original paper
Haematologica 1997; 82:542-549

THE PATTERNS OF IL2, IFN-γ, IL4 AND IL5 GENE EXPRESSION IN HODGKIN'S DISEASE AND REACTIVE LYMPH NODES ARE SIMILAR

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ABSTRACT

Background and Objective. The lymph nodes involved in *classic* Hodgkin's disease (HD), i.e. mixed cellularity (MC) and nodular sclerosis (NS) subtypes, usually contain few (1-2%) Reed-Sternberg (RS) cells scattered in a background of lymphocytes, eosinophils, plasma cells and neutrophils. CD4+ T-lymphocytes are increased in number, express activation markers and cluster around RS cells. The presence of eosinophilia in most HD patients and the presence of hyper-IgE in a subset of them may suggest that activated lymph node T cells release large amounts of IL5 and IL4, respectively.

Methods. The expression of four T-cell-associated cytokine genes, i.e interleukin (IL)2, IL4, IL5 and interferon (IFN)-γ, in frozen sections of 14 HD (7 MC, 7 NS) and 10 reactive lymph nodes was investigated by qualitative and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). T-cell clones were also raised from purified CD4+ lymphocytes of one HD lymph node and one reactive lymph node and tested for IL2, IL4, IL5 and IFN-γ secretion in culture supernatants by immunoassays.

Results. The transcripts of all the cytokine genes were detected in every lymph node irrespective of the HD or reactive nature. HD or reactive lymph node-derived CD4+ T-cell clones released the four cytokines according to a predominant T-helper (Th)0-type pattern. In more than half of the lymph nodes of either HD or reactive nature, there was a predominance of IL4 over IFN-γ mRNA production (Th2-type pattern). In the remaining HD or reactive lymphadenopathies, either a balanced IL4/IFN-γ mRNA ratio (Th0-type pattern) or a predominance of IFN-γ over IL4 mRNA expression (Th1-type pattern) was observed.

Interpretation and Conclusions. The overall pattern of cytokine gene expression in classic HD is similar to that detected in reactive lymph nodes. Further studies are needed to determine whether differences in the absolute concentrations of cytokines released in HD versus reactive lymph nodes and the long-standing course of HD versus the self-limiting nature of reactive adenopathies may explain certain peculiar features of HD, such as eosinophilia, for example.
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Key words: Hodgkin's disease, cytokines, lymph nodes, PCR

Hodgkin's disease (HD) is classified according to four histologic subtypes: nodular sclerosis (NS), mixed cellularity (MC), lymphocyte predominance (LP) and lymphocyte depletion (LD).[1] Recently, these entities have been included in the *Revised European-American Lymphoma Classification* (REAL) together with a provisional entity, the lymphocyte-rich classic HD.[2] Epstein-Barr virus (EBV) may be involved in the pathogenesis of the disease, as the EBV genome can be detected in the neoplastic cells from 40-60% of the NS or MC cases.[3,4]

Reed-Sternberg (RS) cells are always present in the NS, MC and LD histologic subtypes, whereas they are less represented in the LP variant.[1,2] RS cells usually constitute 1-2% of the total lymph node cellularity. Lymphocytes, histiocytes, plasma cells, eosinophils and neutrophils account for the background cellularity.[1,2] CD4+ T lymphocytes in HD lymph nodes are increased in number and express activation markers.[5-10]

The production of IL2, IL4, IL5 and IFN-γ allows for the identification of three discrete subsets of human CD4+ T cells, namely T helper (Th)1, Th2 and Th0 cells.[11-17] Th1 cells produce IL2 and IFN-γ, but little or no IL4 or IL5.[11-14] Conversely, Th2 cells are specialized in the production of IL4 and IL5 rather than IL2 or IFN-γ.[11-14] Lastly, Th0 cells dis-

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Acknowledgements: the authors wish to thank Mrs. Elda Accettulli for her precious secretarial assistance. This study was supported by grants from the Consiglio Nazionale delle Ricerche, A.C.R.O. '95 (PF 39) to V.P., M.F. and C.D.B., Associazione Italiana per la Ricerca sul Cancro to V.P., M.F. and C.D.B. and Ministero della Sanità to V.P.
Received April 14, 1997; accepted July 9, 1997.

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play an unrestricted profile of cytokine production.[13,16-17] In disease states characterized by an accumulation of activated T-cells, the patterns of cytokines produced can play a major role in the pathophysiology of the underlying disease as well as in its clinical manifestations.

We reasoned that *in vivo* activated T-cells present in HD lymph nodes might be an important source of cytokines, and hypothesized that such cells were polarized towards a Th2 phenotype in view of the prominent eosinophilia that represent a histological hallmark of the disease. For this reason, we investigated the qualitative and quantitative profiles of *in vivo* expression of four T cell-associated cytokine genes (IL2, IL4, IL5 and IFN-γ genes) in frozen lymph node tissue sections from 14 HD patients (7 with NS, 7 with MC subtypes) and, for comparison, from 10 patients with reactive adenopathies. We selected the NS and MC subtypes since they represent the histologic variants of HD that are most frequently observed (*classic* HD). The choice of the cytokine genes for study was based on the above-mentioned premises.

Patients and Methods

Patients

Fourteen patients with HD (age range 16-66, 8 males and 6 females) were studied. Seven of them had the MC subtype, whereas the remaining 7 had the NS histologic subtype. Ten patients with reactive lymphadenopathies (age range 20-67, 6 males and 4 females) were also studied. HD was diagnosed by routine clinical, histologic and immunophenotypic criteria.[1,2] In particular, an effaced lymph node architecture characterized by the presence of the multicleated RS cells or mononucleated Hodgkin's cells scattered in an abundance of normal, reactive cells was considered diagnostic for HD.[1,2] The histologic features of the lymph node samples from the 10 patients with reactive lymphadenopathies were the following: 8 displayed follicular hyperplasia, 1 showed paracortical hyperplasia, 1 dysplayed combined follicular and paracortical hyperplasia. All patients were untreated at the time of the study.

Reverse transcriptase polymerase chain reaction (RT-PCR) for cytokine mRNA detection

The expression of IL2, IL4, IL5 and IFN-γ genes was investigated using the RT-PCR procedure. Fifteen to twenty 15mm-thick cryostatic sections were cut from frozen lymph node samples, pooled and used for total RNA extraction by a modified Chomczynsky-Sacchi method.[18] RNA was then reverse transcribed into cDNA for PCR amplification using the 1*st* Strand cDNA Synthesis Kit (Clontech Laboratories Inc., Palo Alto, CA, USA) and the oligo dT primer supplied with the kit according to the instructions of the manufacturer. The resulting cDNA mixture was diluted for a final volume of 100 µL, and 2 µL of the diluted samples were amplified with forward (F) and reverse (R) primers specific for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) housekeeping gene to check the efficiency of reverse transcription.

Qualitative RT-PCR

Five µL of the cDNA mixture for each sample were amplified using 25 pmoles of each cytokine specific primer and 2U of *Taq* polimerase (Clontech Laboratories Inc., Palo Alto, CA, USA) by 35-38 cycles of denaturation at 94°C for 1 min, annealing at 55°C (IL2, IFN-γ), 65°C (IL4) for 2 min or 59°C for 1 min (IL5) and extension at 72°C for 1 min in a total volume of 50 µL. The sequences of the primers used are the following:[19] IL2 F primer ACT CAC CAG GAT GCT CAC AT, IL2 R primer AGG TAA TCC ATC TGT TCA GA; IL4 F primer ATG GGT CTC ACC TCC CAA CTG C, IL4 R primer TTC CTG TCG AGC CGT TTC AG; IL5 F primer ATG AGG ATG CTT CTG CAT TTG, IL5 R primer CTA TTA TCC ACT CGG TGT TCA; IFN-γ F primer AGT TAT ATC TTG GCT TTT CA, IFN-γ R primer ACC GAA TAA TTA GTC AGC TT. PCR products were analyzed by electrophoresis on 2% agarose gel and visualized by etidium bromide staining. The specificity of the amplification products has been unambiguously demonstrated in a previous study with antigen specific T-cell clones where the correspondence between cytokine transcript detection and protein secretion was found to be absolute.[20]

Quantitative RT-PCR

In order to quantify the relative amount of each cytokine mRNA in the individual lymph node samples, a competitive PCR procedure was used.[21,22] The construction of the competitor template was carried out as published[21] with minor modifications. Competitors were designed introducing a 30 bp long sequence into the product of amplification to distinguish the cDNA product from the internal standard.

A typical competitive PCR experiment is performed by the addition of decreasing amounts of a known competitor to a fixed amount of the cDNA to be quantified, and the ratio between the final amplification products for the two species is evaluated for each point.[21] From this ratio, which precisely reflects the ratio between the initial amount of the two templates, the amount of unknown cDNA can be evaluated. Using this procedure, it is possible to quantify the absolute concentrations of cytokine mRNAs within a given lymph node sample, but not to compare such concentrations among different samples. The latter comparison can be obtained by the calculation of the ratio between the absolute concentrations of two cytokine

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mRNAs (e.g. IL4 and IFN-γ mRNA) estimated in the individual samples.

The competitive PCR experiments were usually carried out as follows: five reactions with a 5 fold decreasing amount of competitor from 1.6×10^{-3} to 2.56×10^{-6} attomoles and 1 µL of cDNA were performed. The amplification profile and the experimental conditions were as described above, the number of cycles ranged from 36 to 39. The primers used were the same employed for qualitative PCR with the exception of the IL4 F primer CCG AGT TGA CCG TAA CAG AC and the IL5 F primer GAA ATT CCC ACA AGT GCA TTG. Fifteen µL of each reaction were resolved on a 12% polyacrylamide gel, stained with etidium bromide, photographed, digitalized by computer scanning and analyzed using the NIH Image 1.49 software. The ratio between the competitor value and the cDNA value relative to each competitor dilution was calculated. The experimental points detected by the competitor/cDNA ratio and the relative competitor concentration are distributed linearly. A linear interpolation of the experimental points obtained has been calculated; the resultant line gives the x value corresponding to y=1, that represents the relative amount of mRNA. This mathematical procedure was carried out using a computer program kindly written by Dr. M.G. Calevo, Institute G. Gaslini, Genoa, Italy. When necessary, the range of the competitor dilutions was widened. Some experiments were repeated randomly to verify the reproducibility of the method.

Cell separation

Cell suspensions were obtained from four HD lymph nodes and three reactive lymph nodes by gentle teasing of lymph node tissue. Mononuclear cells (MNC) were subsequently purified by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and washed three times in RPMI 1640 medium (Gibco, Grand Island, NY, USA). In three cases, two with HD and one with reactive lymphadenopathy, for whom sufficient amounts of fresh lymph node tissue were available, the following cell separation procedure was carried out. Cell suspensions were first prepared by gentle teasing of lymph node tissue. $CD4^+$ T cells were isolated from MNC by immunomagnetic separation with Dynabeads (Unipath, Milan, Italy). Briefly, MNC were first incubated with CD19, CD8 and CD14 monoclonal antibodies (mAbs, see below) and next with Dynabeads that had been coated with a goat anti-mouse IgG antiserum (Dako, Glorstup, Denmark) according to the manufacturer's instructions. MNC were subsequently exposed to a magnet and cells that had not bound to the beads were recovered in the supernatants. These negatively selected cell fractions consistently contained more than 95% $CD4^+$ cells.

Cell culture and cytokine assays

Purified lymph node $CD4^+$ T-cells were suspended at the concentration of 1×10^6/mL in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 2 mM sodium pyruvate, 100 IU/mL penicillin (Gibco, Paisley, Scotland) (complete medium) and 50 IU/mL recombinant (r)IL2 (Glaxo, Geneva, Switzerland). Cells were subsequently cultured for 15 days in 24 well plates (Corning, Staffordshire, UK) and washed and cloned by limiting dilution.[20] In brief, cells were seeded in 96-well U-bottom plates (Corning) at the concentration of 0.5 cell/well in 0.2 mL of complete medium supplemented with 20 U/mL rIL2 in the presence of 10^5 gamma-irradiated (60 Gy) allogeneic peripheral blood MNC and 1% v/v PHA-P (Wellcome, Burroughs, UK).[20] Cells were fed weekly with fresh complete medium containing 50 U/mL rIL2. After 15-20 days, each well was checked microscopically and proliferating microcultures were expanded in rIL2-containing medium.[20] The mean cloning efficiency obtained in these experiments was approximately 20%. Thereafter, cloned T-cells were washed three times, resuspended in complete medium and plated (1×10^6/mL) in 24 well plates in the presence or absence of 1% PHA-P and 5 ng/mL phorbol myristate acetate (PMA; Sigma Chemical Company, St. Louis, MO, USA). After 48 hours, culture supernatants were harvested and IL2, IL4, IL5 and IFN-γ concentrations were assayed by ELISA kits. The IL2 and IL4 kits were from Amersham (Buckinghamshire, England) and can detect a minimum of 6 and 2 pg/mL, respectively. The IFN-γ kit, which has a threshold of detection of 20 pg/mL, was from Medgenix Diagnostic, Fleurus, Belgium. The IL5 kit, that was constructed using anti-human IL5 antibodies and rIL5 as a standard, both kindly provided by Genzyme, detected a minimum of 30 pg/mL cytokine. $CD4^+$ T-cell clones were subdivided into three groups based upon the IL4/IFN-γ ratio, calculated by dividing the concentrations of the two cytokines detected in clone supernatants.[15,20] An IL4/IFN-γ ratio ranging from 0.1 to 10 was taken as evidence for Th0-type of the clones; when the same ratio was higher than 10 or lower than 0.1, the clones were defined as Th2-type or Th1-type, respectively.[20]

Surface marker analysis

Lymph nodes purified by Ficoll Hypaque gradients were stained with the following murine mAbs: OKT3 (CD3), OKT4 (CD4) and OKT8 (CD8) from Ortho, Raritan, NJ, USA; Leu 12 (CD19), IL2R (CD25), Leu 11b (CD16), Leu 23 (CD69) and HLA-DR from Becton-Dickinson, San José, CA, USA; UCHL1 (CD45RO) and MY4 (CD14) from Coulter, Hialeah, FL, USA. Cells were analyzed by indirect immunofluorescence using a FACScan

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(Becton-Dickinson). Cellular debris was excluded from the counts by setting the gate on a forward and side scatter dot plot.

Results

Analysis of IL2, IL4, IL5 and IFN-γ gene expression in HD and reactive lymph nodes by qualitative PCR

Frozen lymph node sections from fourteen HD patients (seven with the MC and seven with the NS histotypes) and from ten patients with reactive lymphadenopathies were analyzed for the presence of IL2, IL4, IL5 and IFN-γ mRNA by RT-PCR. As shown in Figure 1, the IL2, IL4, IL5 and IFN-γ genes were found to be expressed in the lymph nodes from all the patients with either HD or reactive lymphadenopathies.

Next, we investigated by RT-PCR whether $CD4^+$ T-cells isolated from two representative HD (1 NS, 1 MC) lymph nodes expressed cytokine mRNAs. IL2, IL4, IL5 and IFN-γ transcripts were detected in both cell fractions (data not shown).

Cytokine gene expression takes place in activated rather than resting T cells. Therefore we looked for the presence of the former cells by immunophenotypic analysis of MNC suspensions from four HD and three reactive lymph nodes. HD lymph nodes were found to contain higher amounts of $CD4^+$ cells than reactive lymph nodes (see the representative experiment of Figure 2A). Furthermore, a large proportion of $CD4^+$ cells from either HD (Figure 2B) or reactive (not shown) lymph nodes expressed the CD45RO, CD69, HLA-DR or CD25 activation markers.

In order to investigate whether the cytokine transcripts detected in lymph node tissue sections and purified $CD4^+$ lymph node cells were translated into proteins, the following experiments were performed. $CD4^+$ T-cells isolated from the lymph nodes of one patient with HD , MC subtype (case #4 of Table 2) and one patient with reactive lymphadenopathy (case #20 of Table 2) were cultured with low dose rIL2. This strategy allows to expand primarily *in vivo* activated T-cells. Thereafter, T-cell blasts were cloned by limiting dilution. Altogether, 28 $CD4^+$ T-cell clones were obtained from the HD lymph nodes and 18 from the reactive lymph nodes. Cloned T cells were stimulated with PHA and PMA for 48 hours. Supernatants were then collected and tested for the presence of IL2, IL4, IL5 and IFN-γ by ELISA. As shown in Table 1, the four cytokines (whose transcripts had been detected in lymph node tissue sections) were present in the culture supernatants from virtually all of the clones.

Based upon the IL4/IFN-γ ratio (see *Materials and Methods*), 22 out of 28 (78.5%) clones from the HD lymph node and 11 out of 18 (61.1%) clones from the reactive lymph node displayed a Th0-type pattern of cytokine production (Table 1). Five out of twenty-eight (17.9%) HD clones and 3 out of 18 (16.6%) clones from the reactive lymph node were classified as Th1-type (Table 1). Lastly, 1 out of 28 (3.6%) and 4 out of 18 (22.2%) clones from the HD and the reactive lymph nodes, respectively, had a Th2-type phenotype (Table 1). Thus, the patterns of cytokine production by $CD4^+$ T-cell clones from either the HD or the reactive lymph node were similar. Table 1 also shows that the majority of $CD4^+$ cell clones from both sources secreted abundant quantities of IL2 and IL5. Furthermore, some clones that could be defined as Th1-type or Th2-type according to the IL4/IFN-γ ratio displayed

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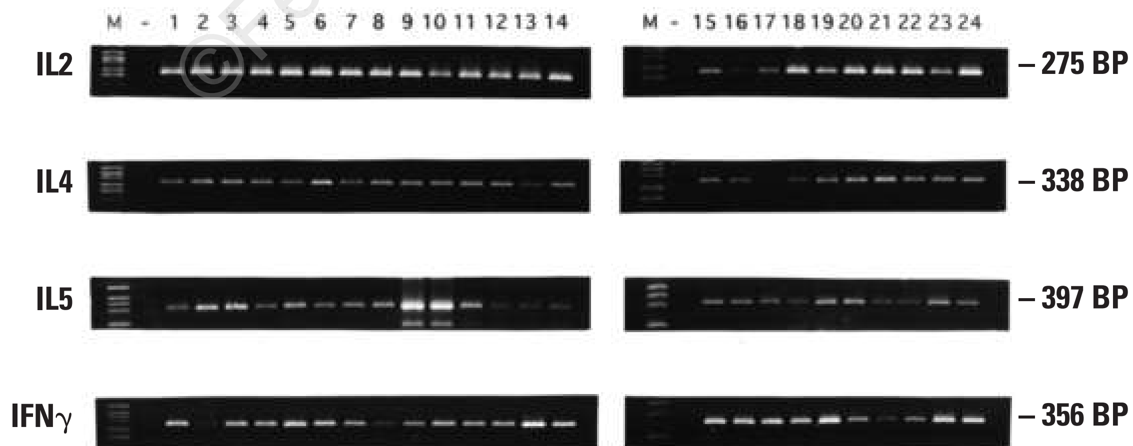

Figure 1. RT-PCR analysis of IL2, IL4, IL5 and IFN-γ gene expression in HD and reactive lymph nodes. M = markers of known sizes. Numbers from 1 to 24 indicate the lymph nodes studied: 1-7 HD lymph nodes, MC subtype; 8-14 HD lymph nodes, NS subtype; 15-24 reactive lymph nodes. The size of the expected amplification product for each cytokine is shown on the right side of the Figure.

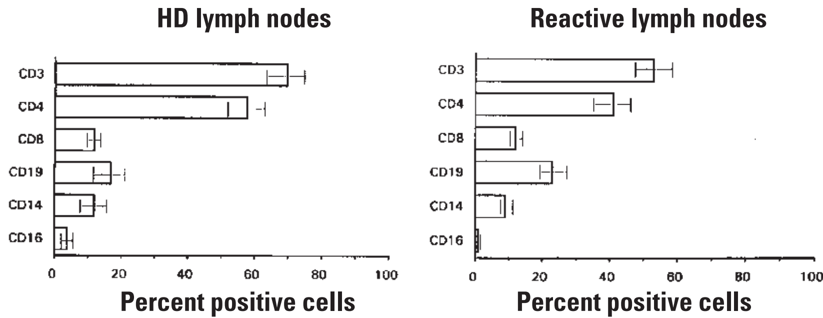

Figure 2A. Immunophenotypic analysis of MNC suspensions isolated from HD and reactive lymph nodes. Results are percent positive cells and represent the means ± SD from four experiments with HD lymph nodes and three with reactive lymph nodes.

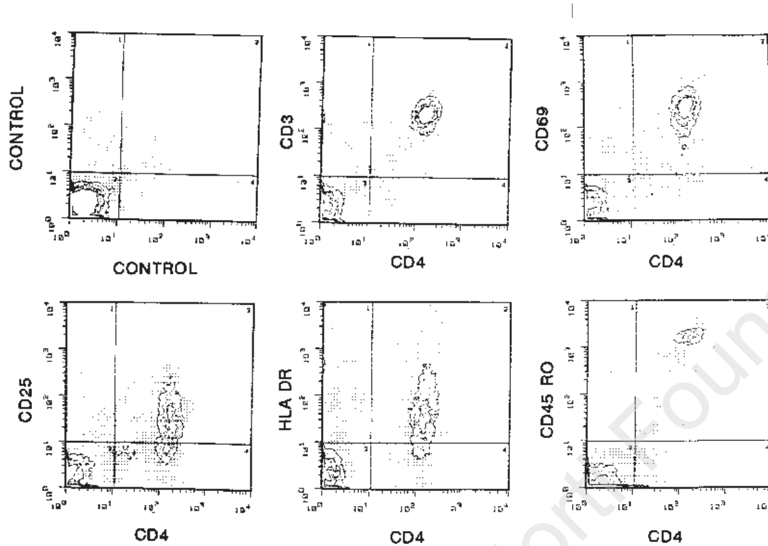

Figure 2B. Immunophenotypic analysis of MNC from one representative HD lymph node (out of four studied) showing the coexpression of activation markers (CD69, CD25, HLA-DR and CD45R0) on CD3+, CD4+ lymphocytes.

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profiles of IL2 and/or IL5 release that were not consistent with such classification[14] (see, for example, in Table 1 the 3.11 and 3.26 Th1-type clones from the HD lymph node which did not produce any IL2 or the 1.04, 1.11 and 1.13 Th2-type cell clones from the reactive lymph node that produced high amounts of IL2).

Quantitation of IL2, IL4, IL5 and IFN-γ gene expression in HD and reactive lymph nodes by competitive RT-PCR

Although the experiments above demonstrated that activated T cells from HD or reactive lymph nodes released cytokines, they gave only an indirect estimate of the relative amounts of cytokines produced *in vivo* in the single lymph nodes.

In order to gain more insight into this issue, we investigated the patterns of IL2, IL4, IL5 and IFN-γ gene expression in frozen lymph node sections using a competitive RT-PCR procedure.[20,22] The results of such experiments are summarized in Table 2, which shows the concentrations of each cytokine mRNA together with the IL4/IFN-γ mRNA ratio, obtained by dividing the amounts of IL4 mRNA by those of IFN-γ mRNA for each sample. This ratio allows the comparison of results from different samples. Lymph nodes were subdivided into three groups, namely, those with an IL4/IFN-γ ratio lower than 0.5, those with a ratio from 0.5 to 1.5 and those with a ratio above 1.5. These groups were arbitrarily defined as having a Th1-type, Th0-type or Th2-type pattern of cytokine gene expression, respectively. Based on these criteria, 8 out of 14 (57.2%) HD lymph nodes displayed a Th2-type pattern, 3 out of 14 (21.4%) a Th1-type pattern and 3/14 (21.4%) a Th0-type pattern (Table 2); 5 out of 7 (71.4%) lymph nodes from the MC subtype had a Th2-type pattern, 1 out of 7 (14.3%) a Th1-type pattern and 1 out of 7 (14.3%) a Th0-type pattern (Table 2); 4 out of 7 lymph nodes (57.1%) from the NS subtype displayed a Th2-type pattern, 2 out of 7 (28.6%) a Th1-type pattern and 1/7 (14.3%) a Th0-type pattern (Table 2); 7 out of 10 (70%) reactive lymph nodes had a Th2-type pattern, 2 out of 10 (20%) had a Th1-type pattern and 1 out of 10 (10%) displayed a Th0-type pattern (Table 2). Notably, the Th1-type pattern was detected in the only reactive adenopathy with paracortical hyperplasia as well as in an additional case with combined follicular and paracortical hyperplasia. Of the 8 reactive adenopathies with follicular hyperplasia, seven displayed a Th2-type pattern and one showed a Th0-

Table 1. Cytokine production by CD4+ T-cell clones raised from HD and reactive lymph nodes.

Clones from		*Cytokines (pg/mL)**				*IL4/IFN-γ*
		IL2	*IL4*	*IL5*	*IFN-γ*	*ratio*
HD lymph node 3	3.01	1800	1800	10000	800	2.25
	3.02	2000	130	800	130	0.065
	3.03	2000	3000	5000	900	3.33
	3.04	2000	500	3000	3000	0.16
	3.05	2000	130	500	600	0.21
	3.06	2000	530	1200	500	1.06
	3.07	2000	170	1000	600	0.28
	3.08	80	230	2400	400	0.57
	3.09	2100	900	1200	500	1.80
	3.10	2200	1500	280	700	2.14
	3.11	< 6	25	< 30	350	0.07
	3.12	2500	300	70	450	0.66
	3.13	2100	250	1000	200	1.25
	3.14	< 6	450	300	30	15.00
	3.15	< 6	150	140	750	0.20
	3.16	1100	2500	1600	350	7.14
	3.17	2200	2200	1400	250	8.80
	3.18	1500	250	1800	180	1.38
	3.19	< 6	120	30	900	0.15
	3.20	2000	250	700	800	0.31
	3.21	2300	120	600	1600	0.07
	3.22	1500	1000	1500	300	3.33
	3.23	< 6	2600	1200	300	8.66
	3.24	40	20	600	1000	0.02
	3.25	1400	20	< 30	1600	0.01
	3.26	< 6	20	1500	1400	0.01
	3.27	2100	120	1500	700	0.17
	3.28	2000	1300	7000	900	1.44
Reactive lymph node	1.01	2000	50	280	600	0.08
	1.02	100	2500	1800	180	13.80
	1.03	2100	450	700	350	1.28
	1.04	2200	2000	500	50	40.00
	1.05	60	480	800	550	0.87
	1.06	1900	450	60	720	0.62
	1.07	< 6	200	500	350	0.57
	1.08	1500	900	80	850	1.05
	1.09	1500	900	550	800	1.12
	1.10	2000	80	260	400	0.20
	1.11	1000	2200	1200	45	48.80
	1.12	< 6	1500	1000	380	3.94
	1.13	1800	1200	< 30	35	34.20
	1.14	< 6	80	< 30	780	0.10
	1.15	10	30	< 30	450	0.06
	1.16	600	400	400	1500	0.26
	1.17	60	250	60	1500	0.16
	1.18	2100	40	400	800	0.05

**Cloned T-cells were cultured for 48 h with PHA and PMA. Cytokines were subsequently assayed in culture supernatants by ELISA.*

type pattern. In conclusion, these experiments showed that the Th2-,Th1- and Th0-type patterns of cytokine gene expression occurred with similar frequency in the HD and reactive lymph nodes as well as in the MC and NS subtypes of HD.

Discussion

Although the lineage and the clonality of RS cells have long eluded definition,[23] a number of reports based upon immunophenotypic[24-28] and molecular[29-39] studies point to a B-cell origin of a proportion of HD cases. Clonal rearrangements of T-cell receptor (TcR) genes have been detected only occasionally in HD lymph nodes,[32-35,40-42] suggesting that T-lymphocytes are reactive rather than neoplastic.

The numeric increase of CD4+ T-lymphocytes in most HD lymph nodes, together with the frequent expression of activation markers,[5-10] may suggest that activated CD4+ T-cells participate in the pathogenesis of some disease manifestations.[43]

To gain more insight into this issue we have investigated here the expression of four T-cell-associated cytokine genes, i.e. IL2, IL4, IL5 and IFN-γ, in frozen lymph node samples from patients with either HD or reactive adenopathies. The rationale for these experiments stems from previous studies showing that antigen specific T-cell responses are driven by the locally available cytokines and their relative ratios.[11-17,19] In excess of IL4, antibody production prevails, whereas in excess of IFN-γ, cell mediated immunity is predominantly elicited.[11-17,19] Such phenomena occur because IL4 and IFN-γ induce the differentiation of Th0 cells into the Th2 or Th1 specialized subsets, respectively.[13] Thus, it may be expected that the knowledge of the relative amounts of T-cell associated cytokines produced *in vivo* in the individual lymph nodes allows a better understanding of HD pathophysiology.

Table 2. Quantitation of cytokine mRNA expressed in HD and reactive lymph node tissue sections by competitive PCR.

Lymph node sample		*Cytokines (attomoles x 10^{-5})*				*IL4/IFN-γ*
		IL2	*IL4*	*IL5*	*IFN-γ*	*ratio*
HD MC	1	12.8	240	3.8	2.4	100
	2	2.6	5.8	16.4	26	0.2
	3	24	38	1.18	17.4	2.2
	4	< 0.26	1.58	19	3.4	0.5
	5	0.38	22	1.22	20	1.1
	6	1.7	3.4	4.2	1.4	2.4
	7	35.4	46.8	2.6	6.4	7.3
HD NS	8	0.4	0.7	0.96	0.44	1.6
	9	40	116	0.32	7.6	8.5
	10	8.2	28	0.22	2.8	10
	11	5.4	3.4	1.98	8	0.4
	12	140	6.2	6.8	34	0.2
	13	11.2	30	14.4	3.2	9.4
	14	2.6	1.8	0.5	2.2	0.8
Reactive lympho-adenopathies	15	18.4	26.5	9.2	1.7	15
	16	8.4	0.58	1	2.4	0.2
	17	5	1.94	2.4	9	0.2
	18	18	100	22	3.2	31
	19	104	92	83.4	21.4	4.3
	20	1.8	600	18	360	1.7
	21	25.2	56	24	23.3	2.4
	22	96.7	61	62	9.4	6.5
	23	2.4	15.4	3.6	30.8	0.5
	24	62.8	207.5	127	90.2	2.3

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IL2, IL4, IL5 and IFN-γ mRNA were detected in all lymph nodes tested irrespective of their HD or reactive origin. The investigation of cytokine gene expression in HD lymph nodes by qualitative RT-PCR has been addressed in a previous study[44] which, however, led to completely different conclusions. In particular, IL4 or IL5 transcripts were not detected in any of the eight lymph nodes studied; IL2 mRNA was found in one sample only, whereas IFN-γ mRNA was identified in most cases. The reasons for the discrepancies between the results of the study above and those of the present report are not immediately apparent, but may be related to a number of variables, such as the design of cytokine primers for RT-PCR, the experimental conditions for cDNA amplification or even patient selection. $CD4^+$ cells with activation markers were detected in both HD[5-10] and reactive lymph nodes; furthermore, $CD4^+$ lymphocytes isolated from two HD lymph nodes expressed the same cytokine genes as the unfractionated cells present in frozen lymph node sections. Therefore, it appeared reasonable to hypothesize that *in vivo* activated $CD4^+$ cells were the major source of cytokine transcripts. Indeed, T cell clones raised from the latter cells released IL2, IL4, IL5 and IFN-γ with similar patterns in HD and reactive lymphadenopathies. In the clones from both sources, the Th0-type pattern was more frequently observed than the Th1- or Th2-types.

In spite of the useful information provided, the clonal analysis of *in vitro* expanded $CD4^+$ cells suffered from the following limitations. First, it was carried out only with two lymph node samples for which sufficient fresh tissue was available. Second, the clones tested for cytokine production, although representing the progeny of *in vivo* activated $CD4^+$ cells, outgrew from long term cultures where a certain cell selection had occurred. Therefore, in subsequent experiments, we addressed the issue of the direct quantitation of cytokine gene expression in frozen lymph node tissue sections by developing a competitive RT-PCR procedure.[20,22] Such a technique has the advantage of being applicable to the analysis of archival tissue samples. Furthermore, the quantitative information obtained by this approach may complement those achieved by other semi-quantitative procedures, such as *in situ* hybridization or PCR.

Heterogeneous patterns of IL2, IL4, IL5 and IFN-γ gene expression were detected both in HD and in reactive lymph nodes. However, the majority of the samples from either source displayed a Th2-type pattern of cytokine gene expression, with a higher production of IL4 than IFN-γ mRNA. The Th2-, Th1- and Th0-type profiles were detected with similar frequencies in the MC and NS HD subtypes. In addition, no correlation was found in the individual HD cases between the pattern of cytokine gene expression and any clinical or laboratory feature.

In evaluating these results, it should be stressed that cell types other than activated $CD4^+$ T lymphocytes could have contributed to some extent in the accumulation of cytokine transcripts. For example, RS cells have been shown to express a large numer of cytokine genes including the IL5 gene.[45-57] IL2-[58] or IL12-[59] treated NK cells are potent producers of IFN-γ, and IL12 has been identified in HD lymph nodes by immunocytochemistry.[60] $CD8^+$ T-lymphocytes can synthesize all of the cytokines here investigated upon appropriate stimulation. The latter issue may be of particular relevance in certain HD cases characterized by the presence of EBV-infected RS cells and of activated $CD8^+$ lymphocytes in the lymph nodes involved.[61] Although the issue of the relationship between EBV infection of RS cells and cytokine gene expression in lymph node cells was not addressed here, previous evidence would indicate that EBV^+ and EBV^- cases display comparable patterns of cytokine mRNA accumulation.[61]

Based on the present results, the possibility that the absolute concentrations of cytokine mRNA reached in the microenvironment of HD lymph nodes were higher than those achieved in the reactive lymph nodes cannot be disregarded. Such a hypothesis may be corroborated by the notion that *in vivo* activated $CD4^+$ cells, i.e. the main source of cytokine mRNAs and proteins, are more numerous in HD lymph nodes than in reactive lymph nodes. In this perspective, the release of large amounts of IL5 might account for eosinophilia,[62] which is a hallmark of HD, but not reactive lymphadenopathies. Likewise, serum hyper-IgE, which is detected in approximately one third of HD cases but is unusual in patients with reactive adenopathies, might be an expression of the higher production of IL4[63] in the lymphoid tissues of the former rather than of the latter patients. Further studies on these issues are now in progress.

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