodies to TCRD could not be applied successfully to paraffin biopsy specimens. However, similar cases have been described as TCR silent PTCL by Theodorou et al.³⁴ Alternatively, such cases may represent in-frame TCR γ rearrangements in TCR β -expressing cells where, for technical reasons, we were unable to detect TCR β clonal rearrangements, since in-frame TCR γ rearrangements have been described in TCRAB thymocytes at low frequency.³⁵

TCR β PCR products from 11 cases were sequenced and functional and nonfunctional TCR β rearrangements were found in 9 and 4 cases respectively. BV4S1 and BV2S1 genes were predominantly used in 3 and 4 cases respectively, while the remaining cases used BV5S1, BV6S1, BV13S3 and BV17S1. These data suggest an association in AILD that has not been observed in previous studies of T cell leukemias and lymphomas.³⁶ These data also explain the discrepancy between TCR β protocols in this study, as McCarthy's primers lack homology with BV families V2, V4, and V8.²⁴ In three cases functional TCR β gene rearrangements were detected in the absence of TCR γ gene rearrangements, contrary to expectation where nonfunctional TCR γ genes are usually associated with functional TCR β gene rearrangements.³⁷ This finding may also result from lack of primer homology for all TCR γ rearrangements. D segment analysis was uninformative but suggested chromosomal inversion in case 18, direct VJ joining in cases 7 and 10, and extensive exonucleolytic nibbling in case 16.^{38–40}

IgH clonal and oligoclonal products from 8 cases were sequenced and functional IgH sequences were identified in all cases, including two sequences each in cases 10 and 18 and multiple sequences in case 15. Although Ig light chain restricted populations of B cells could not be detected immunohistology, these data provide unequivocal molecular evidence for the presence of one or more B cell clones in these AILD biopsies. These data also confirm and extend the historical Southern blot findings that suggest that clones of B cells may be a common finding in AILD.⁸ The origin of these B cell clones was assessed by alignment of sequences to germline IgH genes for the presence of somatic mutations. Evidence for significant clustering of replacement amino acids in the CD2 region was found in 1 clonal sequence (case 11) indicative of antigen selection in the germinal center and another 7 clones (cases 6, 10, 13, 15, 18, 19, and 20) had varying degrees of replacement and silent mutations (0.6–10.1%) consistent with germinal center B cells.³¹ Three clones from 3 cases (cases 10, 15, and 18) harbored unmutated V genes consistent with naive B cells. Serial studies using the CDR3 sequence as a clonal marker may reveal the significance of these minor B cell clones in disease progression following presentation. The association with case 17 described in this report is complex, as the IgH clonal rearrangement was not detected in the original EBV-positive biopsy and its presence was

associated with an EBV-negative follicular center cell lymphoma, which may represent an independent event in a patient with T cell disease.

EBV was found in 75% of our cases by *in situ* hybridization for EBERs, which is lower than the 96 to 97% positivity reported previously.^{40,41} EBV was found in both B and T cells, but we were unable to assign a definite lineage to a high proportion of EBV-positive cells. However, the facts that i) the number of positive cells in all but two of our positive cases was greater than would be expected in reactive tissue and ii) the infected cell nuclei were larger than average suggest that there was active proliferation of EBV or EBV-infected cells in these nodes. Although Weiss et al⁴¹ have suggested that the presence of EBV+ B cells may explain the paradoxical occurrence of B cell lymphoma in a primary T cell lymphoproliferative disorder, we did not find a strong correlation between EBV and B cell clones in the majority of cases in this study or with the emergence of a B cell tumor in case 17. Nevertheless, EBV was associated with B cell oligoclonal in cases 1, 10, and 15, and we cannot exclude a role for EBV in these cases. In the majority of cases, however, the presence of EBV may simply reflect the disordered immunoregulation that characterizes this disease.

In this study we have demonstrated functional T and B cell oligoclonal in 6/20 (30%) cases. These data are compatible with previous cytogenetic studies of AILD and contrast with the reported frequency of unrelated clones of 0.6% in malignant lymphoma.^{9,17,43} Furthermore, oligoclonal were not found by PCR or sequence analysis in the reactive controls or the T cell tumors used to establish the PCR methods in this study. In addition, B cell oligoclonal have not been found in diffuse large B cell lymphomas or in reactive tonsils⁴⁴ (C. Ottensmeier, personal communication). Our data do not support the concept of AILD as a clearly defined PTCL but offer clear experimental evidence in support of the observation of Lipford et al,¹³ who concluded that "AILD is a disease of proliferating lymphoid clones in which supervening malignant lymphomas may develop by a process of clonal selection."

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