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Interleukin-8 in Hodgkin's Disease

Preferential Expression by Reactive Cells and Association with Neutrophil Density

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Hodgkin's disease (HD) shows rare neoplastic Hodgkin and Reed-Sternberg cells embedded in an abundant reactive infiltrate containing, among other cell types, neutrophilic granulocytes. Interleukin (IL)-8 is chemotactic for neutrophils. The expression of IL-8 was tested by* in situ *hybridization with ^{35}S-labeled IL-8-specific RNA probes on 38 cases of HD. Reactive lesions, non-Hodgkin's lymphomas of B and T phenotype, and Langerhans' cell histiocytosis served as controls. IL-8 expression was observed in Hodgkin and Reed-Sternberg cells in 3 of 33 cases of classical HD and in reactive cells in 20 of 33 HD cases as evidenced by combined isotopic* in situ *hybridization and immunohistology for the demonstration of cell-type-characteristic antigens or enzyme histochemistry for chloroacetate esterase. IL-8-positive cells were more numerous in cases of nodular sclerosing HD as compared with the mixed cellularity histotype ($P = 0.01$). The number of IL-8-positive cells and the density of neutrophils were positively correlated ($P < 0.01$). In 5 cases of lymphocyte-predominant HD, IL-8 expression was not displayed. Non-Hodgkin's lymphoma cases contained IL-8 transcripts only in 1 of 23 cases in sparse reactive cells. In 4 of 7 cases of Langerhans' cell histiocytosis, IL-8-specific signals were displayed in S100-negative cells. In conclusion, IL-8 expression in HD is largely confined to reactive cells and associated with infiltration by neutrophils. Elaboration of other cytokines by Hodgkin and Reed-Sternberg cells and reactive cells may explain the frequent expression of this cytokine in HD, particularly in the nodular sclerosing type. (Am J Pathol 1996, 148:1229–1236)

Interleukin (IL)-8 is a cytokine that strongly attracts and activates neutrophils.[1–3] In addition, it is chemotactic for T lymphocytes[4] and induces angiogenesis.[5–7] It is produced by a wide range of cell types including monocytes/macrophages,[1] fibroblasts,[3] mesangial[8] and endothelial cells,[3] keratinocytes,[3] cortical renal epithelial cells,[9] and even neutrophils[10] themselves. In most instances IL-8 is not expressed constitutively but induced by lipopolysaccharide or cytokines such as IL-1 and tumor necrosis factor (TNF)-α.[1–3] IL-8 has been proposed to be involved in the pathogenesis of inflammatory diseases such as ulcerative colitis[11] and rheumatoid arthritis,[12] infectious diseases,[13–16] and aortic aneurysm[17] as well as in the neutrophil accumulation of neoplastic lesions such as gastric carcinoma,[14] transitional cell carcinoma, and renal cell carcinoma.[18] Recently, this cytokine has been reported to function as an autocrine growth factor for melanoma cell lines.[19]

Several lines of evidence suggest that IL-8 may also play a role in the pathogenesis of Hodgkin's disease (HD). First, IL-8 is induced after treatment with phorbol esters in some but not all HD-derived cell lines.[20–22] Second, increased levels of this cytokine have been detected in sera of a substantial proportion of HD patients.[23,24] Third, histological investigation of many HD cases discloses a variably

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dense neutrophilic component in the reactive infiltrate.[25] As neutrophil chemotaxis is one of the major functions of IL-8,[1–3] it is conceivable that accumulation of these cells in HD could be caused by secretion of elevated amounts of IL-8.

In vivo expression, however, of IL-8 in tissue specimens of HD has not been examined. To analyze whether IL-8 transcripts occur in tissues infiltrated by HD and to characterize the potential producer cells by morphology we investigated tissue specimens of 38 HD cases as well as various control groups consisting of tonsils, mycobacteriosis, Langerhans' cell histiocytosis, and non-Hodgkin's lymphoma cases by isotopic *in situ* hybridization (ISH) with a ^{35}S-labeled RNA probe specific for IL-8. Double-labeling procedures were applied for the simultaneous detection of EBER molecules and cytokines in HD and the sequential detection of cell-type-characteristic/specific molecules (S100, CD68, and chloroacetate esterase) and cytokine transcripts in HD and Langerhans' cell histiocytosis. Furthermore, transcript levels of IL-8 in HD were compared with the density of the neutrophilic infiltrate and blood vessels to verify a possible correlation between these parameters.

Materials and Methods

Tissues

Formol-fixed, paraffin-embedded biopsy specimens of 38 cases of HD were drawn from the files of the Institute of Pathology, Klinikum Benjamin Franklin, Berlin (17 cases of nodular sclerosis, 16 cases of mixed cellularity, and 5 cases of the lymphocyte-predominant subtype of HD; 36 lymph nodes, 1 spleen, and 1 pharyngeal biopsy). All specimens were obtained before the initiation of therapy. Controls consisted of 6 paraffin-embedded tonsils, (3 of which displayed slight to moderate follicular hyperplasia and 3 of which showed acute ulcerative tonsilitis), 8 cases of mycobacteriosis, 10 lymphonodal lymphoblastic lymphomas (5 of B and 5 of T phenotype), 4 lymphonodal B cell chronic lymphatic leukemias, 4 lymphonodal follicular center cell lymphomas (grade I or II), 5 T cell lymphomas of angioimmunoblastic lymphadenopathy type, and 7 cases of Langerhans' cell histiocytosis.

Immunohistology

Four-micron sections of paraffin-embedded tissue blocks were stained by the immunoalkaline phosphatase method.[26] The primary monoclonal antibodies were Ber-H2 (CD30), L26 (CD20), βF1 (T cell antigen receptor β-chain), PGM-1 (CD68), C3D1 (CD15), JC70A (CD31), and NP57 (specific for neutrophil elastase). For the detection of S100 protein a polyclonal antibody was used. With the exception of βF1, which was from T-Cell Sciences (Cambridge, MA), all antibodies were purchased from DAKO (Glostrup, Denmark). Ber-H2, L26, and βF1 were used after microwave irradiation (10 minutes in 10 mmol/L citrate buffer at 650 W) for antigen retrieval in paraffin sections.

Plasmids

cRNA probes were prepared after subcloning of cytokine gene fragments in the run-off transcription vector pGEM1 (Promega Biotec, Heidelberg, Germany). The IL-8 probe, kindly provided by Dr. Kunzendorf, Universitätsklinikum Benjamin Franklin, Berlin, was an amplification product covering the entire coding sequence and produced by reverse transcription and polymerase chain reaction of RNA induced in human peripheral blood cells by mitogen. The IL-6 cDNA probe was the 0.6-kb *Eco*RI/*Pst*I fragment of pXM309, kindly provided by Genetics Institute[27] (Boston, MA) and was used as a control probe to ensure the presence of RNA in tissue specimens. The nucleic acid sequences of the cytokine probes were determined on the DNA sequencer 373 (Applied Biosystems, Foster City, CA) and conformed to published data.[1,27] After linearization of the pGEM constructs with appropriate restriction enzymes, anti-sense and sense (control) RNA probes were generated by run-off transcription with incorporation of ^{35}S-labeled nucleotides yielding an average specificity of 1.3×10^9 cpm/mg as described.[28]

In Situ *Hybridization*

ISH was performed as previously described.[28] Prolonged exposure times (up to 9 weeks) were used to ensure maximal sensitivity. The incubation of sections with *Micrococcus* nuclease (Boehringer Mannheim, Mannheim, Germany) before ISH resulted in the extinction of the specific autoradiographic signal, confirming that RNA molecules were the target of ISH.[29] Sections hybridized with sense probes showed only weak nonspecific background (not shown). Sequential immunohistology and ISH were performed as previously described using the enhanced polymer one-step-staining system (DAKO).[30] Detection of chloroacetate esterase was done according to protocol provided by the manufacturer (Sigma Chemical Co., St. Louis, MO) using distilled water treated with diethylproxycarbonate

Table 1. *IL-8, Neutrophilic Granulocytes, and Vessels in Classical Hodgkin's Disease*

Case	Subtype	IL-8	Neutrophils	Vessels
1	NS	6.6	136	19.3
2	NS	2.2	46.5	13.6
3	NS	1.6	112	15.1
4	NS	1.3	49.3	10
5	NS	1.2	36.9	12.3
6	MC	1.1	5.4	20.1
7	NS	0.9	4.7	16.3
8	MC	0.6	73.3	10.1
9	NS	0.5	19.8	13.8
10	NS	0.2	23.3	14.2
11	NS	0.1	9	12.4
12	MC	0.1	6.6	8.4
13	MC	0.1	3.1	19.5
14	NS	0.1	11.1	15.0
15	MC	0	6.2	18.8
16	MC	0	1.8	ND
17	MC	0	18.9	ND
18	NS	0	3.8	15.4
19	NS	0	29.1	ND

Results are expressed as number of IL-8-positive cells, neutrophilic granulocytes, or CD31-positive vessels per high power field. NS, nodular sclerosing type of HD; MC, mixed cellularity type; ND, not determined.

(0.1%; Sigma) and combined with ISH to simultaneously label neutrophils and detect cytokine transcripts. Simultaneous demonstration of EBER and IL-8 transcripts was carried out as reported earlier.[31]

Evaluation

Enumeration of neutrophils and blood vessels in HD was aided by immunohistological staining with the NP57 and JC70A antibody, respectively. For the evaluation of ISH, cells containing more than 20 grains were scored positive. This corresponded in all cases to more than four times background signal. Results were expressed in terms of the number of positive cells per high power field (Table 1). Areas containing necrosis and intravascular cells were not evaluated. Rank correlation according to Kendall and Wilcoxon's rank sum tests were used for statistical analysis.

Results

Classical Hodgkin's Disease

Of 33 cases, 20 contained highly variable amounts of IL-8 transcripts (numerical evaluation of one ISH series is presented in Table 1). In only 3 of the 33 cases (Figure 1, a–c), a minority of Hodgkin and Reed-Sternberg (H&RS) cells were found to be labeled with the IL-8 probe. Some of the IL-8-labeled cells proved to be macrophages and neutrophils as evidenced by expression of CD68 or chloroacetate esterase in double-labeling experiments (Figure 1, d and e). The remainder of the labeled reactive cells had the morphology of mesenchymal cells. Expression of IL-8 in one ISH series of 19 HD cases was correlated with histological subtype, neutrophil density, and density of blood vessels. IL-8 expression was associated with the nodular sclerosis subtype as compared with the mixed cellularity subtype ($P = 0.01$). Neutrophils were present in all of these 19 cases in highly variable amounts. Three cases (14, 17, and 19 in Table 1) displayed moderate amounts of neutrophils but had no or only very low numbers of IL-8-positive cells. Application of statistical methods showed a weak correlation between the density of neutrophils and the number of cells labeled with the IL-8 probe ($\tau = 0.47$, $P < 0.01$). There was no evident association between the number of neutrophils and histological subtype ($0.1 > P > 0.05$), expression of IL-8 and vessel density, or vessel density and histological subtype. All cases displayed IL-6-specific signals in H&RS cells and/or reactive cells conforming to previously described findings.[30]

Table 2. *IL-8 Expression in Other Tumors*

	Positive cases
Lymphocyte-predominant HD	0/5
Lymphoblastic lymphoma	1/10*
Chronic lymphatic leukemia	0/4
Follicular center cell lymphoma	0/4
T cell lymphoma of angioimmunoblastic lymphadenopathy-type	0/5
Langerhans' cell histiocytosis	4/7*

*Labeling of reactive cells only.

Lymphocyte-Predominant Hodgkin's Disease

Neutrophils were present in only very small numbers (average of <1 per high power field) in all five cases of this disease entity. ISH did not reveal any IL-8-specific signals, even after prolonged exposure time of up to 9 weeks, although IL-6-specific signals were observed in reactive and/or neoplastic cells again in agreement with previous findings.[30]

Non-Hodgkin's Lymphomas

Tissues of this group did not contain detectable amounts of IL-8 transcripts (Table 2) with the exception of one case of lymphoblastic lymphoma showing

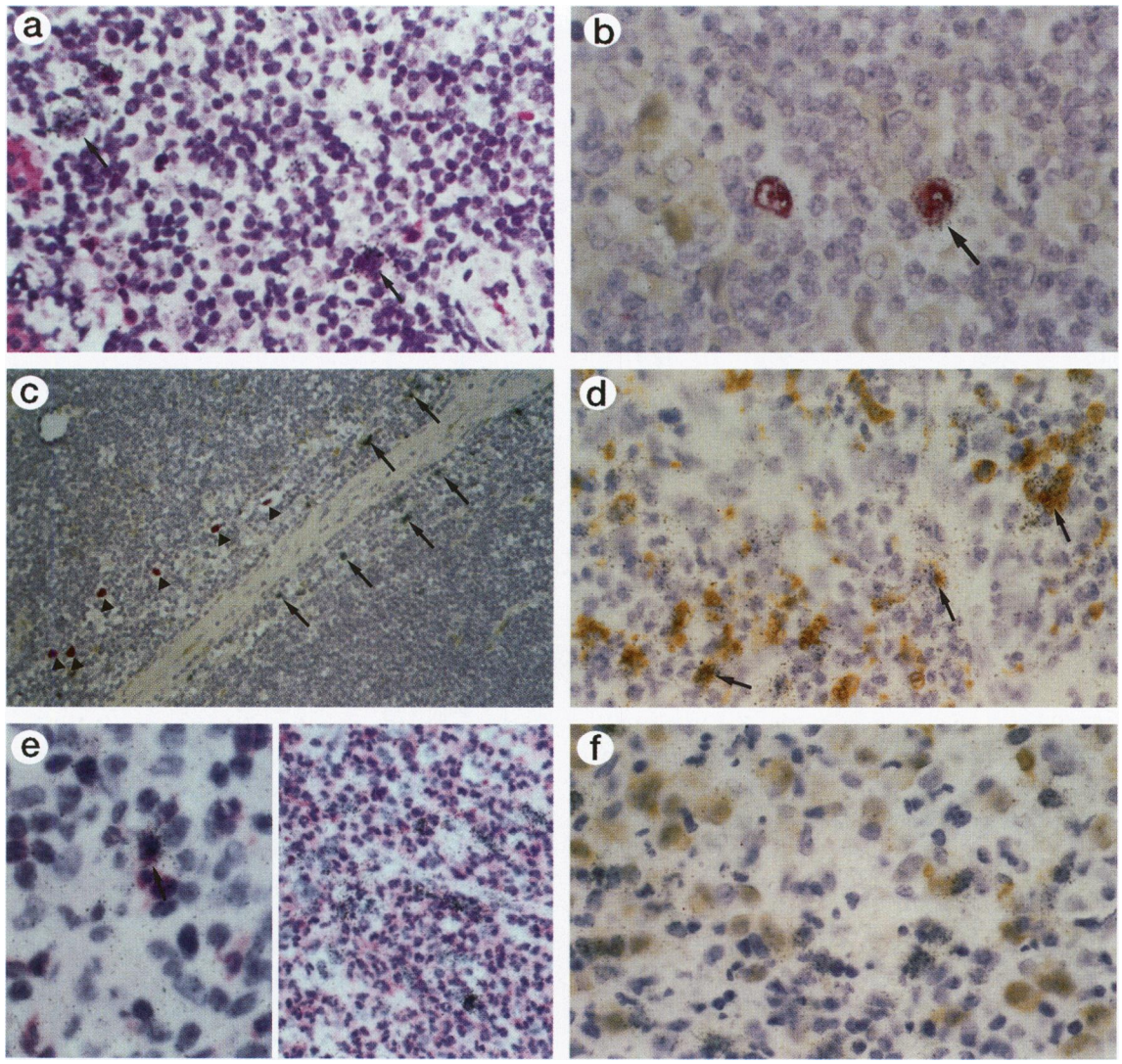

Figure 1. a *to* e: *Hodgkin's disease. Detection of IL-8 transcripts in tumor cells* (arrows) *in two different cases.* a: *ISH.* b: *Simultaneous ISH for cytokine transcripts and EBER.* c: *Another HD case displays IL-8 expression in reactive cells* (arrows) *but not in EBER*$^{+}$ *H&RS cells* (arrowheads). d *and* e: *CD68*$^{+}$ *macrophages* (d) *and chloroacetate-esterase-positive neutrophilic granulocyte* (e, left side) *contain IL-8 transcripts* (arrows). *Strong expression of IL-8 in an area of dense neutrophilic infiltration* (e, right side; *simultaneous ISH and histochemistry for chloroacetate esterase*). f: *Langerhans' cell histiocytosis. IL-8-specific signals are present in S100-negative reactive cells (combined immunohistology for protein S100 (peroxidase, brown color) and ISH). Original magnifications,* ×*280* (a *and* d), ×*480* (b), ×*100* (c), ×*400* (e, left side), *and* (f), *and* ×*160* (e, right side).

sparse reactive cells with the morphology of macrophages to be IL-8 positive (Figure 2a). Infiltration by neutrophils was not evident in these cases with the exception of peripheral T cell lymphoma of angioimmunoblastic lymphadenopathy type cases containing sparse neutrophils. Again, hybridization of the IL-6 probe to reactive cells demonstrated the presence of sufficient amounts of RNA in these tissues.

Tonsils

High levels of IL-8 transcripts were observed in areas adjacent to ulceration in ulcerative tonsilitis (not shown). Tonsils with follicular hyperplasia displayed low levels of IL-8 in squamous epithelium in the vicinity of the few neutrophils present in these cases. Other parts of the tonsils did not contain IL-8-specific transcripts conforming to previously published data.[31]

Mycobacterioses

IL-8-specific signals were found in all cases of this group around areas of necrosis. In five of these cases, expression of high levels of IL-8 transcripts were noted (Figure 2b), whereas in the other three cases only few scattered cells were positive. The

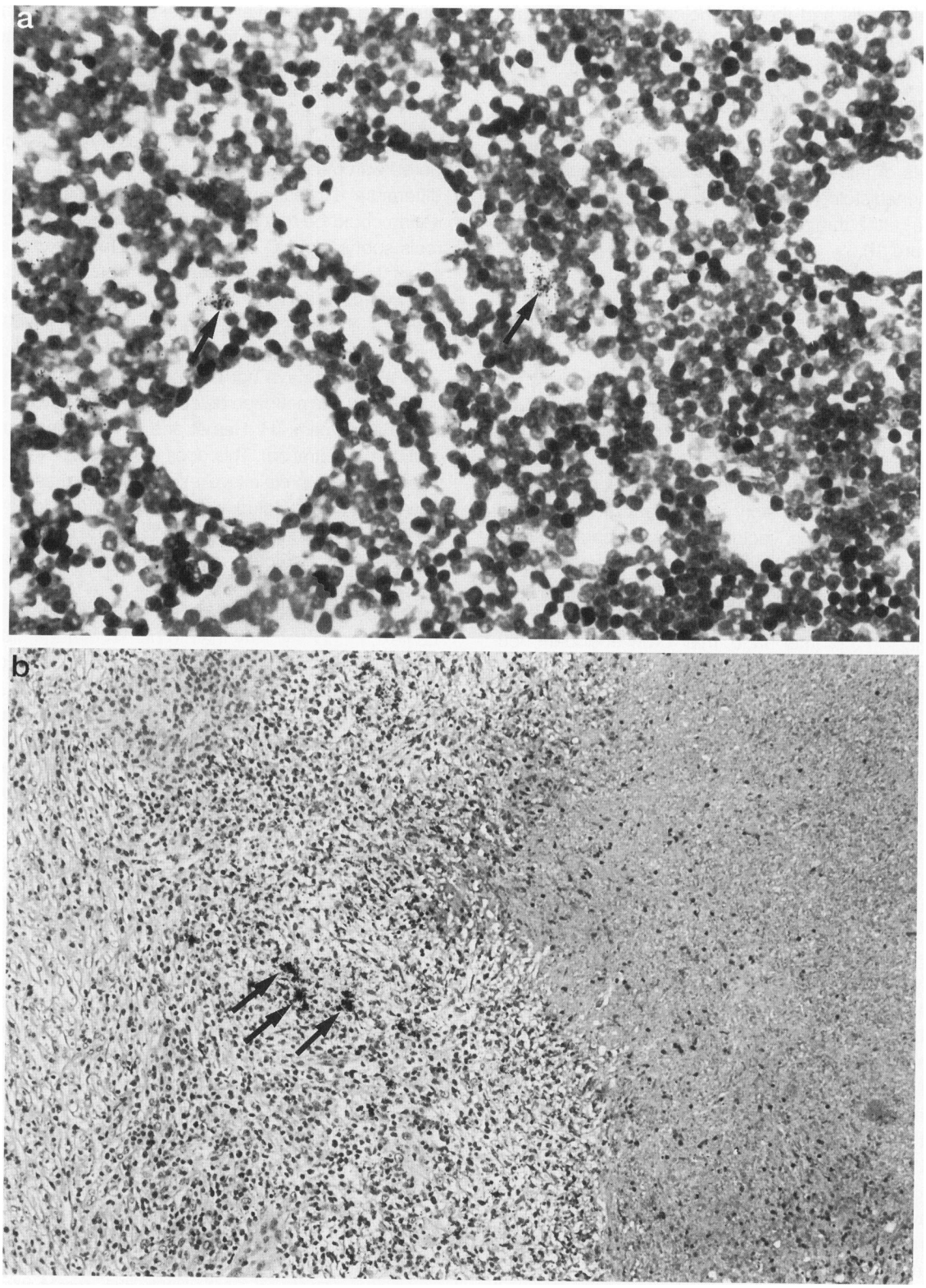

Figure 2. *Labeling of reactive cells with the IL-8 probe in a case of T-lymphoblastic lymphoma* (a) *and expression of IL-8 at the margin of caseous necrosis* (side) *in mycobacteriosis* (b). Arrows *mark labeled cells. Original magnification,* ×250 (a) *and* ×120 (b).

labeled cells had the morphology of macrophages and mesenchymal cells.

Langerhans' Cell Histiocytosis

IL-8-specific signals were observed in four of seven cases of this entity. As evidenced by simultaneous immunohistology and ISH these signals, however, were attributable to S100-negative reactive cells (Figure 1f).

Discussion

IL-8 is a cytokine of the C-X-C chemokine family, which strongly attracts and activates neutrophilic granulocytes.[1–3] Enhanced production of this cytokine has been found in inflammatory disorders such as rheumatoid arthritis[12] and ulcerative colitis,[11] infectious diseases,[13–16] and inflammatory skin diseases.[32] In addition, expression of this factor in gastric carcinomas,[14] urothelial and renal cell carcinomas,[18] and brain tumors[16] is thought to account in part for the inflammatory infiltrate seen in these tumors.

HD, on the other hand, may show abundant neutrophilic granulocytes in the reactive infiltrate.[25] Furthermore, elevated IL-8 levels have been detected in sera of HD patients in 27[24] and 46%[23] of cases. In one of these studies,[24] elevated IL-8 serum levels showed an association with the presence of B symptoms and a weak association with the nodular sclerosis subtype of HD. Finally, some HD-derived cell lines produce IL-8 mainly after stimulation with phorbol esters.[20–22] All of these data suggest a role of IL-8 in the pathogenesis of HD.

At variance with the findings on HD-derived cell lines, H&RS cells *in vivo* expressed this cytokine in only 3 of 33 classical HD cases investigated with an isotopically labeled anti-sense probe specific for IL-8. However, in the majority of cases (61%), reactive cells including macrophages, mesenchymal cells, and less frequently neutrophils were labeled. Therefore, expression of IL-8 in tissues is obviously not necessarily associated with elevated serum levels (27 or 46% of patients[23,24]). This is most likely because expression of low levels of IL-8 in tissues may not be accompanied by an elevation of IL-8 in sera. As cytokine transcripts are not necessarily translated into a protein product, we also performed immunohistological investigations for the presence of IL-8 protein, the results of which were inconclusive, as three different antibodies[14,33,34] produced three different staining patterns both in HD and tonsils (data not shown). The above quoted serological studies in HD, however, suggest that the RNA transcripts detected in our study may well be translated into a protein product and secreted.

Interestingly, IL-8 expression both at the tissue level (this study) and at the serum level[24] is enhanced in the nodular sclerosis subtype as compared with other subtypes of HD. The reason for this differential expression is not clear. A possible explanation could be that H&RS cells in the nodular sclerosis subtype of HD secrete soluble factors that account for both the elevated expression of IL-8 in reactive cells and the induction of fibrosis. IL-8 expression is induced by IL-1 and TNF-α.[1–3] However, differences in the expression of IL-1α[35] and TNF-α[30] in H&RS cells have not been observed at the RNA level. IL-1β has not been detected in H&RS cells in HD tissue specimens (H. Herbst and H. D. Foss, manuscript in preparation). This does not preclude that differences at the protein level exist, as the expression of several cytokines including TNF-α is also regulated by post-transcriptional mechanisms.[36] On the other hand, as TNF-α has also been reported to induce fibrosis,[37] analysis at the protein level of freshly isolated H&RS cells are clearly necessary to clarify this issue. In this context, it is noteworthy that, to our knowledge, fibrogenic activity has not been reported for IL-8[1–3] and this cytokine, therefore, is not likely to participate in the accumulation of collagen in the nodular sclerosis subtype of HD. On the contrary, IL-8 has even been shown to inhibit collagen expression in synovial fibroblasts from patients with rheumatoid arthritis[38] and could therefore partially counteract fibrogenic factors in the nodular sclerosis subtype of HD.

The number of IL-8-positive cells in tissues infiltrated by HD correlated weakly with the density of neutrophils. Interestingly, all cases with dense neutrophilic infiltrates except one also displayed elevated tissue levels of IL-8, suggesting that in these cases IL-8 may indeed be involved in the attraction of neutrophils. The absence of IL-8 in lymphocyte-predominant HD and in the vast majority of non-Hodgkin's lymphoma cases, which also did not display prominent infiltration by neutrophils, further supports this hypothesis. IL-8 transcripts were also observed in some cases of Langerhans' cell histiocytosis, another disease entity associated with neutrophil infiltration. Three HD cases with moderately dense neutrophilic infiltrates, however, had only very few or no IL-8-positive cells. As loss of significant amounts of RNA was excluded by the demonstration of IL-6-specific signals, the accumulation of neutrophils in these cases is probably due to other factors including cytokines such as granulocyte colony-stimulating factor, granulocyte-macrophage colony-

stimulating factor, or members of the C-X-C chemokine family other than IL-8, some of which have been also detected in HD-derived cell lines.[20] Our results in HD are comparable to those obtained in pleural effusions of different etiology.[13] In this study, although IL-8 expression was associated with elevated levels of neutrophils, IL-8 accounted only for part of the neutrophilic chemotactic activity.[13] IL-8 has angiogenic properties.[5–7] In our HD cases, however, there was no association between vessel density and the number of IL-8-positive cells. In addition, cases of T cell lymphoma of angioimmunoblastic lymphadenopathy type, which is associated with prominent angiogenesis, did not show elevated IL-8 transcript levels. This suggests that angiogenesis in the lymphoma entities studied may be related to the secretion of molecules other than IL-8. Indeed, we found expression of high levels of vascular endothelial growth factor in cases of HD and AILD-TCL (H. D. Foss, unpublished results).

In conclusion, whereas H&RS cells in the vast majority of HD cases do not express IL-8, this cytokine can be frequently detected in reactive cells in HD. Intralesional IL-8 expression may thus account for the elevated serum levels described in other studies.[23,24] Expression of IL-8 may cause the accumulation of neutrophilic infiltrates, in part, of HD cases and is associated with the nodular sclerosis subtype of HD. However, at least in some HD cases, this feature is likely due to the activity of other cytokines as well, the precise nature of which remains to be clarified.

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