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RESEARCH ARTICLES

Improved Detection of Chromosomal Abnormalities in Chronic Lymphocytic Leukemia by Conventional Cytogenetics Using CpG Oligonucleotide and Interleukin-2 Stimulation: A Belgian Multicentric Study

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We performed a multicentric study to assess the impact of two different culture procedures on the detection of chromosomal abnormalities in 217 consecutive unselected cases with chronic lymphocytic leukemia (CLL) referred for routine analysis either at the time of diagnosis ($n = 172$) or during disease evolution ($n = 45$). Parallel cultures of peripheral blood or bone marrow were set up with the addition of either the conventional B-cell mitogen 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) or a combination of CpG oligonucleotide (CpG) and interleukin-2 (IL-2). Cytogenetic analyses were performed on both cultures. Clonal abnormalities were identified in 116 cases (53%). In 78 cases (36%), the aberrant clone was detected in both cultures. Among these, the percentages of aberrant metaphases were similar in both conditions in 17 cases, higher in the CpG/IL-2 culture in 43 cases, and higher in the TPA culture in 18 cases. Clonal aberrations were detected in only one culture, either in CpG/IL-2 or TPA in 33 (15%) and 5 (2%) cases, respectively. Taken together, abnormal karyotypes were observed in 51% with CpG/IL-2 and 38% with TPA ($P < 0.0001$). Application of FISH ($n = 201$) allowed the detection of abnormalities not visible by conventional cytogenetic analysis in 80 cases: del(13q) ($n = 71$), del(11q) ($n = 5$), +12 ($n = 2$), del(14q) ($n = 1$), and del(17p) ($n = 1$). In conclusion, our results confirm that CpG/IL-2 stimulation increases the detection rate of chromosomal abnormalities in CLL compared with TPA and that further improvement can be obtained by FISH. However, neither conventional cytogenetics nor FISH detected all aberrations, demonstrating the complementary nature of these techniques. © 2009 Wiley-Liss, Inc.

INTRODUCTION

Numerous studies have shown that the presence, number, and type of chromosomal aberrations represent an independent predictor of prognosis in B-cell chronic lymphocytic leukemia (CLL) (Juliusson et al., 1990; Döhner et al., 2000; Mayr et al., 2006; Van Den Neste et al., 2007). Therefore, cytogenetic analysis is routinely performed in this disease entity. However, CLL lymphocytes have a poor mitotic index in vitro. Several mitogens, such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), lipopolysaccharide (LPS), pokeweed mitogen (PWM), and Epstein-Barr virus, have been added to the cultures to improve the yield of aberrant metaphases, revealing abnormal karyotypes in 40–50% of cases (Juliusson et al., 1990). The rate of detection can be increased to 80% by interphase FISH (Döhner et al., 2000). However, the latter technique provides only partial information confined to the chromosomal regions examined. For this reason, there

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TABLE 1. Results Obtained by Conventional Cytogenetic Analysis with TPA or CpG/IL-2 Stimulation: Review of the Literature and Comparison with Present Data

Study/center	Diagnosis	Number of cases	Median number of metaphases analyzed	Success[a]		Clonal aberrations	
				CpG/IL-2	TPA	CpG/IL-2	TPA
Dicker et al. (2006)	CLL	132	NR	95.0%	ND	81.0%	ND
Haferlach et al. (2007)	CLL	506	20	98.8%	ND	83.0%	ND
Struski et al. (2009)	CLL/LPD	80/52 (132)	26/23	86%/94%	96%/96%	79%/76%	70%/67%
Present study	CLL	217	20	79%	83%	51.2%	38.2%
Center 1	CLL	78	22	96%	96%	53%	41%
Center 2	CLL	62	15	52%	60%	48%	39%
Center 3	CLL	36	25	97%	94%	56%	39%
Center 4	CLL	21	35	86%	100%	76%	48%
Center 5	CLL	20	20	55%	65%	20%	15%

[a]Success is defined as ≥10 normal metaphases or the presence of any clonal abnormality and is expressed as percentage of total number of cases. CLL, chronic lymphocytic leukemia; CpG/IL-2, CpG-oligonucleotide and interleukin-2; LPD, lymphoproliferative disorder; ND, not done; NR, not reported; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

has been great interest in improving culture methods. CD40 ligand-induced cell cycle stimulation resulted in the detection of abnormalities in 89% of cases compared with 22% after stimulation with conventional mitogens (TPA, LPS, and PWM) (Buhmann et al., 2002). Because this technique is labor-intensive and expensive, it is not applicable for routine analysis. More recently, the combination of an immunostimulatory CpG oligonucleotide (CpG) combined with interleukin-2 (IL-2) has been reported (Dicker et al., 2006; Mayr et al., 2006; Haferlach et al., 2007), identifying aberrations in 81-83% of cases, a percentage that is almost equivalent to interphase FISH (Table 1). Until now, only one group compared the results obtained by this new procedure (CpG/IL-2) with TPA (Struski et al., 2009). Therefore, we performed a multicentric study to assess the impact of these different culture procedures on the detection of clonal abnormalities in a routine setting. The aim of the present study was to confirm CpG/IL-2 stimulation as a gold standard for conventional cytogenetic assessment in CLL.

MATERIALS AND METHODS

Patients and Samples

This was a retrospective (for three centers) and prospective (for two centers) multicentric study performed in five Belgian genetic centers currently using CpG/IL-2 for stimulation of CLL cell cultures. Between October 2007 and November 2008, 217 consecutive unselected cases, referred for routine analysis of CLL either at diagnosis (n = 172) or during follow-up (n = 45), were enrolled. Inclusion criteria were (1) diagnosis of CLL and (2) assessment by parallel cultures for each patient as explained below. CLL was diagnosed based on immunophenotype [Matutes-Catovsky score of at least 3/5 (Matutes et al., 1994)] and morphology (n = 198). However, in 19 patients, referred for work-up of CLL, definitive confirmation of diagnosis could not be obtained from the referring hospital. The median age was 66 years (range 36–94 years) and the male:female ratio was 115:102.

Conventional Cytogenetic Analyses

Sample cellularity was determined using a Coulter counter (Beckman Coulter, Analis, Namur, Belgium). Details on culture condition are shown in Table 2. In each center, 1.0–2.0 × 10^6 cells/ml from peripheral blood (n = 97) or bone marrow (n = 120) were added to RPMI 1640 medium with HEPES and Ultraglutamine (Lonza, Verviers, Belgium), supplemented with FetalClone II (HyClone Laboratories, Logan, UT), 4 ml penicillin and streptomycin (Gibco, Invitrogen Corporation, Merelbeke, Belgium). No cell separation procedure was applied. Dual cultures were set up with the addition of either TPA (Sigma-Aldrich, Steinheim, Germany) or CpG (Tib MolBiol, Berlin, Germany) and IL-2 (PeproTech, London, UK). The following final concentrations were used: (1) TPA: 0.1 μg/ml (two centers), 0.05 μg/ml (one center), 0.01 μg/ml (two centers) and (2) CpG: 1 μM and IL-2: 100 U/ml (all centers). According to local practice policies, different concentrations TPA were used, as the study was retrospective for three centers. Samples were incubated for 72 hr at 37°C and 5% CO_2 in a fully humidified atmosphere. Colcemid (Gibco, Invitrogen Corporation, Merelbeke,

TABLE 2. Techniques Used for Conventional Cytogenetic Analysis with TPA and CpG/IL-2: Review of the Literature and Comparison with Present Data

Study/center	Medium	Separation procedures	Tissue	Cells added (number/ml)	Total volume (ml)	Final concentration				Colcemide: time of addition/culture duration after addition
						TPA (μg/ml)	CpG (μM)	IL-2 (U/ml)	Culture duration	
Decker et al. (2000b)	RPMI 1640 + Hepes/ 10% FCS/Penicillin-Streptomycin/ Na-Pyruvate/L-glutamine/ L-asparigine/2-mercaptoethanol/ MEM nonessential amino acids	Centrifugation over Ficoll-Hypaque layer + anti-CD2/anti-CD14 magnetic beads	PB	1.0×10^6	1	ND	2	ND	72h	ND
Decker et al. (2002)	As above	As above	PB	1.0×10^6	5	ND	1	100	8h	ND
Mayr et al. (2006)	Iscove medium/ 10% FCS/ Penicilline-Streptomycin (for CD40L cultures)	Ficoll-Hypaque density? (for CD40L cultures)	PB	2.5×10^6 (for CD40L cultures)[a]	NR	ND	1	50	72 hr	72 hr/NR
Dicker et al. (2006)	RPMI 1640 + Hepes + Ultraglutamine/ Penicillin-Streptomycin/ 20% FCS	ND	BM or PB	10×10^{6a}	NR	ND	2	200	72 hr	48 hr/24 hr
Haferlach et al. (2007)	As above	ND	BM or PB	10×10^{6a}	NR	ND	2	200	72 hr	48 hr/24 hr
Struski et al. (2009)	NR	ND	NR	$1.0–2.0 \times 10^6$	NR	0.05	1	20	72 hr	72 hr/20 min
Present study	RPMI 1640 (+Hepes + Glutamine + FCS + Penicillin-Streptomycin)	ND	BM or PB	$1.0–2.0 \times 10^6$	5–10	0.01–10	1	100	72 hr	72 hr/10–60 min
Center 1	RPMI 1640 + glutamax/18% FCS	ND	BM or PB	1.0×10^6	5	0.1	1	100	72 (−120) hr	72 hr/45 min
Center 2	RPMI 1640 + L-glutamine/ Penicillin-Streptomycin/FCS	ND	BM or PB	NR	5	10	1	100	72 hr	72h/60min
Center 3	RPMI 1640 + Hepes + Ultraglutamine/Penicillin-Streptomycin/FCS	ND	BM or PB	1.8×10^6	10	0.1	1	100	72 hr	72 hr/50 min
Center 4	As above	ND	BM or PB	1.0×10^6	6	0.01	1	100	72 hr	72 hr/45 min
Center 5	As above	ND	BM or PB	$1.0–2.0 \times 10^6$	10	0.05	1	100	72 hr	72 hr[b]/10 min

[a]Number of cells/culture.
[b]Colchicine instead of Colcemid.
BM, bone marrow; CpG, CpG-oligonucleotide; hr, hours; IL-2, interleukin-2; min, minutes; ND, not done; NR, not reported; PB, peripheral blood; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

TABLE 3. Clinical and Cytogenetic Data per Center in the Present Study

Clinical and cytogenetic data	Center 1	2	3	4	5	Total
Investigated by CCA (*n*)	78	62	36	21	20	217
Investigated by FISH (*n*)	73	58	33	19	18	201
Age (median, yr)	70	65.5	62.5	64	65	66
Gender						
Men (*n*)	35	32	20	14	14	115
Women (*n*)	43	30	16	7	6	102
Diagnosis						
CLL confirmed (*n*)	70	52	35	21	20	198
CLL suspected (*n*)	8	10	1	0	0	19
Time of sample collection						
Diagnosis (*n*)	60	52	32	16	12	172
Follow-up (*n*)	18	10	4	5	8	45
Metaphases analyzed in both cultures (median, *n*)	22	15	25	35	20	20
Metaphases analyzed in TPA culture (median, *n*)	10	7	13	16	9.5	10
Metaphases analyzed in CpG/IL-2 culture (median, *n*)	10	8	10.5	20	10	10
CCA: success rate (%)[a]						
Overall (%)	97	66	100	100	90	89
TPA (%)	96	52	97	86	55	79
CpG/IL-2 (%)	96	60	94	100	65	83
CCA: proliferation capacity (cases investigated; *n*)	0	0	36	21	0	57
>20 metaphases/slide in TPA	0	0	8	15	0	23
>20 metaphases/slide in CpG/IL-2	0	0	13	20	0	33
15–20 metaphases/slide in TPA	0	0	14	0	0	14
15–20 metaphases/slide in CpG/L-2	0	0	12	1	0	13
<15 metaphases/slide in TPA	0	0	14	6	0	20
<15 metaphases/slide in CpG/IL-2	0	0	11	0	0	11
CCA: quality of banding (cases investigated; *n*)	0	0	36	21	16	73
Good in TPA	0	0	8	3	6	17
Good in CpG/IL-2	0	0	16	6	2	24
Intermediate in TPA	0	0	17	7	10	34
Intermediate in CpG/IL-2	0	0	12	12	14	38
Poor in TPA	0	0	11	11	0	22
Poor in CpG/IL-2	0	0	8	3	0	11
CCA and FISH: detection of chromosomal aberrations						
Normal CCA in TPA and CpG/IL-2 (*n*)	35	30	15	5	16	101
Abnormal CCA in TPA and CpG/IL-2 (*n*)	30	22	13	10	3	78
Abnormal CCA in TPA alone (*n*)	2	2	1	0	0	5
Abnormal CCA in CpG/IL-2 alone (*n*)	11	8	7	6	1	33
CCA detection rate TPA (%)	41	39	39	48	15	38
CCA detection rate CpG/IL-2 (%)	53	48	56	76	20	51
FISH detection rate (%)	77	60	64	63	67	68
Combined CCA and FISH detection rate (%)	85	71	73	95	67	78
Type of tissue						
BM (*n*)	25	54	21	13	7	120
PB (*n*)	53	8	15	8	13	97
CCA detection rate, BM (%)	48	48	57	69	43	52
CCA detection rate, PB (%)	58	75	60	88	8	56
CCA: proportion of abnormal metaphases per case						
Highest in TPA (*n*)	7	5	3	2	1	18
Highest in CpG/IL-2 (*n*)	12	13	8	8	2	43
Equal in CpG/IL-2 and TPA (*n*)	11	4	2	0	0	17
CCA: abnormalities per culture						
TPA: 1 aberration	16	15	6	4	2	43
CpG/IL-2: 1 aberration	20	18	10	8	2	58
TPA: 2 aberrations	6	7	2	2	1	18
CpG/IL-2: 2 aberrations	9	8	2	4	0	23
TPA: ≥3 aberrations (complex karyotype)	10	2	6	4	0	22
CpG/IL-2: ≥3 aberrations (complex karyotype)	12	4	8	4	2	30

(Continued)

TABLE 3. Clinical and Cytogenetic Data per Center in the Present Study (Continued)

Clinical and cytogenetic data	Center 1	2	3	4	5	Total
CCA: detection of specific clonal aberrations						
del(13q) TPA (*n*)	4	10	4	0	0	18
del(13q) CpG/IL-2 (*n*)	6	14	9	0	2	31
+12 TPA (*n*)	13	3	4	2	0	22
+12 CpG/IL-2 (*n*)	14	4	4	6	0	28
del(11q) TPA (*n*)	9	6	4	2	1	22
del(11q) CpG/IL-2 (*n*)	10	5	3	2	1	21
del(17p) TPA (*n*)	3	0	2	2	0	7
del(17p) CpG/IL-2 (*n*)	3	0	2	2	1	8
del(6q) TPA (*n*)	3	1	0	0	0	4
del(6q) CpG/IL-2 (*n*)	6	1	0	0	1	8
14q32 aberration TPA (*n*)	2	4	0	2	0	8
14q32 aberration CpG/IL-2 (*n*)	2	5	0	2	0	9
Translocations TPA (*n*)	15	5	8	2	0	30
Translocations CpG/IL-2 (*n*)	25	9	11	6	2	53
CCA: nonclonal aberrations (cases investigated; *n*)	0	62	36	21	20	139
Nonclonal aberrations in TPA (*n*)	0	18	9	11	6	44
Nonclonal aberrations in CpG/IL-2 (*n*)	0	12	9	10	3	34
Nonclonal translocations TPA (*n*)	0	3	4	0	4	11
Nonclonal translocations CpG/IL-2 (*n*)	0	4	5	0	2	11

[a]Success is defined as ≥10 normal metaphases or the presence of any clonal abnormality and is expressed as percentage of total number of cases. BM, bone marrow; CCA, conventional cytogenetic analysis; CLL, chronic lymphocytic leukemia; CpG/IL-2, CpG-oligonucleotide and interleukin-2; FISH, fluorescence in situ hybridization; *n*, number; PB, peripheral blood; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

Belgium) was added for 10-60 min before harvest. In one center, colchicine (Fluka, Sigma-Aldrich, Bornem, Belgium) was used instead of Colcemid. Chromosome preparation, staining and metaphase analyses of both cultures were performed according to standard protocols.

In one center, parallel control cultures were set up with peripheral blood from five healthy volunteers, using identical culture conditions as for the patient samples.

Chromosomal aberrations

Karyotypes were described according to ISCN 2005 (Shaffer and Tommerup, 2005). Miscellaneous aberrations were defined as those distinct from del(6q), del(11q), +12, del(13q), 14q32 aberrations, del(17p), and translocations. Additional material of unknown origin (add) was included in the group of miscellaneous aberrations and was not counted as a translocation. Clonality was defined as the presence of structural aberrations or gain of an entire chromosome in at least two metaphases or loss of an entire chromosome in at least three metaphases. When a particular aberration was observed in only one metaphase, it was considered as clonal when confirmed in at least one metaphase in the parallel culture (or two metaphases in case of loss of a whole chromosome) or in a significant proportion of interphase nuclei by interphase FISH. Failure was defined as less than 10 normal metaphases in the culture under consideration, whereas success was defined as 10 or more normal metaphases or the presence of a clonal abnormality. As the analyses of the results were in part retrospective, a total of only 15–20 metaphases was analyzed in two centers (Centers 2 and 5), not reaching the optimum of 10 metaphases per culture.

Proliferation capacity

Proliferation capacity was estimated through a manual count of metaphases per slide. The cases were classified into three groups: <15 mitoses/slide, 15–20 mitoses/slide, and >20 mitoses/slide.

Quality of banding

The cases were classified into three groups: good, intermediate, and poor, according to the quality of banding, with respect to the number of chromosomal bands per haploid karyotype, i.e., >300 bands, 200–300 bands, and <200 bands, respectively. The number of bands was estimated with idiograms of banding patterns (Shaffer and Tommerup, 2005).

Interphase FISH Analyses

For historical and practical reasons, FISH was performed on cultured material in most Belgian centers. Only in Center 4 (all cases) and occasionally in other centers, uncultured material was used. The following commercially available probes (all from Vysis/Abbott, Downers Grove, IL) were used to investigate loci commonly involved in CLL: 6q21 [LSI MYB], 11q22 [LSI ATM], centromere 12 [CEP 12], 13q14 [LSI 13 RB1 combined with LSI D13S319], 14q32 [LSI IgH dual color/break apart], and 17p13 [LSI TP53 combined with CEP17]. At the time of diagnosis, FISH was performed in all centers on samples from patients aged less than 70 years. In Center 1, the panel included six probes (see above), whereas in the remaining four centers, the panel included four probes, i.e., 11q22, centromere 12, 13q14, and 17p13. On follow-up samples and in patients aged more than 70 years, interphase FISH was performed according to local practice policy. The analyses were performed either on uncultured samples or on stored cytogenetic specimens harvested after stimulation with TPA or CpG/IL-2. Cut-off values were established in each center for each probe. For uncultured and cultured samples and for peripheral blood and bone marrow, the same cut-offs were used.

Statistical Analyses

Unadjusted differences between TPA and CpG/IL-2 were assessed using the McNemar test with continuity correction. This test was also applied for testing proliferation capacity and quality of banding (McNemar, 1947; Agresti, 2002).

Differences, adjusted for center and tissue, were estimated using subject-specific estimates in generalized linear mixed models with a random intercept for patient and a logit or log link as appropriate, using maximum likelihood (Agresti, 2002). Adaptive Gaus-Hermite quadrature with 7 points was used to integrate over the random intercept, unless otherwise indicated (Agresti, 2002; Bates et al., 2008). Estimates are given compared with a baseline level, which is "Center 1" for center, "bone marrow" for tissue and "CpG/IL-2" for method. Details are shown in Supporting Information Tables 1–9. Results were considered significant at the 0.05 level.

Some individual aberrations were found to have a low overall prevalence. For these aberrations, the models adjusted for center and tissue were numerically unstable because of imbalances in the results (indicated in text by *). We therefore only report unadjusted results using the McNemar test for these (Agresti, 2002).

RESULTS

Conventional Cytogenetic Analysis

Chromosomal aberrations

A median of 20 metaphases (range 3–61) could be cytogenetically analyzed in 214 of 217 cases (99%). Failure (defined above) occurred in 42 cases after TPA (success rate of 81%) and in 35 cases after CpG/IL-2 culture (success rate of 84%). No significant difference in failure/success rate for either stimulation technique was observed ($P = 0.082$). Centers 2 and 5 were significantly more likely to have a failure; however, this can be explained by local practice policies concerning the number of analyzed metaphases per culture. Of note, Center 4 analyzing the highest number of metaphases per culture had the highest detection rate of abnormalities. Detailed results per center are shown in Table 3.

Clonal abnormalities were observed in 116 cases (53%). In 78 cases (36%), the aberrant clone was detected in both cultures. Of these, the percentages of aberrant metaphases were similar in both cultures in 17 and higher in CpG/IL-2 and TPA cultures in 43 and 18 cases, respectively. One aberrant clone was detected in 46 of these 78 cases. All but one clone were identical in both culture conditions. Of 32 cases with more than one subclone, the subclones were related in both cultures in 26 cases. One additional unrelated subclone was found only after stimulation with CpG/IL-2 or TPA in four and one cases, respectively. In one case, all subclones observed in CpG/IL-2 and TPA cultures were unrelated. In 33 and five additional cases, a clonal abnormality was detected only in CpG/IL-2 or TPA cultures, respectively. Thus, the percentage of abnormal karyotypes with CpG/IL-2 and TPA was 51% and 38%, respectively ($P < 0.0001$). In addition, CpG/IL-2 cultures were significantly more likely to yield a higher number of aberrations ($P = 0.0004$). Details on number of aberrations per culture and on karyotype complexity are shown in Table 3.

The following abnormalities were detected after CpG/IL-2 and TPA stimulation, respectively: del(13q) ($n = 31$ vs. 18, $P = 0.0059$*), +12

TABLE 4. Clonal Translocations Detected by Conventional Cytogenetic Analysis

Breakpoint	Translocation	Case no.	Center	Breakpoint	Translocation	Case no.	Center
1 p34	t(1;9)(p34;p15)[a]	98	2	q13	t(5;12)(q31;q13)[a]	182	4
q12	t(1;14)(q12;p11.2)[a,b]	211	5	13 q10	der(13;21)(q10;q10)[a]	182	4
q21	t(1;2)(q21;q11)	46	1	q13	t(2;13)(q33;q13)[a]	142	3
q25	der(2)t(1;2)(q25;q37)	156	3	q13	t(6;13)(p11;q13)	150	3
q25	der(17)t(1;17)(q25;q21) ins(17;?)(q21;?)	51	1	q13	der(7)t(7;13)(p12;q21), der(13)t(7;13)(p12;q13)[a]	163	3
q31	t(1;9)(q31;q33)	159	3	q14	t(2;13)(q11;q14)[a]	30	1
2 p21	t(2;15)(p21;q15)[a]	58	1	q14	t(2;13)(q33;q14)	114	2
p13	t(2;14)(p13;q32)[c]	120	2	q14	t(6;13)(q13;q14)	48	1
p12	t(2;8)(p12;p11)[a]	15	1	q14	t(8;13)(q24;q14)	164	3
p12	der(17)t(2;17)(p12;p11)	66	1	q14	t(9;13)(q?32;q14)[a]	58	1
p11.2	t(2;18)(p11.2;q23)	188	4	q14	?t(13;14)(q14;q24)[c,d]	52	1
q?11	t(2;6)(q?11;p21)	22	1	q14	t(13;17)(q14;q22)[a]	102	2
q11	t(2;13)(q11;q14)[a]	30	1	q14	t(13;17)(q14;q25)[a]	68	1
q21	t(1;2)(q21;q11)	46	1	q14	t(13;18)(q14;q22)[a]	57	1
q24	der(2)del(2)(q21q24) t(2;11)(q24;q14), der(11) t(2;11)(q24;q14)	174	3	q15	t(6;13)(q14;q15)[a]	68	1
				q21	der(7)t(7;13)(p12;q21), der(13)t(7;13)(p12;q13)	163	3
q33	t(2;13)(q33;q14)	114	2	14 p11.2	t(1;14)(q12;p11.2)[a,b]	211	5
q33	t(2;13)(q33;q13)[a]	142	3	q24	?t(13;14)(q14;q24)[c,d]	52	1
q37	der(2)t(1;2)(q25;q37)	156	3	q32	t(2;14)(p13;q32)[c]	120	2
3 q26	t(3;5)(q26;q22)	79	2	q32	t(8;14)(q24;q32)	44	1
4 p11.2	der(17)t(4;17)(q12;p11.2)[a,b]	211	5	q32	t(11;14)(q13;q32)[a]	137	2
p10	der(4;17)(p10;q10)	182	4	q32	t(14;18)(q32;q21)[c]	45	1
q21	der(9)t(4;9)(q21;q33)[a]	171	3	q32	t(14;18)(q32;q21)[a]	96	2
q27	t(4;6)(q27;q24)	150	3	q32	t(14;18)(q32;q21)	129	2
q31	t(4;9)(q31;p23)[a]	131	2	q32	t(14;19)(q32;q13)	92	2
5 q22	t(3;5)(q26;q22)	79	2	q32	t(14;19)(q32;q13)[a]	43	1
q31	t(5;12)(q31;q13)[a]	182	4	15 q15	t(2;15)(p21;q15)[a]	58	1
6 p21-22	t(6;18)(p21–22;q11.2)[a]	58	1	16 q24	t(16;18)(q24;q22)[a]	186	4
p21	t(2;6)(q?11;p21)	22	1	q10	der(4;17)(p10;q10)	182	4
p21	t(6;12)(p21;p13)	31	1	17 p13	t(17;17)(p13;q11.2)[a]	15	1
p11	t(6;13)(p11;q13)	150	3	p11.2	der(17)t(4;17)(q12;p11.2)[a,b]	211	5
p10	?der(6;17)(p10;q10)[a]	179	4	p11	der(17)t(2;17)(p12;p11)	66	1
q13	t(6;13)(q13;q14)	48	1	p11	der(17)t(8;17)(q13;p11)	67	1
q14	t(6;13)(q14;q15)[a]	68	1	p10	?der(6;17)(p10;q10)[a]	179	4
q23	t(6;22)(q23;q11–13)[a]	53	1	q11	der(8)t(8;17)(p11;q11)	8	1
q24	t(4;6)(q27;q24)	150	3	q11	der(18)t(17;18)(q11;p11)	151	3
7 p12	der(7)t(7;13)(p12;q21), der(13)t(7;13)(p12;q13)[a]	163	3	q11.2	t(17;17)(p13;q11.2)[a]	15	1
8 p23	der(8)t(X;8)(p21.2;p23)	35	1	q21	der(17)t(1;17)(q25;q21)ins(17;?)(q21;?)	51	1
p21	der(8)t(8;8)(p21;q24)[a]	76	1	q22	t(13;17)(q14;q22)[a]	102	2
p11	der(8)t(8;17)(p11;q11)	8	1	q25	t(13;17)(q14;q25)[a]	68	1
q13	der(17)t(8;17)(q13;p11)	67	1	18 p11	der(18)t(17;18)(q11;p11)	151	3
q24	der(8)t(8;8)(p21;q24)[a]	76	1	p11	der(18)t(18;20)(p11;q11)	159	3
q24	t(8;13)(q24;q14)	164	3	q11.2	t(6;18)(p21-22;q11.2)[a]	58	1
q24	t(8;14)(q24;q32)	44	1	q22	t(13;18)(q14;q22)[a]	57	1
q24	psu dic(8;17)(q24;?)	67	1	q21	t(14;18)(q32;q21)[c]	45	1
9 p23	t(4;9)(q31;p23)[a]	131	2	q21	t(14;18)(q32;q21)[a]	96	2
p15	t(1;9)(p34;p15)[a]	98	2	q21	t(14;18)(q32;q21)	129	2
q?32	t(9;13)(q?32;q14)[a]	58	1	q22	t(16;18)(q24;q22)[a]	186	4
q33	t(1;9)(q31;q33)	159	3	q23	t(2;18)(p11.2;q23)	188	4
q33	der(9)t(4;9)(q21;q33)[a]	171	3	19 q13	t(14;19)(q32;q13)[a]	43	1
11 q?11	der(11)t(11;11)(q?11;?)	51	1	q13	t(14;19)(q32;q13)	92	2
q13	t(11;14)(q13;q32)[a]	137	2	20 q11	der(18)t(18;20)(p11;q11)	159	3
q13-21	der(11)t(8;11)(?;q13-21)	67	1	21 q10	der(13;21)(q10;q10)[a]	182	4
q14	der(2)del(2)(q21q24)t(2;11)(q24;q14), der(11)t(2;11)(q24;q14)	174	3	22 q11–13	t(6;22)(q23;q11-13)[a]	53	1
12 p13	t(6;12)(p21;p13)	31	1	X p21.2	der(8)t(X;8)(p21.2;p23)	35	1

[a]Only in CpG/IL-2 culture.
[b]Nonclonal, but previously detected at the time of diagnosis.
[c]Only in TPA culture.
[d]This translocation was present in a tetraploid subclone, which could not be fully analyzed.

(n = 28 vs. 22, P = 0.11*), del(11q) (n = 21 vs. 22, P = 1*), 14q32 aberrations (n = 9 vs. 8, including deletions, translocations, and add P = 0.8), del(17p) (n = 8 vs. 7, P = 1*) and del(6q) (n = 8 vs. 4, P = 0.13*). Translocations after CpG/IL-2 and TPA stimulation were observed in 53 versus 30 cultures (P = 0.027), respectively (Tables 3 and 4). The increase in detection rate after CpG/IL-2 stimulation was similar for both balanced (n = 32 vs. 17) and unbalanced (n = 21 vs. 13) translocations. Translocations associated with B-cell lymphoproliferative disorders were observed in eight cases: t(14;18)(q32;q21) (n = 3), t(14;19)(q32;q13) (n = 2) and one each of t(2;14)(p13;q32), t(8;14)(q24;q32), and t(11;14)(q13;q32). We did not attempt to review all karyotypes and small variations in breakpoint designations are to be expected because different banding techniques (G, R or Q) were used in different centers. Nevertheless, the breakpoint 13q13-14 appears to be highly recurrent, being observed in 13 translocations. Some of these, such as t(2;13)(q33;q13-14), could be recurrent in different patients. Miscellaneous aberrations were detected in 48 versus 38 cases (P = 0.030) after CpG/IL-2 and TPA stimulation, respectively.

In peripheral blood from five healthy volunteers, 50 metaphases were examined per culture per case. In cultures stimulated with CpG/IL-2, no aberrations were detected, except for a nonclonal t(3;7)(q23;q35) and gain of a nonclonal marker in one control. Similar results were obtained with TPA, i.e., a nonclonal translocation t(7;14)(q33;q12) and nonclonal trisomy 3 in one control each.

Data on structural and numerical nonclonal aberrations were available in four centers (n = 139). Miscellaneous nonclonal abnormalities were observed in 44 cases after TPA and in 34 cases after CpG/IL-2 stimulation (P = 0.13). Among these, nonclonal translocations were detected in a higher proportion in CpG/IL-2 than in TPA cultures (P = 0.75), i.e., in 11 (32%) versus 11 cases (25%), respectively (Table 3).

Proliferation capacity

Proliferation capacity was assessed in a total of 57 cases in two centers (Table 3). The mitotic index was slightly higher in CpG/IL-2 than in TPA cultures: >20 mitoses/slide were seen in 33 versus 23, 15–20 mitoses/slide in 13 versus 14, and <15 mitoses/slide in 11 versus 20 cases, respectively. However, this increase was not significant (P = 0.094).

Quality of banding

Quality of banding was assessed in a total of 73 cases in three centers (Table 3). There was a tendency for superior quality in CpG/IL-2 cultures compared with TPA cultures. Indeed, the quality was good in 24 versus 17, intermediate in 38 versus 34, and poor in 11 versus 22 cases after CpG/IL-2 and TPA stimulation, respectively (P = 0.063).

Interphase FISH Results

Interphase FISH investigations were performed in 201 cases (93%), either on uncultured samples (n = 24) or on fixed cells obtained after culture with TPA (n = 78) or CpG/IL-2 (n = 99). A full probe panel of four to six commonly involved regions was applied in 188 cases, whereas in 13 cases only one to three regions were analyzed.

One or more abnormalities were detected in 68% of cases. The combination of cytogenetics and FISH increased the detection rate to 78% (Table 3). In 24% of the cases with normal conventional cytogenetics, FISH detected an aberration. Conversely, FISH failed to detect any aberration, while conventional cytogenetics was abnormal in 10% of the cases. In 66% of all cases, both FISH and conventional cytogenetics were concordantly abnormal (44%) or normal (22%) (data not shown). However, some changes established by conventional cytogenetics and FISH were not overlapping. Indeed, interphase FISH missed a substantial proportion of abnormalities. Of 263 aberrations found in 217 CpG/IL-2 stimulated cultures, 183 and 168 aberrations could not be detected by the classical FISH panel with four and six probes, respectively. For TPA stimulated cultures, 131 and 119 of 194 aberrations could not be detected by the classical FISH panel with four and six probes, respectively.

Recurrent aberrations not identified by conventional cytogenetics were detected in 80 cases: del(13q) (n = 71), del(11q) (n = 5), +12 (n = 2), del(14q) (n = 1), and del(17p) (n = 1). Conversely, FISH failed to detect del(11q), del(13q), del(6q), +12, and del(17p) in six, two, one, one, and one cases, respectively, in which the specific change was visible at the karyotypic level. This may reflect a selective proliferative response of

the CLL clone or inappropriate location of the FISH probe.

DISCUSSION

Oligonucleotides (ODN) containing a CpG motif stimulate cells of the immune system, and Toll-like receptor 9 (TLR9) has been identified as the receptor for CpG. In humans, the only cell types known to express TLR9 are B cells and plasmacytoid dendritic cells (PDCs) (Hornung et al., 2002). As malignancies of PDCs are extremely rare, CpG-mediated activation may be applicable only for B-cell malignancies. It has been established that CpG stimulates a broad spectrum of B-cell malignancies, i.e., CLL, small lymphocytic lymphoma, marginal zone lymphoma, follicular lymphoma, diffuse large B-cell lymphoma, and mantle cell lymphoma (Jahrsdorfer et al., 2005a). After endosomal uptake, CpG-ODN activates stress kinase pathways (Häcker et al., 1998) and NFkB within minutes (Sparwasser et al., 1997). As a consequence, B cells enter the cell cycle, secrete immunoglobulin, and are rescued from spontaneous (Yi et al., 1998) or Fas-mediated (Wang et al., 1997) apoptosis. Moreover, this process results in up-regulation of costimulatory and surface molecules (e.g., CD20), and therefore may provide a target for antibody therapy (Jahrsdorfer et al., 2005a). Although CpG induces proliferation in the above-mentioned B-cell malignancies, proliferation is weaker compared with normal B cells and, at least in CLL, it is followed by increased apoptosis (Jahrsdorfer et al., 2005a). This apoptotic phenomenon has been documented particularly in CLL samples with del(13q) as a single aberration. In samples with other recurrent aberrations (del(17p), del(11q), and trisomy 12) the effect on apoptosis is more heterogeneous. CLL apoptosis depends in part on caspase activation and is accompanied by up-regulation of Fas as well as of the Fas ligand (Jahrsdorfer et al., 2005 a,b).

The lower proliferative response to CpG-ODN for CLL cells compared with normal B cells can be overcome by addition of IL-2. Indeed, compared with normal B cells, CpG causes a stronger induction of the IL-2 receptor α chain (CD25) in CLL, resulting in higher numbers of IL-2 receptors with a stronger affinity. Costimulation with CpG and IL-2 might alter IL-2 signaling in CLL cells in addition to increase cytokine production and surface molecule expression (Decker et al., 2000a). Recently introduced culture methods with CpG/IL-2 improved proliferation capacity of CLL cells and yielded detection rates of cytogenetic aberrations comparable with interphase FISH, i.e., 81–83% (Table 1) (Dicker et al., 2006; Haferlach et al., 2007). However, in our centers, the overall detection rate of chromosomal aberrations was lower (51%). Although this represents a significant improvement (i.e., an increase of 13%) compared with TPA ($P < 0.0001$), detection rates $\geq$80% could not be reached. A possible explanation for this observation is that no cell separation procedures (Decker et al., 2000b; 2002) were applied. However, Struski et al. (2009) and Dicker et al. (2006), who did not enrich B-cell populations (personal communication) nevertheless also achieved detection rates of 80%. Until now, only one group (Struski et al., 2009) reported comparative data on stimulation with TPA and CpG/IL-2, showing an increase in detection rate of 9% versus 13% in our study.

Interphase FISH identified abnormalities in 68%. In 24% of the cases with a normal karyotype, FISH detected at least one aberration. Conversely, in 10%, FISH failed to detect any abnormality, despite abnormal conventional cytogenetics. The combination of cytogenetics and FISH could increase the detection rate to 78%. Of note, the abnormalities observed by conventional cytogenetics and FISH, using classical CLL probe panels, were not always overlapping. This illustrates the need for a combined routine analysis, including both techniques.

Interestingly, in some cases aberrations were detected in only one culture condition, i.e., CpG/IL-2 ($n = 33$) or TPA ($n = 5$). Of note, CpG/IL-2 cultures resulted in a higher detection rate of translocations ($P = 0.027$) and del(13q) ($P = 0.0059$*). Most of the aberrations that were undetected at the karyotypic level but detected by FISH were small deletions involving 13q14 ($n = 71/80$), which often are cytogenetically cryptic. While del(13q) is easily detected by FISH, translocations which represent an indicator of poor prognosis in CLL (Mayr et al., 2006; Van Den Neste et al., 2007) can escape FISH detection. This illustrates the importance of performing conventional cytogenetics in addition to FISH. Stimulation with CpG/IL-2 resulted in better quality of banding and a higher proliferation capacity; however, these results were not significant ($P = 0.063$ and $P = 0.094$, respectively). The latter contrasts with the data by Struski et al. (2009), who reported a gain of 20 metaphases on average after TPA stimulation versus CpG/IL-2 stimulation.

Another question to address is whether abnormalities found after CpG/IL-2 stimulation might be related to activation-induced cytidine deaminase (AID). CpG stimulation of CLL and normal B cells induces expression of AID, an enzyme that is linked to the development of cytogenetic abnormalities (Capolunghi et al., 2008). However, culturing B cells of healthy blood donors with CpG/IL-2 did not induce clonal abnormalities, thus validating CpG/IL-2 as a tool for the cytogenetic analysis of CLL (Wu et al., 2008). Dicker et al. (2006) also showed that normal karyotypes were obtained after CpG/IL-2 stimulation of samples of some CLL patients and of healthy individuals and that sequential analysis yielded the same aberrations in two tested cases. Accordingly, we did not detect clonal aberrations in samples from five healthy donors and most abnormalities in patient samples were found in both CpG/IL-2 and TPA cultures. Moreover, nonclonal aberrations were not more prevalent after CpG/IL-2 cultures compared with TPA cultures. This indicates that CpG/IL-2 does not induce clonal cytogenetic changes. The detected abnormalities hence represent true CLL-associated aberrations.

In conclusion, we confirm that the detection rate of clonal chromosomal abnormalities, especially of translocations and del(13q), is superior after CpG/IL-2 stimulation compared with TPA. The combination of both culture techniques does not result in a considerable increase of the detection ratio. Therefore, CpG/IL-2 should be preferred for routine conventional cytogenetic analysis of CLL. However, our detection rate was lower than previously reported, possibly reflecting differences in patient characteristics, inclusion criteria, transport delay or in sample quality in routine settings. Finally, neither conventional cytogenetics nor CLL-specific FISH detected all aberrations, demonstrating the complementary nature of both techniques.

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