PCR Analysis of Immunoglobulin Heavy Chain (IgH) and TcR- γ Chain Gene Rearrangements in the Diagnosis of Lymphoproliferative Disorders: Results of a Study of 525 Cases

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This report summarizes a cumulative 4-year experience in polymerase chain reaction (PCR) analysis of immunoglobulin heavy chain (IgH) and TcR- γ chain gene rearrangements in 525 cases of lymphoproliferative disorders. Because the sensitivity of the PCR methodology was found to be tissue dependent, in the study of the presence of clonal cell population in tissues containing a small number of polyclonal lymphocytes, such as skin and gastrointestinal biopsy specimens, we used the multiple-PCR run approach. In this latter methodology, we repeat the PCR reaction from the same sample at least three times to confirm the reproducibility of the results. In the study of 273 cases of B- or T-cell lymphomas with characteristic immunomorphological and clinical features, a clonal IgH or TcR- γ chain gene rearrangement was detected in approximately 80% of cases. A clonal rearrangement involving both IgH and TcR- γ chain genes was found in 10% of cases of both B-cell and T-cell lymphomas. The study of 167 cases of nonneoplastic lymphoid tissue samples showed the presence of clonally rearranged cell populations for IgH or TcR- γ genes in 3 and 9% of cases, respectively. We also applied PCR for the study of 85 cases of lymphoproliferations with no definite diagnosis (*i.e.*, benign *versus* malignant) after immunomorphological analysis. In 65 cases (76%), the correlation of immunomorphological features with the presence (48 cases) or the absence (17 cases) of clonal lymphoid cell populations led to a definite diagnosis. In almost all these cases, the final diagnosis was found to be in agreement with the clinical course. In the 20 remaining cases (24%), no definite diagnosis could be made. We also assessed the value of PCR in detecting bcl-2/J_H gene rearrangement as an additional clonal marker in the diagnosis of follicular lymphoma. Bcl-2/J_H rearrangement and/or IgH gene rearrangement was found in approximately 85% (71/85) of follicular lymphoma cases studied.

KEY WORDS: bcl-2/J_H, Clonality, IgH, Lymphoid proliferations, Multiple-PCR run approach, PCR, TcR- γ .

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Immunoglobulin (Ig) and T-cell receptor (TcR) gene rearrangement studies have been successfully applied to investigate the clonality and cell lineage of various lymphoid malignancies (1). Consensus oligonucleotide primers directed to the Framework 3 (FRIII) and, to a lesser extent, Framework 2 (FRII) and Framework 1 (FRI) regions of the IgH genes have been used to detect B-cell clonality (2–5). The detection rate in previous studies varies from 50 to nearly 100% (6–8). However, recent studies have shown that the detection rate of IgH rearrangement was closely related to the cell origin of malignant lymphomas (1). Thus, tumors derived from naive lymphocytes, also designated pre-germinal center (pre-GC) naive B cells, express unmutated V_H-region genes and show a high detection rate of clonal IgH gene rearrangement by polymerase chain reaction (PCR). In this category are precursor B-lymphoblastic leukemia/lymphoma, mantle cell lymphoma, and a subset of chronic lymphocytic leukemia (9, 10). The other group of tumors derived from memory B cell, generated in the GC and characterized by cells bearing somatically hypermu-

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tated V_H-region genes (GC and post-GC memory B cells), give a lower rate of clonality by PCR methodology. In the latter group are the majority of non-Hodgkin's B-cell lymphoma subtypes such as lymphoplasmacytic, mucosa-associated lymphoid tissue (MALT) type, follicle-center lymphomas, hairy cell leukemia, diffuse large-cell lymphomas, Burkitt's lymphomas, multiple myeloma, and some chronic lymphocytic leukemias (9, 10). In these latter cases, the mutations at binding sites of the primers account for the lower amplification rate of IgH gene rearrangements (4). Therefore, the use of more than one appropriate set of primers in these lymphomas has been shown to yield higher detection rates varying from 80 to 90% (11–13).

To evaluate T-cell receptor gene rearrangements, T-cell receptor- γ chain (TcR- γ) gene rearrangement is frequently investigated because it occurs at an early stage of T-cell development and can be found in almost all T-lymphoid neoplasms (14, 15). The detection rate of clonal TcR- γ gene rearrangement in non-Hodgkin's T-cell lymphoma, using different sets of primers specific for the V γ and J γ gene regions, varies from 60 to 100% (16–19).

This report summarizes our cumulative 4-year experience using PCR for detecting IgH and TcR- γ as well as bcl-2/J_H gene rearrangements in lymphoproliferative disorders. We also tried to define the optimal sets of primers for detecting clonality in a broad morphologic spectrum of B- and T-cell lymphomas. The detection rate was also correlated with the cell origin of malignant lymphoma (*i.e.*, pre-GC or GC and post-GC) subtypes. In addition, to assess the diagnostic value of PCR in difficult cases (*i.e.*, benign *versus* malignant), we investigated 85 cases of lymphoproliferations with no definite diagnosis after immunomorphological analysis.

MATERIALS AND METHODS

Patients and Specimens Studied

The material used in the present study was collected in the tissue bank of the Department of Pathology at Purpan hospital in Toulouse, France from February 1987 to December 1997. We started the use of the PCR methodology for a diagnostic purpose in our laboratory at the beginning of 1994, and the results included in this report are based on a cumulative 4-year experience of PCR; that is, to the end of 1997. The specimens available in our tissue bank before 1994 were used to increase the number of well-characterized neoplastic and non-neoplastic lesions and to perform clinicopathologic correlations. Seven hundred four samples from 525 patients with various lymphoid disorders were included (sample numbers were as follows: lymph nodes, 272; tonsils, 22; spleens, 14; bone marrows,

146; skin biopsies, 141; gastrointestinal tract samples, 50; and various nonlymphoid tissue specimens, 59).

The diagnosis of lymphoma subtypes was based on immunomorphologic criteria according to the Revised European-American Lymphoma classification (20; Table 1). These patients included 290 men and 235 women and were aged from 1 to 93 years, with a majority of adult patients ($n = 448$ [85%]).

On the basis of their immunomorphologic features, these cases were classified as non-Hodgkin's lymphomas ($n = 273$) of B-cell ($n = 183$) or T-cell phenotype ($n = 90$; Table 1; 21, 22). We also applied PCR to 85 cases of lymphoproliferations with no definite diagnosis (benign *versus* malignant) after immunomorphological analysis. For the latter cases, clinical findings and follow-up were correlated with immunohistologic features and PCR findings. Beside malignant lymphoproliferative disorders, 167 cases of various nonneoplastic lymphoid disorders were also investigated. Clonal non-

TABLE 1. Results of IgH and TcR- γ Gene Rearrangements in 273 Cases of Non-Hodgkin's Lymphomas of B-Cell ($n = 183$) and T-Cell ($n = 90$) Phenotype Classified According to the REAL Classification

Type	IgH+/Tested	TcR- γ +/Tested*
B-cell lymphoma:		
Pre-GC B-cell:		
Precursor B-lymphoblastic leukemia	2+/2	0+/2
Chronic lymphocytic leukemia**	11+/12	0+/12
Mantle cell	12+/12	0+/12
Total	25+/26 (96 %)	0+/24 (0 %)
GC and post-GC B-cell:		
Lymphoplasmacytoid	3+/4	1+/4
Follicle center	65+/85	8+/85
MALT type	11+/14	2+/14
Myeloma	0+/1	0+/1
Diffuse large cell	30+/38	5+/38
Burkitt	0+/1	0+/1
Total	109+/143 (76%)	16+/143 (11%)
Unclassified:	10+/14	2+/14
Total	144+/183 (79%)	18+/183 (10%)
Type	TcR- γ +/Tested	IgH+/Tested***
T-cell lymphoma:		
Chronic lymphocytic leukemia	2+/3	0+/3
Mycosis Fungoides/Sezary syndrome	12+/12	1+/12
Peripheral T-cell	10+/10	1+/10
Angioimmunoblastic T-cell	10+/12	3+/12
Angiocentric T-cell	1+/1	0+/1
Lymphoblastic	0+/2	0+/2
Unclassified	20+/21	2+/21
Anaplastic large cell	19+/29	3+/29
Total	74+/90 (82 %)	10+/90 (11 %)

* TcR- γ gene rearrangement is associated with IgH gene rearrangement in these cases.

** Recent studies have demonstrated that there are 2 subtypes of CLL: one derives from pre-GC B-cell and the other from post-GC memory B-cells (9, 10).

*** IgH gene rearrangement is associated with TcR- γ gene rearrangement in these cases.

Hodgkin's B- (OCI LY8; 23) or T-cell lymphoma (CEM and HSB-2; ATCC CCL 119 and ATCC CCL 120.1) cell lines were used as positive controls for IgH and TcR- γ gene rearrangements, respectively (22). In addition, OCI LY8 positive for the major breakpoint region (MBR) of the t(14;18)(q32;q21) translocation and a follicular lymphoma (FL) positive for the translocation involving the minor cluster region (mcr) were used as positive controls for bcl-2/J_H rearrangements.

Tissue specimens were received fresh in our laboratory. For every sample, except for bone marrow biopsies, one half was snap-frozen in liquid nitrogen and stored at -80°C for immunostaining on cryostat sections and DNA extraction, and the other half was fixed in Duboscq-Brasil (ethanol-based Bouin's fluid) for routine morphological diagnosis and immunostaining on paraffin-embedded tissue sections. For bone marrow biopsy samples, the first half was fixed in acetone and embedded in paraffin using the ModAMeX method described elsewhere, which was developed for simultaneous study of cell morphology and fixation-sensitive leukocyte differentiation antigens (21). The ModAMeX method was also used for DNA extraction.

DNA Isolation and PCR Analysis of Tissue Samples

DNA extraction

Total cellular DNA from tissue biopsy samples was extracted from 10 frozen or ModAMeX sections (each 10 μm thick) using standard technique (22). For small biopsy specimens, DNA was extracted

from tissue sections using proteinase K digestion in buffer (100 μL Tris 50 mM, EDTA 10 mM, NaCl 100 mM; pH 8; 24). Amplification of a fragment of the housekeeping gene c-raf-1 (258-bp fragment; 25) or β -actin (606-bp fragment; 26) was used as a positive control for successful amplification of the extracted DNA (Table 2). In addition, all standard precautions were taken to guard against cross-contamination of amplified DNA (27). In each experiment, a polyclonal DNA (reactive lymphoid tissues) and negative (sterile water [blank]) controls were systematically included.

Primer Design

Analysis of IgH gene rearrangements was performed by seminested PCR (sn-PCR) according to the method of Trainor *et al.* (2) and Ramasamy *et al.* (3), using FR_{II}-V_H and FR_{III}-V_H-specific primer amplifications (Table 2). In addition, the consensus FR1c primer described by Aubin *et al.* (5), directed against a highly conserved region in the FRI region shared by the 7 V_H families, was employed for the FRI-J_H amplification using the same methodology described for the two other V_H framework-specific primers.

The demonstration of TcR- γ gene rearrangements was performed by using two rounds of multiplex PCR with a set of seven primers (22, 28; Table 2). These primers were used in two mixes: Mix 1 contained V γ I cons in conjunction with J γ 2S2, JP and JP1/2 primers, and Mix 2 contained V γ 9.2, V γ 10, and V γ 11 in association with J γ 2S2, JP and JP1/2 primers. For each sample, two PCR amplifications were performed, using first Mix 1 and then

TABLE 2. Sequences of Primers and Probes

House keeping genes:	
1-Actin	: 5'-TCA TGT TTG AGA CCT TCA A-3'
2-Actin	: 5'-GTC TTT GCG GAT GTC CAC G-3'
Raf 8+	: 5'-GAT GCA ATT CGA AGT CAC AGC G-3'
Raf 9-	: 5'-TTT TCT CCT GGG TCC CAG ATA-3'
t(14;18) translocation:	
MBR3+	: 5'-TTT GAC CTT TAG AGA GTT GCT TTA CG-3'
MC8	: 5'-GAC TCC TTT ACG TGC TGG TAC C-3'
JH-	: 5'-ACC TGA GGA GAC GGT GAC C-3'
MBR-P+	: 5'-GCC TGT TTC AAC ACA GAC CCA C -3'
MC12-F+	: 5'-GAT GGC TTT GCT GAG AGG TAT TG-3'
Ig heavy chain (IgH) genes:	
FR1c	: 5'-AGG TGC AGC TG(G/C) (A/T)G(G/C) AGT C(G/A/T)G G-3'
FR2a	: 5'-TGG (A/G)TC CG(C/A) CAG (G/C)C(T/C) (T/C)CN GG-3'
FR3a	: 5'-ACA CGG C(C/T)(G/C) TGT ATT ACT GT-3'
LJH	: 5'-TGA GGA GAC GGT GAC C-3'
VLJH	: 5'-GTG ACC AGG GTN CCT TGG CCC CAG-3'
TcR- γ genes:	
V γ I cons	: 5'-CTG GTA CCT ACA CCA GGA GGG GAA-3'
V γ 9.2	: 5'-GAA AGG AAT CTG GCA TTC CG-3'
V γ 10	: 5'-GCA GCA TGG GTA AGA CAA GC-3'
V γ 11	: 5'-GAT TGC TCA GGT GGG AAG AC-3'
J γ 2S2	: 5'-CCT GTG ACA ACA AGT GTT GT-3'
JP	: 5'-TTG TTC CGG GAC CAA ATA CC-3'
JP1/2	: 5'-CCA GGT GAA GTT ACT ATG AG-3'

Mix 2, respectively. The reamplification was done with the same mix as the first cycle.

The presence of the t(14;18) translocation (bcl-2/J_H rearrangement) was also detected by PCR. The MBR was amplified using oligonucleotide primer MBR3+, and the J_H consensus sequence (JH- primer; 29) and the mcr were detected by MC8 primer (30) with the JH- primer.

PCR conditions

For each initial amplification, either 0.5 µg of extracted DNA template or 2.5 µL of the tissue lysate, in a total of 50 µL PCR reaction mixture, was subjected to 35 cycles of PCR amplifications using AmpliTaq Gold DNA polymerase in a Perkin-Elmer DNA thermal cycler (Perkin-Elmer Corporation, Norwalk, CT) as described elsewhere (22, 31). The 35 cycles were performed using conditions adapted for each amplimer set. The PCR cycle consisted of denaturation at 93° C; annealing at 55° C for TcR-γ and bcl-2/J_H, at 54° C for FR1c/J_H, FR3a/J_H, and c-raf-1, and at 50° C for β-actin and FR2a/J_H amplimers; followed by extension at 72° C. The length of each step of amplification cycle was 1 minute. For all PCR amplifications, an initial step of 10 minutes at 93° C to denature the DNA templates and to activate the AmpliTaq Gold enzyme and a final period of 10 minutes at 72° C to complete the reaction were performed. For reamplification of the IgH and TcR-γ gene PCR products, 1% of the first round was used as a template and subjected to another 35-cycle PCR amplification. Then, 20 µL of each amplified product was applied to a polyacrylamide (8% for FR3a/J_H, 6% for FR2a/J_H and TcR-γ, and 4 or 5% for FR1c/J_H) or to a 2% agarose (for bcl-2/J_H, c-raf-1, and β-actin amplification fragments) gel electrophoresis and visualized by staining with ethidium bromide. For TcR-γ gene rearrangements, electrophoresis of the first and the second PCR amplification products was systematically performed. In effect, even if the two rounds of PCR amplification improve the sensitivity of the detection, in some rare cases, the band detected after the first PCR can be masked by the increased background generated by the polyclonal T-cell population present in the sample (17).

Amplifications of bcl-2/J_H PCR fragments were confirmed by hybridization with an internal oligonucleotide specific for bcl-2-MBR (MBR-P+ probe) or the bcl-2-mcr (MC12-F+ probe) of the t(14;18), obtained from the sequences reported by Crescenzi *et al.* (32) and Ngan *et al.* (30), at 63° C after alkaline transfer of PCR products to Hybond-N+ membranes (Amersham Pharmacia Biotech Europe GmbH, Saclay, France). These probes were end labeled with digoxigenin-11-dUTP 3' before hybridization, and their fixation was revealed by an alkaline phosphatase-conjugated anti-digoxigenin

antibody, followed by BromoChloroIndoyl Phosphate-nitro blue tetrazolium incubation (Roche Diagnostics, Meylan, France). All probe sequences are shown in Table 2.

Sensitivity of PCR

As reported elsewhere, the sensitivity of the IgH and TcR-γ PCR for the detection of a clonally rearranged cell population is tissue dependent (24, 33, 34). Indeed, this sensitivity is dependent on the number of polyclonal cells present in the sample, which is coamplified with the clonal cell population if present. Because the number of polyclonal cell differs between tissues, the origin of the tissue examined should be taken into account in the interpretation of the results. In our previous reports, we demonstrated that using the sn-PCR assay for the detection of IgH gene rearrangement, a clonal-rearranged B-cell population of approximately ≥1% of total cells is detectable in a background of lymph node-based polyclonal B-cells (22). In the absence of polyclonal cell population, this technique was sufficiently sensitive to produce a detectable band from a single IgH gene-rearranged B-cell (24). The applications of the same conditions of PCR amplifications for TcR-γ genes yielded comparable results (22 and data not shown). The sensitivity of IgH and TcR-γ PCR gene amplifications for the detection of clonal cell populations in bone marrow samples was intermediate between the above-mentioned values. Using FR1c/J_H, FR2a/J_H, and FR3a/J_H PCR amplifications, a clonal B-cell population was detectable when it constituted ≥0.1% of the total bone marrow mononuclear cell populations, whereas a clonal T-cell population was detectable when it represented ≥0.5% and ≥0.1% of the total DNA for TcR-γ/mix-I and TcR-γ/mix-II, respectively. Finally, for the detection of the t(14;18) chromosomal translocation, using the technique reported in this study, we were able to detect t(14;18)-positive DNA representing ≥0.1% of the total DNA analyzed; in other words, this method detects 50 positive cells (equivalent to 0.5 ng) diluted in 50,000 cells (equivalent to 0.5 µg DNA used for each PCR amplification).

RESULTS

IgH-Chain and TcR-γ Gene Rearrangement Studies of B-Cell and T-Cell Lymphomas

In the group of 183 well-characterized B-cell lymphomas, a clonal IgH gene rearrangement was detected in 144 cases (79%). Clonal rearrangements of IgH chain genes resulted in one or two predominant amplification products within the expected range of size; in other words, 330 to 350 bp, 230 to

270 bp (Fig. 1), and 70 to 110 bp for FR1c/J_H (5), FR2a/J_H, and FR3a/J_H (2, 3), respectively. For the study of IgH gene rearrangements in tissue specimens with a relatively small polyclonal B-cell population, such as skin and gastrointestinal samples, we used the multiple sn-PCR experiment (24). Three to five FR1c/J_H, FR2a/J_H, and FR3a/J_H PCR amplifications were performed from the same sample, and the demonstration of a band of the same size in all the different PCR runs was considered as indicative of a clonal cell population (Figs. 2 and 3). As shown in Table 1, in precursor B-lymphoblastic leukemia/lymphoma, in chronic lymphocytic leukemia, and in mantle cell lymphoma, cases that usually lack mutations in the V_H gene segments, the detection rate of clonally rearranged IgH genes was nearly 100% (10). In this group of lymphomas, the three sets of primers, used in this study for IgH gene rearrangement analysis, were usually all positive, indicating the unmutated status of the V_H segments in these cases (4). By contrast, in the other B-cell lymphoma types, considered by the presence of mutated V_H genes to be of GC or post-GC memory B-cell origin (10), a clonal IgH gene rearrangement was detected in only 76% of cases. As expected, in this latter group of lymphomas, the positivity of each set of primers was much lower because among the 143 cases studied, a clonal IgH gene rearrangement was found in 78 (55%), 94 (66%), and 45 (31%) of the cases, using FR1c/J_H, FR2a/J_H, and FR3a/J_H primers, respectively. It was of note that using the three sets of primers, the detection rate of clonality reached 76% (109/143) compared with the highest rate obtained; that is, 66% using only FR2a/J_H. However, cases bearing a clonal IgH rearrangement (109 cases) were detected using only FR1c/J_H and FR2a/J_H primers, and PCR using FR3a/J_H did not increase the detection rate of positive cases. By comparison, the detection rate of IgH rearrange-

FIGURE 1. Examples of polymerase chain reaction analysis of IgH chain gene rearrangements using FR2a/J_H primers in 10 lymph node specimens (1–10). Reactions were subjected to 6% polyacrylamide gel electrophoresis and stained with ethidium bromide. Lane ϕ : size markers in base pairs (bp). Lane BL (blank): Polymerase chain reaction amplification without DNA template. Lane C: Positive control (B-cell lymphoma). Lane P: Polyclonal control (reactive lymph node). Samples 1, 2, 4, 5, 6, 8, and 9 show one or two rearranged bands between 230 and 270 bp, indicating the presence of clonally IgH-rearranged cell population in these lymph node specimens. The other samples (3, 7, and 10) show a polyclonal smear.

FIGURE 2. Multiple seminested polymerase chain reaction (PCR) analysis of gastric samples from three cases (1–3) of suspected MALT lymphoma using FR2a/J_H primers. Reactions were subjected to electrophoresis on a 6% polyacrylamide gel and stained with ethidium bromide. Lane ϕ : the DNA size markers in base pairs (bp). Lane BL (blank): A PCR amplification without DNA template to rule out contamination. Lanes C and P are a positive B-cell lymphoma cell line and a polyclonal control (polyclonal lymph node DNA), respectively. In Case 1, the three PCR runs performed from each sample showed the presence of a rearranged band of the same size within the expected range of size (230–270 bp). These findings confirmed the presence of clonal B population in this sample. Cases 2 and 3 show either different rearranged bands in each of the three PCR runs performed from this sample (Case 2) or from polyclonal smear (the three PCR runs; Case 3). These results indicate the reactive nature of the B-cell populations in these two cases.

FIGURE 3. Multiple seminested polymerase chain reaction (PCR) analysis of skin samples from three cases of suspected primary cutaneous lymphomas using FR1c/J_H primers. Reactions were subjected to electrophoresis on a 4% polyacrylamide gel and stained with ethidium bromide. Lane ϕ : DNA size markers in base pairs (bp). Lane BL (blank): PCR amplification without DNA template to rule out contamination. Lanes C and P are a positive B-cell lymphoma cell line and a polyclonal (polyclonal lymph node DNA) control, respectively. In Case 1, PCR shows the presence of one rearranged band of the same size within the expected range of size (330–350 bp) in all the three PCR runs performed from each sample. This finding confirms the presence of a clonal B-cell population in this sample. The final diagnosis was primary cutaneous B-cell lymphoma. Cases 2 and 3 show either different rearranged bands in each of the 3 PCR runs performed from this sample (Case 2) or from polyclonal smear (the three PCR runs; Case 3). These results indicate the reactive nature of the B-cell populations in these two cases. These results were in agreement with the final diagnosis of benign cutaneous lymphoid infiltrates.

ment using FR1c/J_H and FR3a/J_H or using FR2a/J_H and FR3a/J_H was 59% (84/143 cases) and 67% (96/143 cases), respectively. TcR- γ genes were also found to be rearranged in 18 of the 144 cases showing clonally IgH rearranged genes (Table 1). No IgH or TcR- γ gene rearrangement was found in the remaining 39 cases (21%) of B-cell lymphomas studied.

Of the 90 T-cell lymphomas diagnosed on immunomorphologic criteria, a TcR- γ gene rearrangement was detectable in 74 cases (82%; Table 1). As expected, PCR products for TcR- γ chain gene rearrangements were approximately of 230 and 125 bp

for the Mix 1 (Fig. 4) and Mix 2 primer pairs, respectively (22). The anaplastic large-cell lymphomas showed a lower detection rate of clonality (19/29 [65.5%]) in comparison with the other types of T-cell lymphomas (55/61 [90%]). In 10 of the 74 positive cases, TcR- γ gene rearrangement was associated with IgH gene rearrangement (Table 1). As demonstrated in Table 1, the angioimmunoblastic T-cell lymphomas gave a higher rate of rearrangements involving both IgH and TcR- γ (3/12; 25%) in comparison with the other T-cell subtypes (7/78; 9%). The remaining 16 cases (18%) investigated showed a polyclonal pattern.

Detection of IgH-Chain and TcR- γ Gene Rearrangements in Cases with No Definite Diagnosis after Immunomorphologic Study

In 85 cases including 49 skin lesions and 36 non-cutaneous lymphoid disorders (20 lymph node samples, 9 bone marrow biopsies, and 7 other miscellaneous tissue specimens), no definite diagnosis could be made after histopathologic examination associated with immunohistochemistry (Table 3).

Skin tissue samples

For the 49 skin tissue samples, the suggested diagnosis after immunomorphological studies was as follows (Table 3).

- (1) Primary cutaneous T-cell lymphomas *versus* nonneoplastic T-cell infiltrate was a question in 37 cases. Gene rearrangement study showed a clonal TcR- γ gene rearrangement in 21 cases, a clonal birearranged cell population ([TcR- γ /IgH] +) in 1 case, and a clonal IgH gene rearrangement in another 1. In the 14 remaining cases, gene rearrangement study yielded polyclonal patterns. Among the 21 TcR- γ -positive cases, the correlation between immunomorphology, PCR results, and

clinical findings led to the final diagnosis of primary cutaneous T-cell lymphoma in 15 cases, whereas in 4 cases, the lymphoid infiltrate was considered nonneoplastic and related to lymphomatoid papulosis in 3 cases and to pseudolymphoma in 1 case (35). None of these patients developed a lymphoma after 3 to 4 years of follow-up. In the two remaining cases, no definite diagnosis was made. No definite diagnosis was made in the case showing clonally rearranged TcR- γ and IgH genes. In the 14 PCR-negative cases, 9 cases were finally considered to be nonneoplastic lymphoid infiltrates, and one case remained without definite diagnosis, whereas the four remaining cases proved to be cutaneous T-cell lymphoma on the basis of the clinical follow-up, despite the absence of a detectable clonally rearranged lymphoid cell population. The last case with IgH-positive rearrangement was found in a clinical context of psoriasis.

- (2) In 11 cases, consisting of a predominant B-cell population, the immunomorphological features raised the question of malignant *versus* reactive B-cell infiltrate. Gene rearrangement analysis confirmed the clonal nature (IgH+) of the B-cell population in nine of these cases. In the two remaining cases, PCR demonstrated a polyclonal pattern in one case and a TcR- γ -positive cell population in the remaining one. The correlation between immunohistological, gene rearrangement analysis-based, and clinical findings allowed a definite diagnosis of primary cutaneous B-cell lymphoma in eight of the nine IgH-positive cases. In the remaining case, no definite diagnosis was made. The one polyclonal case was considered to be benign cutaneous lymphoid infiltrate, and no definite diagnosis was made in the TcR- γ -positive case.
- (3) In one case, the lymphoid infiltrate was of mixed B and T cells, and the question regarded malignant *versus* reactive infiltrate. Both IgH and TcR- γ were rearranged. Clinical presentation and follow-up supported a neoplastic process, and the final diagnosis was a primary CBCL.

FIGURE 4. Examples of PCR analysis of TcR- γ gene rearrangements using TcR- γ /Mix 1 PCR amplification in 10 cases of suspected T-cell lymphomas (Lanes 1–10). Lane C: Positive control (T-cell lymphoma). Lane ϕ : Size markers. Lane BL (blank): PCR amplification without DNA template. Lane P is a polyclonal control (reactive lymph node). Tissue Samples 1, 2, 4, and 6 through 9 show the presence of a clonal TcR- γ -positive cell population indicated by the presence of one or two bands of approximately 230 bp. The other samples (3, 5, and 10) show a polyclonal smear.

Overall, the results of gene rearrangement studies were contributory to the diagnosis in 34 cases (70%), of which 24 cases were considered to be malignant and 10 cases, benign lymphoid infiltrates. In the 15 (30%) remaining cases, gene rearrangement analysis was of no help to elucidate the nature of the lesion (*i.e.*, benign *versus* malignant; Table 3).

TABLE 3. Diagnostic Value of PCR in Lymphoproliferations with No Definite Diagnosis after Immunomorphologic Studies (85 Cases)

PCR Analysis	No. of Cases	Final Diagnosis
Cutaneous infiltrates (49 cases)		
1. Cutaneous infiltrates with a predominant population of T-cells (37 cases)		
TcR- γ R	15	CTCL
TcR- γ R	4	Non-neoplastic infiltrate
TcR- γ R	2	No definite diagnosis
TcR- γ R and IgH R	1	No definite diagnosis
Polyclonal pattern	9	Reactive infiltrate
Polyclonal pattern	4	The clinical follow-up supported the diagnosis of CTCL
Polyclonal pattern	1	No definite diagnosis
IgH R	1	Reactive infiltrate
2. Cutaneous infiltrates with a predominant population of B-cells (11 cases)		
IgH R	8	CBCL
IgH	1	No definite diagnosis
TcR- γ R	1	No definite diagnosis
Polyclonal pattern	1	Reactive infiltrate
3. Cutaneous infiltrates with a mixture of T- and B-cells		
TcR- γ and IgH R	1	CBCL
Non-cutaneous infiltrates (36 cases)		
1. Lymphoid infiltrates with a predominant population of B-cells (11 cases)		
IgH R	7	B-cell lymphoma
IgH R and TcR- γ R	4	B-cell lymphoma
2. Lymphoid infiltrates with a predominant population of T-cells (8 cases)		
TcR- γ R	5	T-cell lymphoma
TcR- γ R and IgH R	3	T-cell lymphoma
3. Lymphoid infiltrates with a mixture of T- and B-cells (17 cases)		
IgH R	2	B-cell lymphoma
IgH R	3	No definite diagnosis
TcR- γ R	2	T-cell lymphoma
TcR- γ R	1	Reactive infiltrate
IgH R and TcR- γ R	1	B-cell lymphoma
Polyclonal pattern	7	Reactive infiltrate
Polyclonal pattern	1	No definite diagnosis in a patient with Gougerot-Sjögren syndrome who developed a B-cell lymphoma 2 years later

R, rearranged; CTCL, cutaneous T-cell lymphoma; CBCL, cutaneous B-cell lymphoma.

No definite diagnosis: means that no conclusion could be made taking into account immunomorphological features, PCR results and clinical follow-up.

Noncutaneous lymphoid proliferations

In the 36 cases of noncutaneous lymphoid proliferations, the diagnosis uncertainties were as follows (Table 3).

- (1) in 11 cases, the immunomorphologic feature was suggestive of a B-cell lymphoma. A clonal IgH gene rearrangement was detected in all of these cases, associated with TcR- γ gene rearrangement in four of them. The diagnosis of B-cell lymphomas was made and confirmed by the clinical evolution.
- (2) In eight cases, the immunomorphologic characteristics of the infiltrate was evocative of a T-cell lymphoma. A clonal TcR- γ gene rearrangement was detected in all cases, associated with an IgH gene rearrangement in three of them, and led to a final diagnosis of T-cell lymphoma. The clinical follow-up results were concordant with such a diagnosis.
- (3) In the remaining 17 cases, after immunohistological examination, we were unable to decide between a malignant or reactive lymphoid disorder. In nine cases, a clonal gene rearrangement involving IgH ($n = 5$), TcR- γ

($n = 3$), or both IgH and TcR- γ ($n = 1$) was detected. Only two of the five cases showing a clonal IgH gene rearrangement proved to be a B-cell lymphoma. In the three cases with TcR- γ -positive lymphoid cell populations, the correlation between the immunohistological, gene rearrangement, and clinical findings led to the diagnosis of T-cell lymphoma in two cases and of reactive process in the third. In the case with the birearranged (IgH/TcR- γ)-cell population, immunomorphologic features suggested either an Epstein-Barr-associated B-cell lymphoma or an Epstein-Barr-induced reactive florid immunoblastic proliferation (36). The clonal nature of the lymphoid population led to the diagnosis of diffuse large B-cell lymphoma. The clinical presentation and follow-up were in agreement with the latter diagnosis. In eight cases, no detectable rearrangement was found, and the final diagnosis was reactive lymphoid disorders (Table 3). In seven of these cases, the clinical evolution was in agreement with the diagnosis of reactive infiltrates. In the re-

maintaining case, the diagnosis remained undetermined between malignant lymphoma and reactive process in a clinical context of Gougerot-Sjögren syndrome. This patient developed a diffuse large B-cell lymphoma 2 years later.

Overall, the results of the gene rearrangement studies were contributive in 31 cases (86%), of which 24 corresponded to malignant lymphoid infiltrates and 7, to benign lymphoid infiltrates (Table 3).

Taken together, the results of the PCR monoclonality analysis were contributive in 65/85 cases (76%) in resolving the diagnosis difficulties in these different lymphoid pathologies.

IgH and TcR γ -Chain Gene Rearrangement Studies in 167 Cases of Reactive or Nonneoplastic Lymphoid Disorders

167 cases clinically and immunohistologically proven to be reactive lymphoid proliferations were included in this study. In 20 cases (12%), a clonal rearrangement was detected, involving IgH ($n = 5$; 3%) or TcR- γ ($n = 15$; 9%) genes. The clinical context was extremely variable and included autoimmune diseases, infectious states, neoplastic processes, and various cutaneous lesions.

Detection of Rearranged bcl-2/J_H and/or IgH Gene Chain in FL

Among the 85 cases of FL, 40 cases (47%) were found to have an amplifiable t(14;18) translocation (MBR: 39 cases; mcr: 1 case). The PCR products were expected to range from approximately 150 to 400 bp for t(14;18)-MBR (29) and from 500 to 1000 bp for t(14;18)-mcr (30). Rearranged IgH genes were found in 65/85 (76%) cases (34 cases [bcl-2/J_H]⁺ and 31 cases [bcl-2/J_H][−]). Thus, in FL, the use of IgH and bcl-2/J_H gene rearrangement analyses allowed the detection of monoclonality in 71/85 (84%) of cases. No bcl-2/J_H or IgH gene rearrangement was found in 14 cases (16%). The efficacy of each of the three sets of primers used for IgH gene rearrangement analysis was variable from one set to another. Of the 65 IgH-positive cases, 28 cases (43%), 18 cases (28%), and 19 cases (29%) were positive for only one, two, or three of the sets of primers, respectively. As mentioned above, all IgH-positive cases were detected with the association of FR1c/J_H and FR2a/J_H, and the use of the FR3a/J_H did not increase the number of positive cases.

DISCUSSION

In this study, PCR amplification of the IgH gene rearrangement was performed with three sets of

primers (2, 3, 5, 37). We demonstrated that IgH gene rearrangement was detectable in approximately 80% of B-cell non-Hodgkin's lymphomas. These results are comparable to those reported in series based on a large number of B-cell lymphomas of different categories (11–13). It appears that the addition of the third primer set, FR3a/J_H, does not increase the number of IgH-positive cases. However, FR3a/J_H primers could be of diagnostic value when the immunomorphological study is inconclusive and there is a differential diagnosis between two types of B-cell lymphomas, such as mantle cell lymphoma *versus* FL. Overall, the positivity of the three sets of primers is suggestive of an unmutated status of the V_H gene of the lymphoma cells (*i.e.*, pre-GC B-cell origin, such as mantle cell lymphoma), whereas the positivity of FR1c/J_H and/or FR2a/J_H together with the negativity of FR3a/J_H is mainly found in lymphomas of GC (such as FL) and post-GC B-cell origin. In FL, the detection of bcl-2/J_H rearrangement is a diagnostic adjunct, but only 50% were found to be positive with sets of primers detecting MBR and mcr, which is comparable to the results reported in other European series (37, 38).

As far as the routine use of PCR methodology is concerned, the results of this study stress the importance of the origin of the tissue sample analyzed. Indeed, the sensitivity of PCR in detecting clonal IgH rearrangement is strongly dependent on the relative number of polyclonal lymphoid cells, which are coamplified with the clonal population if present in the sample (24, 33, 34). As demonstrated in this study, these polyclonal lymphoid cells can be responsible for a pseudoclonal PCR profile (see Figs. 2 and 3). Therefore, when IgH gene rearrangement study was performed using tissue specimens containing a relatively small number of polyclonal B cells (*i.e.*, skin and gastrointestinal biopsies), we used a multiple-PCR strategy that involves three to five PCR reactions from the same sample (24). Using this strategy, the demonstration of the same rearranged band in every experiment is highly indicative of the presence of clonal B-cell population in the analyzed sample. Inversely, when the B-cell population is polyclonal, each PCR experiment gives different rearranged bands in size and in sequence (pseudoclonal pattern; 24 and data not shown).

In T-cell lymphomas, a clonal TcR- γ gene rearrangement was found in approximately 80% of cases, which is comparable to the results reported in the literature (16–19). The highest percentage of positive cases was found in cutaneous T-cell lymphomas and peripheral T-cell lymphomas (unspecified) and the lowest, in anaplastic large-cell lymphomas. As reported elsewhere, in the skin, the demonstration of a clonal TcR- γ gene rearrange-

ment was found to be of diagnostic value in distinguishing reactive infiltrates rich in T cells from cutaneous T-cell lymphomas (39–41). However, TcR- γ gene rearrangement is not restricted to T-cell lineage and can occur in various B-cell lymphomas, although rarely in mature neoplastic B-cell proliferations (42–44). Of note is the detection of a clonal IgH gene rearrangement in approximately 10% of T-cell lymphomas (45–47), which is of the same degree of magnitude as the detection of TcR- γ rearrangement in B-cell lymphomas (approximately 5% of cases; 2, 47, 48). It is noteworthy that the presence of clonal TcR- γ gene rearrangement was mainly found in B-cell lymphoma subtypes, such as follicular, MALT-type, and T-cell-rich B-cell lymphomas, which are known to contain large numbers of T lymphocytes admixed with neoplastic B cells. The detection of such a rearrangement may simply reflect the presence of oligoclonal expansion of tumor-infiltrating T-lymphocytes rather than a dual rearrangement involving neoplastic B-cells (49–51). In such circumstances, only single-cell gene rearrangement study of tumor-infiltrating T-lymphocytes in non-Hodgkin's B-cell lymphoma-affected tissues could address this issue (52). Clonal populations of nonneoplastic T-cells have also been described in patients with B-cell chronic lymphocytic leukemia and multiple myeloma (53, 54) and even in healthy elderly humans (55).

In difficult diagnostic situations, PCR results must be combined with clinical presentation and follow-up. In 85 cases, no definite diagnosis could be made after histopathological examination associated with immunohistochemistry. Overall, the results of the gene rearrangement studies were contributive in approximately 75% and were in agreement with the follow-up (see Table 3). As previously reported, in 12% of lymphoid infiltrates proved to be clinically and immunohistologically reactive, we found a clonal rearrangement involving IgH (3% of cases) or TcR- γ (9%) genes. The majority of patients presented with autoimmune diseases, including Sjögren syndrome, systemic lupus erythematosus, and rheumatoid arthritis, which are characterized by polyclonal B-cell activation and autoantibodies (56). Overt B-cell clonal expansion occurs in some cases, suggesting an antigen-driven B-cell expansion (56, 57). In addition, many examples of clonal T-cell expansion in tumor-infiltrating lymphocytes in patients with tumors and autoimmune diseases have been reported (58–60). Furthermore, clonal expansion of T/ $\gamma\delta$ -lymphocytes has been reported to occur in granulomatous diseases such as sarcoidosis and tuberculosis, and some of our cases (data not shown) fell in this category (61, 62). Conversely, the failure to detect a clonal population in an immunomorphological and clinical context highly sug-

gestive of a malignant process (in approximately 10–20% of malignant lymphoid proliferations) should be interpreted with caution. Among the factors responsible for these negative results are sampling errors or a low number of malignant cells under the threshold of PCR detection at the time of the analysis.

In conclusion, the results of this study further emphasize the value of gene rearrangement study in human lymphoid pathology. The use of the multiple-PCR run approach with the same DNA sample should be used as a complementary tool for the analysis of Ig and TcR gene rearrangements, in particular in biopsy specimens containing a small number of polyclonal lymphocytes. The addition of DNA technology to the immunomorphological and clinical findings increases the likelihood of early diagnosis and appropriate therapy.

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Book Review

Ward JM, Mahler JF, Maronpot RR, Sundberg JP, editors: *Pathology of Genetically Engineered Mice*, 394 pp, Ames, IA, Iowa State University Press, 2000 (\$174.95).

This book grew out of a symposium sponsored by the National Institutes of Health in 1999. The editors (three from NIH and one from The Jackson Laboratory) and the publishers deserve to be congratulated for presenting it to us so fast.

The printers cannot keep pace with the progress of science, and thus it is self evident that many data published here have been superseded by newer developments. Nevertheless, this book is an important cornerstone providing a baseline for future endeavors of this kind. Although it is not comprehensive, and accordingly does not cover all the existing mouse models of

human diseases, it still contains a wealth of data. Its illustrations are well chosen, reproduced as high-quality color photographs. The text is succinct but informative and profusely referenced. A basic knowledge of pathology is needed for a good understanding of the pictures, but even those who do not use microscopes on a daily basis will profit from studying the material covered in this book.

This monograph is highly recommended to experimental pathologists dealing with biologically engineered mice, but it will be welcomed by many other scientists in academia and industry as well.

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