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Real-time polymerase chain reaction determination of cytokine mRNA expression profiles in Hodgkin's lymphoma

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A B S T R A C T

Background and Objectives. Classical Hodgkin's lymphoma (HL) is a malignant disorder characterized by a small number of tumor cells and inflammatory cells. Both the tumor cells and the inflammatory cells produce cytokines which are thought to contribute to the clinical parameters of HL. Quantification of these cytokines at a protein level is still somewhat imprecise. We, therefore, used a method to quantify cytokine mRNA expression in HL cell lines and lymph node biopsies.

Design and Methods. We used real-time quantitative polymerase chain reaction (RQ-PCR) to investigate mRNA expression for interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-12p35, IL-12p40, IL-13, IL-15, interferon (IFN)-γ and tumor necrosis factor (TNF-α) in lymph node tissue from 15 patients with classic Hodgkin's lymphoma (c-HL) and one with lymphocyte predominance (LP) HL. HL-derived cell lines L1236, L540, and L428 were also investigated. Reactive lymphatic tissue (n=6) and peripheral blood mononuclear cells (PBMC) from healthy donors (n=4) before and after stimulation were used as controls. In 5 c-HL samples the cytokine expression in T lymphocytes was also studied by flow cytometry.

Results. All c-HL samples (but not LP) expressed IL-13 mRNA. This cytokine was not found in non-stimulated PBMC or in reactive lymphatic tissue. Expression of IL-10, IL-1β, IL-15 and IL-12p35 mRNA was higher in HL samples than in PBMC and reactive lymphatic tissue. Expression of IL-10, IL-1β, TNF-α and IFN-γ mRNA was significantly higher in the EBV+ HL samples (n=6) than in the EBV- cases. All HL cell lines showed high expression of IL-13, IL-12p35, TNF-α and IL-15 mRNA. IFNγ mRNA levels were high in L428 and L540 cells, IL-10 in L1236 cells and L540 cells, IL-5 in L428 cells and IL-4 in L1236 cells.

Interpretation and Conclusions. Cytokine mRNA levels can be measured by RQ-PCR using a limited amount of tissue. This method gives valuable information on biological variation between different HL samples and may contribute to unraveling the complex cytokine network contributing to the clinical and biological heterogeneity of this disease.

Key words: cytokines, Hodgkin's lymphoma, real-time quantitative PCR, EBV, flow cytometry.

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Classical Hodgkin's lymphoma (cHL) is a malignant lymphoproliferative disorder characterized by a small number of tumor cells [Hodgkin and Reed-Sternberg (H-RS) cells] surrounded by inflammatory cells, including T-cells, macrophages, plasma cells, eosinophils and neutrophils.[1,2] This inflammatory infiltrate has been associated with an altered production of cytokines, which play a central role in the immune system by modulating immune responses. Both the H-RS cells and the inflammatory cells have been identified as a source of cytokines in HL infiltrates.[3] However, the complex interactions between these cell populations are far from elucidated. It is believed that all cell populations present contribute to the clinical features of the disease.

Quantification of cytokines on a protein level in tissue samples has been hampered by limited availability of specific antibodies for immunohistochemical detection and by the usually insufficient quantity of tissue for ELISA studies. Therefore, detection of cytokine mRNA expression by Northern blotting analysis and *in situ* hybridization (ISH) has been widely applied to determine cytokine profiles in HL.[3] Most of the previously used detection systems are not reliably quantitative. Since we were interested in the global production of cytokines by all cell types present, we applied a novel, real-time polymerase chain reaction (RQ-PCR) method[4] to quantify cytokine mRNA expression in HL cell lines and lymph nodes. We applied a commercially available Cytokine Gene Expression Plate I [PE Applied Biosystems, Foster City, CA, USA (PEAB)] to measure interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-12p35, IL-12p40, IL-15, interferon (IFN)-γ and tumor

necrosis factor (TNF-α) mRNA levels and separately investigated mRNA levels for IL-13.

Design and Methods

Patients and biopsies

Representative fragments from lymph node biopsies collected during 1997-99 from patients with HL at diagnosis were frozen in liquid nitrogen and stored at –80°C. Histological diagnosis was made according to the WHO classification[5] (11 had mixed cellularity (MC) type HL, 4 had nodular sclerosis (NS) and 1 nodular LP HL). The frequency of H-RS cells and the level of eosinophilic infiltration were determined as previously described.[6] The clinical characteristics of the patients are given in Table 1.

PBMC were obtained from 4 healthy donors by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Lymphocytes were activated at a concentration of 2×10^6 cells/mL in RPMI (Gibco Laboratories, Grand Island, NY, USA) with 10% fetal calf serum (FCS) phorbol 12-myristate 13-acetate (PMA, 10ng/mL, Sigma, St.Louis, USA), ionomycin (Io, 1 μM) Sigma, St.Louis, USA) and phytohemagglutinin (PHA, 2.5 μg/mL, Sigma, St.Louis, USA) for at least 3 hours in 5% CO_2 at 37°C. Three tonsils were obtained from patients undergoing tonsillectomy because of recurrent tonsillitis and 3 samples of carotid lymphatic plaques (CLP) were obtained from patients undergoing carotid endarterectomy. These specimens are examples of reactive lymphatic tissue (RL).

HL cell lines L428, L1236 and L540 (all EBV negative)[7,8] were obtained from Prof. Volker Diehl (Cologne, Germany) and cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FCS, L-glutamine and antibiotics.

Immunohistochemistry (IHC)

Immunostaining for CD3, CD15, CD20, CD30, CD79a [all from DAKO A/S, Glostrup, Denmark (DAKO)] was performed on paraffin sections using avidin-biotin-peroxidase complex (Strept. AB Complex /HRP, DAKO) to confirm the diagnosis. EBV-related latent membrane protein–1 (LMP-1, CS 1-4, DAKO) was detected as described previously.[9] The presence of EBV in LMP-1 positive biopsies was confirmed by *in situ* hybridization for EBV Early RNA (EBER) using the INFORM EBER Kit and Benchmark IHC/ISH Staining Module (Ventana Medical Systems, Tucson, Arizona, USA).

Real-time quantitative PCR

Total RNA was extracted using Ultraspec (Biotex Laboratory, Inc., Houston, TX, USA). c-DNA was prepared from 1 μg of total RNA in a 20 μL volume according to standard methods. PCR reactions were performed in a 96-well plate (Cytokine Gene Expression Plate I, PEAB) and 2 μL of c-DNA were used for amplification by the RQ-PCR ABI Prism TM 7700 Sequence Detection System (PEAB). Reaction components including universal master mix and cycling conditions were identical to those described in User Bulletin # 2 P/N 4303859 (PEAB). All primers and probes were designed by PEAB (Cytokine Gene Expression Plate I - PEAB). The probes were labeled with FAM as the reporter dye and TAMRA as the quencher dye, except for the 18S RNA probe, which was labeled with VIC as the reporter dye and TAMRA as the quencher dye. The system was linked directly to a Power-Macintosh-7200/120 containing data analysis software (ABI Prism™ 7200/7700 Sequence Detection System version1.6.3 P/N 4304880, PEAB).

In order to determine IL-13 mRNA expression, the PCR reaction was performed in a 96-well plate using 2 μL of c-DNA, universal master Mix (PEAB), applying 1.25 μL of primers and probes for IL-13 (PEAB) and 18S control gene, supplemented with RNA-ase/DNA-ase free water to 25 μL volume. The probe for IL-13 was labeled with FAM and 18S was labeled with VIC.

To normalize for differences in RNA extraction and efficiency of the RT, we applied the comparative Ct method using the endogenous reference gene 18S RNA. The relative cytokine mRNA quantification was performed by the comparative ΔΔCt method[10] (Manual Taq-Man Cytokine Expression Plate I, PEAB) in which the levels of cytokine mRNA in each studied sample were compared to the results obtained in the non-stimulated PBMC from healthy donors used as the calibrator. The results in all experimental samples are expressed as an *n*–fold difference relative to the calibrator. For cytokine mRNA not expressed in PBMC the ΔCt values were used. For each quantification assay, samples were tested in duplicate and PBMC from healthy donors were included as controls on each plate. After normalization, comparison of Ct values for duplicate samples showed minimal variation (range 0.1-0.4). All samples had Ct values <23 for the reference gene 18S RNA and were thus considered as having mRNA of good enough quality for further studies. The samples with Ct values >36 were considered as not expressing quantitative amounts of a given cytokine mRNA. The mean ΔCt obtained in non-stimulated PBMC for each cytokine (Table 2) was used as a calibrator in the ΔΔCt method to establish the expression of cytokines in HL samples, cell lines, tonsils and CLP.

Flow cytometric detection of cytokine expression in lymphocytes

Mononuclear cell populations were obtained from 3 reactive tonsils and HL samples from 5 patients by

Table 1. Characteristics of Hodgkin's lymphoma patients and cytokine mRNA expression in HL samples.

Pat	Age	Sex	HL type	Stage	EBV	No H-RS HPF*	Eosino-philia	IL-1α***	IL-1β**	IL-2	IL-4	IL-5	IL-8	IL-10	IL-12 p35	IL-12 p40	IL-15	INFγ	TNFα	IL-13***
1	63	M	MC	IIIB	+	12.5	+	ΔCt=20.7	15.56	3.25	neg	neg	1.31	183.6	1.83	ΔCt=19.9	20.68	210.80	2.41	ΔCt=21.7
2	49	M	NSII	IA	+	8.8	+	neg	11.24	1.68	neg	neg	131.6	903.9	Neg	ΔCt=23.4	13.64	63.56	4.56	ΔCt=22
3	51	M	MC	IA	+	5.9	+	neg	7.94	neg	neg	neg	1.3	155.4	5.30	ΔCt=21.1	0.59	11.47	1.17	ΔCt=17
4	18	M	MC	IIA	+	5.3	+++	neg	0.32	0.62	neg	neg	0.23	51.63	1.61	ΔCt=22.2	1.39	11.16	0.34	ΔCt=18.2
5	26	M	NSI	IIIA	+	18	+	ΔCt=23.2	10.6	13.2	0.95	ΔCt=23	10.5	198.1	0.82	ΔCt=21.1	13.6	18.64	2.46	ΔCt=15.1
6	41	F	NSI	IIB	+	8.8	++	ΔCt=22.2	89.26	8.88	neg	neg	neg	754.8	79.3	neg	192.67	108.4	4.82	ΔCt=21.8
7	44	M	NSII	IIIA	–	8	+	ΔCt=20.7	1.19	1.43	0.37	neg	0.69	25.46	1.15	ΔCt=20.5	8.63	5.70	1.42	ΔCt=18
8	23	M	MC	IIIA	–	25.1	+	ΔCt=23.8	5.54	0.13	neg	neg	0.82	34.78	1.41	ΔCt=22.1	0.55	0.61	1.04	ΔCt=15.2
9	24	M	MC	IA	–	1.6	+	ΔCt=25.8	0.34	neg	neg	neg	neg	39.67	1.37	ΔCt=20.7	0.68	20.68	0.50	ΔCt=20.2
10	54	F	MC	IA	–	3.2	+++	ΔCt=23.9	1.07	0.76	neg	neg	neg	8.17	1.47	ΔCt=20.6	neg	1.87	0.44	ΔCt=18
11	83	F	MC	IIA	–	2.9	+	ΔCt=20.2	3.32	1.27	neg	neg	4.53	155.42	3.14	ΔCt=15.3	18.77	44.32	1.39	ΔCt=23
12	23	M	MC	IIA	–	20	+	neg	0.22	0.72	0.34	neg	0.34	7.36	0.12	neg	1.41	3.66	0.07	ΔCt=19.8
13	56	M	MC	IIA	–	14.1	+	ΔCt=24.4	3.78	2.77	neg	neg	2.01	49.87	0.76	ΔCt=16.2	0.71	22.47	0.95	ΔCt=23.4
14	84	F	MC	IA	–	2.8	+++	ΔCt=23.02	0.31	0.77	neg	neg	0.72	11.31	0.13	ΔCt=24.2	1.85	1.16	0.15	ΔCt=19
15	53	F	NSI	IA	–	10	+++	ΔCt=23.1	0.88	neg	neg	neg	2.75	4.72	0.19	ΔCt=neg	0.85	0.41	0.11	ΔCt=19
16	41	M	LP	IA	–	Not tested	0	ΔCt=19.9	0.71	1.73	neg	neg	0.28	149.1	2.08	ΔCt=19.4	17.03	18.77	1.11	Neg

HPF: high power field **Results of mRNA expression for IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12p35, IL-15, INF-γand TNF-α are given by comparison to those found in PBMC (n-fold expression) * Since no expression of IL-1α, IL-5, IL-12p40 and IL-13 was found in PBMC, the results are given as ΔCt.*

Table 2. Cytokine mRNA expression in non-stimulated and stimulated peripheral blood mononuclear cells (PBMC).

	PBMC-1	PBMC-2	PBMC-3	PBMC-4	Mean PBMC non-stimulated	Mean PBMC stimulated
IL-1α*	neg	neg	neg	neg	neg	13.4
IL-1β	18.4	18.3	17.5	18.3	18.1	8.8
IL-2	22.4	21.5	21.1	23.6	22.2	10.5
IL-4	22.2	23.3	23.7	24.3	23.3	17.7
IL-5	neg	neg	neg	neg	neg	20.1
IL-8	22.4	21.1	24.1	20.9	22.2	5.5
IL-10	22.7	23.2	22.6	22.7	22.7	17.0
IL-12p35	19.7	20.7	21.1	21.7	20.8	22.0
IL-12p40	neg	neg	neg	neg	neg	neg
IL-15	22.6	22.5	21.6	20.1	21.7	21.1
IFN-γ	19.7	21.6	18.9	19.9	20.1	10.6
TNF-α	16.8	17.1	16.2	17.1	16.8	10.5
IL-13	neg	neg	neg	neg	neg	21.0

**The results are given as normalized ΔCt value (ΔCt=Ct FAM -Ct VIC).*

Table 3. Cytokine mRNA expression in HL cell lines and reactive lymphatic tissue.

	L 1236*	L 428*	L 540*	Tonsil*	CLP*
IL-1β°	neg	neg	neg	1.08	1.07
IL-2	neg	neg	neg	0.35	5.5
IL-4	7.4	neg	neg	neg	neg
IL-8	neg	neg	neg	9.86	neg
IL-10	2.3	neg	0.5	19.7	24.7
IL-12p35	25.3	18.38	12.38	0.64	2.16
IL-15	5.6	11.96	17.75	0.64	2.16
IFN-γ	neg	25.45	2.77	0.67	0.8
TNF-α	10.1	5.39	2.39	2.56	0.96
IL-1α#	ΔCt=20.5	neg	neg	ΔCt=19.3	ΔCt=22.3
IL-5	ΔCt=23.9	ΔCt=11.6	ΔCt=24.5	neg	neg
IL-12p40	neg	neg	neg	ΔCt=21.9	ΔCt=25.6
IL-13	ΔCt=16.2	ΔCt=11.8	ΔCt=16.9	neg	1 of 3 was positive with ΔCt=22.4

**The mean value given is for two studies for cell lines and three samples for reactive lymphatic tissue. °Results of mRNA expression for IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12p35, IL-15, INF-γ and TNF-α are given by comparison to those found in PBMC. #Since no expression of IL-1α, IL-5, IL-12p40 and IL-13 was found in PBMC, the results are given as ΔCt .*

mincing with scalpel and Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Cytokine detection by flow cytometry was performed as described previously.[11] Lymphocytes were activated as described above. Monensin (SIGMA, St Louis, USA, 2 μM) was added 60 min before harvesting. Immediately after Ficoll separation (non-stimulated sample) or after stimulation for 3hr, cells were washed twice with buffer (0.9% NaCL, 0.5% BSA, 0.1% $N\alpha N_3$), fixed with paraformaldehyde at 4 °C for 10 min. and permeabilized with 500μL FACS Perm (BD) for 10 min, in the dark. Cells were washed twice and stained by three color direct immunofluorescence with antibodies against the B-cell marker CD22-FITC or T-cell markers CD4-FITC, CD3-PerCP (all from BD) and CD8-TRICOLOR (Caltag, Burlingame, CA, USA), simultaneously with PE-conjugated antibodies to cytokines (IL-2, IL-4, IFN-γ, TNF-α, all from BD-Pharmingen, San Diego, CA, USA). The activation was confirmed by staining with CD69-FITC antibody (BD) (>80% of $CD69^+$ CD4 and CD8 cells). At least 20,000 events were acquired on a FACS-Calibur flow cytometer (BD) using Cell Quest acquisition software. The expression of cytokines in various subsets was evaluated after gating on the population-specific marker expression and lymphoid (low/intermediate) side scatter using Paint-a-Gate-Pro software (BD).

Statistical analysis

Standard descriptive statistics and a non-parametric Mann-Whitney U-test were applied. All values were based on two-tailed analysis and p values <0.05 were considered to be statistically significant.

Results

Cytokine mRNA expression

Normal peripheral blood mononuclear cells

The results for cytokine mRNA expression in non-stimulated PBMC were similar for all 4 samples (Table 2). PBMC, after 3 hr non-specific activation with PMA, ionomycin and PHA, showed increased expression of IL-1β, IL-2, IL-8, IL-10, IFN-γ, and TNF-α mRNA and expressed IL-1α, IL-5 and IL-13 mRNA, but not IL-12p40 (Table 2). No difference in IL-12p35 and IL-15 mRNA expression was detected between before and after stimulation (Table 2).

Reactive lymphatic tissue (RL)

A comparison of cytokine mRNA expression profiles in RL and mRNA levels found in PBMC is presented in Table 3. IL-13 mRNA was detected only in one of 3 CLP samples, but not in tonsils. Levels of IL-10 and IL-1β mRNA were higher in RL than in PBMC. By comparison to PBMC levels, IL-8 mRNA expression was higher in tonsils, but not expressed in CLP and IL-2 was higher in CLP but not in tonsils. For most other cytokines mRNA expression was almost identical in RL and PBMC. Neither IL-4 nor IL-5 mRNA was detected in RL.

Cytokine mRNA expression in HL cell lines

All HL cell lines expressed IL-13, IL-12p35, TNF-α, and IL-15 mRNA at considerably higher levels than did activated PBMC, while no expression of IL-1β, IL-2, IL-

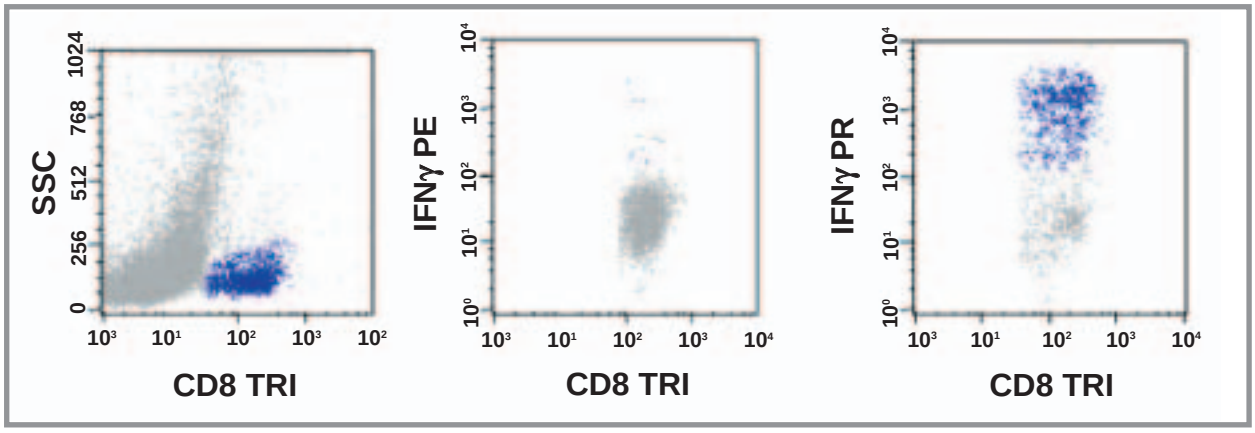

Figure 1. IFNγ protein expression in CD8⁺ T-cells isolated from a c-HL lymph node sample. The left panel shows the gating procedure with the gate set on CD8 expression and side scatter (SSC). The middle panel shows low numbers of IFN-γ-expressing cells before stimulation (0.5% of CD8⁺ cells) and the right panel shows a high number of IFN-γ expressing cells after stimulation with PMA, PHA and ionomycin in the presence of monensin (55% of CD8⁺ cells).

12p40 and IL-8 mRNA was observed (Table 3). Considerable differences were noted between the three tested cell lines. IL-1α and IL-4 mRNA was found in L1236 cells, while no expression could be demonstrated in L540 and L428 cells. IL-10 mRNA was expressed in L1236 and L540 at levels similar to those in PBMC, but was not detectable in L428 cells. Levels of IL-5 mRNA were much higher in L428 than in L540 and L1236 cell lines. INFγ mRNA expression was much higher in L428 cells than in L540, but not detected in L1236.

Cytokine mRNA expression in lymph nodes from HL patients

The levels of expression for all tested cytokines are given in Table 1. IL-13 mRNA expression was found in all tested HL samples except the one of LP subtype. The levels of expression were similar to those found in stimulated PBMC and in HL-cell lines. IL-10 mRNA expression was higher in all HL samples than in PBMC and in 66% of HL samples higher than in RL. IL-1α mRNA was detectable in 75% of HL samples. The expression was similar to that in RL, but lower than in stimulated PBMC. IL-1β mRNA expression was higher in 50% of HL samples than in PBMC and RL. IL-2 mRNA was detectable in 80% of c-HL samples and in 46% (7/15) showed slightly higher levels than those of PBMC. Variable IFN-γ mRNA expression was found in 75% HL samples. TNF-α mRNA levels in most HL samples were similar to or lower than those in PBMC and RL. IL-15 and IL-12p35 mRNA expression was higher in HL samples than in PBMC and RL. IL-8 mRNA expression levels were similar to those in PBMC and lower than those in RL. The expression of IL-12p40 was found, in most c-HL samples, at levels similar to those detected in RL. IL-4 transcripts were found in only 3 c-HL samples (20%) and constituted 0.34-0.95 of the mean level found in PBMC. IL-5 mRNA expression in c-HL samples was under the cut-off level for reliable detection, while low levels (Ct 33.8) were found in case #5.

By comparison to EBV negative samples, EBV positive HL lymph nodes showed significantly higher expression of IL-1β (p=0.018), IFN-γ (p=0.034), IL-10 (p=0.003) and TNFα mRNA (p=0.025). For all other cytokines there was no statistical difference between mRNA expression in EBV+ and EBV- samples.

We observed a tendency towards a somewhat higher expression of TNF-α mRNA in the NS histological subtype (p=0.066). Otherwise the levels of cytokine mRNA expression did not differ between MC and NS histological subtypes. There was no correlation between numbers of H-RS cells in samples or degree of eosinophilia and mRNA expression for various cytokines.

Flow cytometric detection of cytokine expression in T lymphocytes from HL lymph nodes and tonsils

Freshly isolated, non-stimulated T-cells from HL samples and tonsils showed only a small fraction of T-cells positive for IL-2, IL-4, IFN-γ and TNF-α (0.5-3% of CD4 and CD8⁺ cells). After 3h stimulation the percentages of CD8⁺ cells expressing IFN-γ were observed to be high in HL samples (mean 53% CD8/IFN-γ double positive cells, Figure 1) and somewhat lower in the tonsil samples (mean 22%). A slight increase in CD4/IFN-γ double positive cells was noted both in tonsils (mean 11%) and HL samples (mean 12%). A greater increase in percentages of CD4⁺ cells and CD8⁺ expressing TNF-α was found in HL samples (mean 30% and 24%, respectively) than in tonsils (mean 10% and 12% for CD4⁺ and CD8⁺ cells, respectively). No increase in percentages of T cells expressing IL-2 after stimulation was observed in HL samples. However, it was found that 25% of CD4⁺ cells

expressed IL-2 in 1 of the 3 tonsil cell suspensions studied. IL-4-producing cells remained very scarce (<5%) even after stimulation. No production of studied cytokines was observed in B cells. When the results for individual samples were compared, no direct correlation was seen between the levels of cytokine mRNA expression by RQ-PCR and percentages of T cells expressing the studied cytokines on a protein level before and after stimulation.

Discussion

The present results, obtained by a novel quantitative RQ-PCR technique, demonstrate that levels of expression for IL-13, IL-10, IL-1β and IFNγ mRNA are higher in HL lymph node tissue than in PBMC and reactive lymphatic tissue from tonsils and CLP. Several methods have been applied to detect cytokine mRNA expression, such as Northern blotting, *in situ* hybridization, RNA-ase protection assays, c-DNA arrays and reverse transcription PCR.[3,4] The results obtained by these methods are at best semi-quantitative. RQ-PCR is faster, more accurate, more sensitive and allows reliable comparison of mRNA expression levels between various samples.[4,12]

Like the recent data from the c-DNA array methodology, our results were obtained using mRNA from total HL tissue and cell lines considered as representing H-RS cells.[13] Interactions between the H-RS cells and surrounding cells contribute to the clinical picture of HL. Therefore, data providing information about the global tumor microenvironment are of interest. RQ-PCR results corresponding to the average expression of the given RNA in a tissue may not be sensitive enough to detect expression confined to a small population of cells. However, all examined c-HL samples (but not the LP HL sample) showed expression of IL-13 mRNA similar to that found in activated PBMC and in HL cell lines. Previous *in situ* hybridization studies[8,14] suggested that H-RS cells are a primary source of IL-13. Both H-RS cells and surrounding reactive cells frequently express IL-13 receptor. Therefore, an autocrine growth stimulation mechanism has been proposed.[14,15] We could not show any correlation between the level of expression and frequency of H-RS cells in sections from the same lymph node sample. This lack of correlation may be due to the fact that the density of H-RS varies in lymph nodes and the frozen fragments could have a different H-RS cell density than that of samples evaluated by routine histology. However, by studying HL cell lines we found that the level of IL-13 mRNA was higher in L428 cells than in L1246 cells. This is in agreement with the previous report by Oshima and Puri[15] who showed considerably higher IL-13 secretion by L428 cells. The levels of mRNA expression for several cytokines were significantly higher in EBV$^+$ HL samples than in EBV$^-$ HL samples. The association of high IL-10 expression with EBV positivity in HL was previously shown by both *in situ* hybridization and immunohistochemistry.[16-18] It is important to note that the primer set for the IL-10 gene does not include sequences homologous to that of the viral IL-10 gene, thus the results represent the endogenous, human IL-10 mRNA expression.[17,19] IL-10 has been suggested as a candidate for autocrine regulation of EBV-immortalized B cells.[20] It has been postulated that, by expressing IL-10, H-RS cells may evade T-cell recognition.[16] However, *in vitro* studies using IL-10 pre-treated HL derived cell-lines and EBV-specific cytotoxic T-lymphocytes were unable to confirm this hypothesis.[21] Clinical studies have indicated that elevated IL-10 levels in sera of untreated HL patients correlate with an unfavorable prognosis.[22-24] However, gene-profiling studies using a cDNA array method associated up-regulation of IL-10 mRNA within the NS HL subtype with a good prognosis.[13]

We found high expression of IFN-γ mRNA in all c-HL samples, with the highest levels in EBV$^+$ cases. This is in contrast to findings by Dukers *et al.*[18] who, using *in situ* hybridization could not detect any significant difference in numbers of IFN-γ positive cells between EBV$^+$ and EBV- HL samples. Previous studies indicated the dominance of activated CD4$^+$ cells with the Th_2-like phenotype and cytokine expression profile in HL.[25] Our finding of high expression of IFNγ mRNA suggests that there may be a group of patients with predominance of a Th_1-like response in the lymph nodes.

In summary, quantitative real-time PCR showed that cytokine mRNA levels of IL-13, IL-10, IL-1β, IFN-γ, IL-15 and IL12p35 in HL lymph node tissue were higher than those in PBMC and reactive lymphatic tissue. The highest levels of mRNA for several cytokines were found in EBV$^+$ samples, which may be related to both immune response and immune escape mechanisms in EBV$^+$ Hodgkin's lymphoma. Extended studies by RQ-PCR in larger cohorts of patients may help to define the potential association of cytokine mRNA levels *in situ* in HL lymph nodes with clinical features and outcome.

MM, APM, MB: conception and design, MM,MS, AS: RQ-PCR studies; BM, NL: flow cytometry studies, JS: clinical data, MM, APM: analysis and interpretation of data, MM: drafting the manuscript, APM, MB, JS: revising the manuscript, AMP, MB: final approval of the manuscript. The authors reported no potential conflicts of interest.

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