Interleukin-6 Gene Expression in Castleman’s Disease

By M.B. Leger-Ravet, M. Peuchmaur, O. Devergne, J. Audouin, M. Raphael, J. Van Damme, P. Galanaud, J. Diebold, and D. Emilie

Defined by histological criteria, Castleman’s disease (CD) is a clinically and histologically heterogeneous syndrome. The functional status of immune cells in affected tissues may vary between the different forms of the disease. To address this question, the expression of cytokine genes in eight CD lymph nodes was analyzed by in situ hybridization. Two lymph nodes were taken from patients with a localized form of the disease associated with systemic manifestations, two from patients with a localized form without systemic symptoms, and four from patients with a multicentric form. Five lymph nodes exhibiting a benign follicular hyperplasia were used as controls. The interleukin-6 (IL-6) gene was expressed at a very high level in two cases: the two localized forms of CD associated with systemic manifestations. IL-6 gene overexpression occurred inside follicles of these lymph nodes. The morphology of follicular cells hybridizing with the IL-6 probe or labeled with an anti-IL-6 monoclonal antibody suggested that follicular dendritic cells expressed the IL-6 gene. In contrast, no IL-6 gene expression was detected inside follicles of the six other CD lymph nodes or of the five control lymph nodes. In interfollicular areas, IL-6 gene-expressing cells were detected in all lymph nodes by both in situ hybridization and immunohistochemistry. In CD lymph nodes, positive cells were located outside sinuses, in close contact with blood vessels and plasma cells. This distribution was clearly different from that observed in control lymph nodes, in which IL-6 gene-expressing cells were present inside sinuses. A similar difference between CD and control lymph nodes was observed for the distribution of IL-1β and IL-1α gene-expressing cells in interfollicular areas. The morphology of interfollicular IL-6-producing cells was heterogeneous, consistent with that of macrophages, interdigitating cells, lymphocytes, and endothelial cells, and different from that of plasma cells. Taken together these results show that CD is consistently associated with a particular pattern of IL-6 gene expression in interfollicular areas whereas elevated IL-6 gene expression inside follicles only occurs in the localized form of the disease associated with systemic manifestations. The variable pattern of IL-6 gene expression as well as the clinical and histologic heterogeneity of CD indicate that different immune mechanisms may be involved in the different forms of this disease.

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The expression of the IL-6 and other cytokine genes was analyzed in eight CD lymph nodes affected by these different forms of CD. To define CD-specific events, the levels of expression were compared with those detected in five control, hyperplastic lymph nodes.

**PATIENTS AND METHODS**

**Patients.** Seven patients suffering from CD were included in this study. Their main clinical and biologic characteristics are summarized in Table 1. Four patients suffered from a localized form and three patients from a multicentric form of CD. Two lymph nodes obtained at 2-month intervals were available for patient no. 7. All patients were human immunodeficiency virus (HIV)-1 seronegative. One patient (no. 1) was seropositive for anti-HTLV-1 (human T-cell leukemia virus type 1) antibodies by enzyme-linked immunosorbent assay (ELISA) assay and Western blot analysis. This patient originated from Western Africa. No seronegative patients and displaying a benign follicular hyperplasia in the localized form were observed in CD."

As controls, we analyzed five lymph nodes taken from HIV-seronegative patients and displaying a benign follicular hyperplasia without any histologic features characteristic of CD. These control tissues were collected and stored under conditions identical to those used for CD lymph nodes.

**Immunohistochemical analysis.** MoAbs used were: anti-CD22 (pan-B, final dilution: 1/10; Dakopatts, Glostrup, Denmark), anti-igD (final dilution: 1/100; Coultronics, Margency, France), anti-CD38 (OKT10, final dilution: 1/10; Ortho-Diagnostic Systems, Roissy, France), anti-DRC-1 (final dilution: 1/20; Dakopatts), anti-CD68 (KiM7, final dilution: 1/1,000; Behring, Rueil-Malmaison, France), anti-CD1 (OKT6, final dilution: 1/100; Ortho-Diagnostic Systems), and anti-CD3 (Leu4, final dilution: 1/100; Becton Dickinson, Mountain View, CA). These antibodies were used in an indirect three-stage immunoperoxidase protocol as previously described. The anti-IL-6 MoAb was an IgGl obtained after immunization of a mouse with natural human IL-6. The ascitic fluid used had a titer of 1/25,000 against 10 U/mL of IL-6. For immunohistochemical studies, it was used at a final dilution of 1/500 in an indirect five-stage immunoperoxidase protocol.

**Nucleic acid probes.** Several probes were used in the study. The IL-1α, IL-2, and IL-6 probes have been previously described. They correspond to antisense riboprobes recognizing the coding sequences of the relevant mRNA. The probe recognizing IL-1α mRNA was a fragment extending from nucleotide position 396 to 854 of the human IL-1α cDNA cloned in the Hinc2-EcoRI restriction sites of plasmid pGEM-1 giving plasmid pGEM-1-IL-1α. The IL-1α antisense probe was obtained by RNA synthesis with SP6 polymerase (Amersham, Les Ulis, France) using pGEM-1-IL-1α linearized at the EcoRI site. The template used to synthesize J chain specific riboprobes was made by digestion of a human genomic DNA clone with AccI and XbaI. The 1,560-bp fragment obtained was inserted in the AccI-XbaI restriction sites of the plasmid T7T7 Bluescript KS (Stratagene, La Jolla, CA). The J chain antisense probe was obtained after linearization of the recombinant plasmid with AccI and RNA synthesis using T7 polymerase (Amersham). All riboprobes were labeled with 35S uridine triphosphate (UTP) (>1,000 Ci/mmol; Amersham). Sense probes were used as controls and gave no positive signals (data not shown).

**RESULTS**

**IL-6 production in the localized forms of CD associated with systemic manifestations.** IL-6 production was analyzed by in situ hybridization and immunohistochemistry in the lymph node from two patients each presenting a localized form of CD associated with systemic manifestations (lymph

**Table 1. Main Characteristics of CD Patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Form of CD</th>
<th>Systemic Manifestations</th>
<th>Hb (g/dL)</th>
<th>ESR (mm/h)</th>
<th>γ-g (g/L)</th>
<th>Follicles</th>
<th>Germinal Centers</th>
<th>Plasma Cells in Interfollicular Areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31/F</td>
<td>Localized</td>
<td>+</td>
<td>5.4</td>
<td>133</td>
<td>47</td>
<td>Numerous</td>
<td>Large</td>
<td>Numerous</td>
</tr>
<tr>
<td>2</td>
<td>24/F</td>
<td>Localized</td>
<td>+</td>
<td>6.6</td>
<td>120</td>
<td>58</td>
<td>Numerous</td>
<td>Large</td>
<td>Numerous</td>
</tr>
<tr>
<td>3</td>
<td>25/M</td>
<td>Localized</td>
<td>–</td>
<td>16.4</td>
<td>3</td>
<td>13</td>
<td>Rare</td>
<td>Absent</td>
<td>Numerous</td>
</tr>
<tr>
<td>4</td>
<td>15/M</td>
<td>Localized</td>
<td>–</td>
<td>14</td>
<td>3</td>
<td>11</td>
<td>Rare</td>
<td>Small</td>
<td>Rare</td>
</tr>
<tr>
<td>5</td>
<td>57/F</td>
<td>Multicentric</td>
<td>+</td>
<td>5.6</td>
<td>160</td>
<td>69</td>
<td>Rare</td>
<td>Absent</td>
<td>Numerous</td>
</tr>
<tr>
<td>6</td>
<td>70/M</td>
<td>Multicentric</td>
<td>+</td>
<td>12</td>
<td>45</td>
<td>12</td>
<td>Rare</td>
<td>Small</td>
<td>Rare</td>
</tr>
<tr>
<td>7*</td>
<td>53/M</td>
<td>Multicentric</td>
<td>+</td>
<td>5.4</td>
<td>150</td>
<td>52</td>
<td>Rare(a)</td>
<td>Absent</td>
<td>Numerous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rare(b)</td>
<td></td>
<td>Absent</td>
</tr>
</tbody>
</table>

Systemic manifestations included hyperthermia, weight loss, and/or autoimmune manifestations.

Abbreviations: Hb, hemoglobin; ESR, erythrocyte sedimentation rate; γ-g, γ-globulins.

*Two lymph nodes (a and b) were available for patient no. 7.
nodes 1 and 2). These were the only two lymph nodes (from a total of eight CD lymph nodes) to contain numerous and florid hyperplastic follicles (see Table 1).

Large amounts of IL-6 were produced in both cases, and predominantly inside hyperplastic follicles. Both by in situ hybridization and by immunohistochemistry two different areas of follicles were shown to contain cells producing IL-6: the mantle zone and the germinal center (Fig 1, A and B). In the mantle zone, the labeling was mainly distributed in concentric layers, which involved the whole mantle zone thickness (Fig 1, C and D). A number of positive cells were in close contact with vessels penetrating the follicles.

Numerous IL-6–producing cells were also present in germinal centers, and these cells displayed very large cytoplasm. Clustering of these cells resulted in a diffuse pattern of labeling of the germinal centers.

IL-6–producing cells were also observed in interfollicular areas of these two CD lymph nodes. In situ hybridization and immunohistochemistry experiments gave concordant results, showing scattered positive cells (Fig 2, A and B).

In the five control hyperplastic lymph nodes studied in parallel, no IL-6 production was detected inside follicles by immunohistochemistry or in situ hybridization even after a prolonged autoradiographic exposure (up to 56 days).
Fig 2. IL-6 gene expression in interfollicular areas of CD lymph nodes. IL-6 gene-expressing cells were detected in interfollicular areas of CD lymph nodes by both in situ hybridization with an IL-6 specific probe (A) and immunohistochemistry with an anti-IL-6 MoAb (B). Positive cells were similarly distributed in all CD lymph nodes. In each case, three types of IL-6–containing cells were evidenced: (1) large cells bearing processes (arrowhead), which were morphologically similar to macrophages (labeled with an anti-CD68 MoAb, [C]) and interdigitating cells (labeled with an anti-CD1 MoAb, [D]). (2) Small and round cells, which were morphologically related to T and B lymphocytes (labeled with an anti-CD3 MoAb [E] or an anti-CD22 MoAb [not shown], respectively). (3) Elongated cells in an endothelial position (arrow), which were presumably endothelial cells. In contrast, no IL-6–containing cell was morphologically similar to plasma cells labeled with an anti-CD38 MoAb (F). Results shown are from lymph nodes 1 and 2. Autoradiograph exposure was for 36 days in A. Original magnification: ×400 in A to F.
IL-6-producing cells were detected in the interfollicular areas of the five control lymph nodes, but their pattern of distribution was different from that observed in CD lymph nodes (see below).

**IL-6 production in the localized forms of CD without systemic manifestations.** The in situ production of IL-6 was analyzed in the two lymph nodes (nos. 3 and 4) from patients suffering from a localized form of the disease but displaying no systemic manifestations. These lymph nodes contained a limited number of follicles, which had no or small germinal centers.

Using in situ hybridization, no positive cells were detected inside follicles, even after prolonged autoradiographic exposure (up to 56 days) (Fig 1I). Cells hybridizing with the IL-6 probe were exclusively found in interfollicular areas. Similarly, no cell inside the follicles of these two lymph nodes scored positive with the anti-IL-6 MoAb, whereas scattered positive cells were detected in interfollicular areas.

**IL-6 production in the multicentric forms of CD.** IL-6 production in four lymph nodes (nos. 5 to 7b) obtained from patients suffering from a multicentric form of the disease was analyzed. All patients exhibited systemic manifestations. In three cases, follicles were rare and contained no germinal centers. Small germinal centers were present in lymph node no. 6.

No cell hybridized with the IL-6 probe inside follicles of these four lymph nodes, and the anti–IL-6 MoAb did not bind to any of the follicles. In contrast, cells hybridizing with the IL-6 probe or cells labeled with the anti–IL-6 MoAb were readily detected in interfollicular areas.

**Characterization of follicular IL-6-producing cells in lymph nodes 1 and 2.** The pattern of follicular cells labeled with the IL-6–specific probe and with the anti–IL-6 MoAb in lymph nodes 1 and 2 suggested that follicular dendritic cells did produce IL-6. To further characterize positive cells, we compared the morphology of IL-6–producing cells with that of follicular dendritic cells (labeled with an anti–DRC-1 MoAb), of macrophages (labeled with an anti–CD68 MoAb), and of B lymphocytes (labeled with an anti–CD22 MoAb or an anti–IgD MoAb).

The pattern of labeling obtained with the anti–IL-6 MoAb was different from that obtained with the anti–CD68 MoAb, the anti–CD22 MoAb, or the anti–IgD MoAb. In contrast, it was similar to that of follicular dendritic cells (Fig 1F). Both in situ hybridization and immunohistochemical analysis showed concentric processes of IL-6–producing cells in the mantle zones. The only antibody to give this pattern was the antifollicular dendritic cell antibody. The pattern of IL-6–producing cells and follicular dendritic cells was also identical in germinal centers. However, as germinal centers were heavily labeled by the anti–IL-6 MoAb, we cannot exclude that some B lymphocytes and/or macrophages in close contact with follicular dendritic cells participated to IL-6 production.

An additional argument against a predominant role of follicular B lymphocytes in IL-6 production was obtained by comparing the in situ hybridization pattern with the IL-6 probe and with the J chain probe. J chain–expressing cells, corresponding to differentiated B lymphocytes, were scattered and had an opposite distribution when compared with IL-6 gene-expressing cells: they were predominant in interfollicular areas and were rare in the mantle zone. No concentric layers of labeling were detected in this latter location (Fig 1H).

**Characterization of IL-6–producing cells in interfollicular areas of CD lymph nodes.** Cells hybridizing with the IL-6 probe or labeled with the anti–IL-6 MoAb were similarly distributed in interfollicular areas of all CD lymph nodes. To characterize them, the morphology of cells labeled with the anti–IL-6 MoAb was compared with that of other interfollicular cells, and particularly of plasma cells. The findings were similar in all forms of CD.

First, the predominant population in most CD lymph nodes were plasma cells (Fig 2F). They were more numerous than cells hybridizing the IL-6–specific probe or labeled with the anti–IL-6 MoAb (Fig 2, A and B). Therefore, most plasma cells in CD lymph nodes did not express the IL-6 gene. Moreover, we did not observe any IL-6–producing cells with a plasma cell morphology. Three distinct patterns of IL-6–producing cells were detected: (1) large cells bearing processes, which corresponded to macrophages and/or interdigitating cells (Fig 2, C and D); (2) small and round cells, possibly lymphocytes (Fig 2E); and (3) elongated cells in an endothelial position, which were presumably endothelial cells.

In interfollicular areas of CD lymph nodes, IL-6–producing cells were distributed outside sinuses, and often in contact with blood vessels (Fig 2, A and B). In control lymph nodes, cells labeled with the anti–IL-6 MoAb or labeled with the IL-6 probe were present in sinuses (data not shown), as previously reported. Therefore, interfollicular IL-6–producing cells were differently distributed in CD and control lymph nodes.

Thus, although IL-6 gene expression by some plasma cells is not formally disproved, IL-6 production in interfollicular areas of CD lymph nodes mainly occurs in several nonplasma cell populations.

**Quantification of cytokine gene-expressing cells in CD lymph nodes.** The number of cells hybridizing the IL-6 probe in CD lymph nodes was compared with that of the five control, hyperplastic lymph nodes. IL-6 gene-expressing cells in follicles of the CD lymph nodes 1 and 2 could not be counted exactly due to their close clustering. The number of positive cells, expressed per square centimeter of tissue, was determined in interfollicular areas. This number was higher in CD lymph nodes 1 and 2 than in control lymph nodes. It was also higher than the number of IL-6 gene-expressing cells in the six other CD lymph nodes (Table 2).

The expressions of a lymphokine gene (the IL-2 gene) and of two other monokine genes (the IL-1α and IL-18 genes) were then analyzed by in situ hybridization (Table 2).

Cells expressing the IL-2 gene were rare and found mainly in interfollicular areas, outside sinuses. This distribu-
tion and the number of positive cells were similar to those of the five control lymph nodes. This shows that a specific marker of activated T lymphocytes was not overexpressed in any CD lymph nodes.

No IL-1α or IL-1β gene-expressing cell was present inside follicles of CD lymph nodes. Thus, the IL-6 gene was selectively overexpressed in this compartment of CD lymph nodes 1 and 2. In interfollicular areas, IL-1α and IL-1β gene-expressing cells were detected in all cases tested. Interestingly, positive cells were located outside sinuses in CD lymph nodes, whereas they were found predominantly inside sinuses in control lymph nodes. Therefore, the differences in the patterns of monokine gene expressions in CD and control lymph nodes is not a unique feature of IL-6.

**DISCUSSION**

In this work, the in situ production of IL-6 was analyzed in eight CD lymph nodes representative of the various clinical forms of the disease. IL-6 was produced in large amounts only in the lymph nodes of patients suffering from a localized form of CD associated with systemic manifestations. In this situation, IL-6 was mainly produced inside follicles. Follicular IL-6 production did not occur either in the localized CD forms without systemic manifestations or in the multicentric forms. In contrast with the heterogeneity of IL-6 production inside follicles, IL-6 was produced in interfollicular areas of all forms of the disease. The pattern of interfollicular IL-6—producing cells was different from that of control hyperplastic lymph nodes, as it was characterized by positive cells being specifically located outside sinuses, in contact with blood vessels and plasma cells.

These findings extend those of Yoshizaki et al. By immunohistochemistry with an anti—IL-6 MoAb, these investigators observed labeled cells inside follicles of two CD lymph nodes associated with systemic manifestations. Follicular IL-6 gene overexpression in CD is a specific phenomenon, as the IL-6 gene is not expressed inside follicles of normal4 or hyperplastic lymph nodes34 (and this work), or inside follicles of other lymphoid organs.35,37 As suggested by Yoshizaki et al, the increased production of IL-6 may explain a number of clinical manifestations of CD, including autoimmune manifestations and hyper-γ-globulinemia, acute-phase reaction, and hyposideremic microcytic anemia.38,39 The specific role played by IL-6 gene dysregulation in the development of systemic manifestations in localized CD is emphasized by three different observations: (1) In the patients with localized CD, IL-6 overexpression correlated with the presence of extranodal symptoms. (2) Cytokines other than IL-6 were not overexpressed in these patients (and this work). (3) CD is the only known clinical condition for which selective overexpression of IL-6 has been observed.40,41,42 In the multicentric forms of CD the IL-6 gene was not expressed at a higher level than the IL-1β gene. As IL-1β and IL-6 act synergistically on several of their targets, including hepatocytes and B lymphocytes,43,44 their concomitant production in interfollicular areas of numerous lymph nodes may account for the presence of systemic manifestations in this form of the disease.

The role of IL-6 in the development of CD-associated malignancies has previously been suggested.45 However, as B-lymphoid malignancies and Kaposi's sarcoma are not observed in the localized form of CD,46 substantial in situ overproduction of IL-6 may not be sufficient per se to induce such malignancies. This concept is consistent with results from murine models, in which constitutive IL-6 production induces CD-like manifestations including polyclonal B-lymphocyte proliferation but no B-cell malignancies.55,56 The association of multicentric CD and malignancies may reflect the involvement of other pathogenic phenomena in addition to an in situ production of cytokines. Multicentric CD can occur during Hepatitis B Virus, Epstein-Barr virus, and HIV-1 infections.57,58 These pathogens have been associated with a number of malignancies, including B-lymphocyte neoplasia and Kaposi's sarcoma.

Interestingly, the two lymph nodes in which the IL-6 gene was expressed at a high level inside follicles were the only ones to contain numerous florid hyperplastic follicles. The large size of follicles was due to an accumulation of both B lymphocytes and follicular dendritic cells. Hyperplasia of

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**Table 2. Numbers of Cytokine Gene-Expressing Cells in CD Lymph Nodes**

<table>
<thead>
<tr>
<th>Lymph Node</th>
<th>CD Form</th>
<th>Systemic Manifestations</th>
<th>Interfollicular Areas</th>
<th>Follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IL-6</td>
<td>IL-1β</td>
</tr>
<tr>
<td>1</td>
<td>Localized</td>
<td>+</td>
<td>3,321</td>
<td>459</td>
</tr>
<tr>
<td>2</td>
<td>Localized</td>
<td>+</td>
<td>4,026</td>
<td>286</td>
</tr>
<tr>
<td>3</td>
<td>Localized</td>
<td>—</td>
<td>99</td>
<td>173</td>
</tr>
<tr>
<td>4</td>
<td>Localized</td>
<td>—</td>
<td>790</td>
<td>27,400</td>
</tr>
<tr>
<td>5</td>
<td>Multicentric</td>
<td>+</td>
<td>488</td>
<td>129</td>
</tr>
<tr>
<td>6</td>
<td>Multicentric</td>
<td>+</td>
<td>533</td>
<td>520</td>
</tr>
<tr>
<td>7a</td>
<td>Multicentric</td>
<td>+</td>
<td>1,853</td>
<td>6,080</td>
</tr>
<tr>
<td>7b</td>
<td>Multicentric</td>
<td>+</td>
<td>2,993</td>
<td>7,400</td>
</tr>
</tbody>
</table>

Control lymph nodes 677 ± 152 5,100 ± 1,193 180 ± 45 257 ± 47 — — — +

The number of cytokine gene-expressing cells was determined in interfollicular areas and in follicles of each of the eight CD lymph nodes. Results are expressed as the number of positive cells per cm² of tissue in interfollicular areas. A semi-quantitative method was used for follicles (+, abundant cells; +, rare cells). Results were compared with those (mean ± SEM) from five control hyperplastic lymph nodes studied in parallel.

Abbreviation: NT, not tested.
folicular dendritic cells has already been reported in CD.\textsuperscript{37} This dual accumulation suggested that these two cell populations may be the major contributors to the pathologic process occurring in CD florid follicles. In conditions other than CD, no IL-6 gene expression has been shown inside follicles,\textsuperscript{24,25,27} and follicular dendritic cells may stimulate germinal center B-lymphocyte proliferation by cell-to-cell contact rather than by producing cytokines.\textsuperscript{38-40} Our results suggest that in the localized forms of CD associated with systemic manifestations, follicular dendritic cells may at least partly stimulate follicular B lymphocytes by producing IL-6.

IL-6-producing cells were present in interfollicular areas of all CD lymph nodes. Their distribution was similar (outside sinuses), and clearly differed from that of IL-6-producing cells in control hyperplastic lymph nodes. Numerous plasma cells were present in the interfollicular compartment of CD lymph nodes. However, none of the IL-6-containing cells evidenced by immunohistochemistry displayed a plasma cell morphology. Rather they corresponded to macrophages, interdigitating cells, lymphocytes, and endothelial cells. These IL-6-producing cells were in close contact with blood vessels, which are abundant in interfollicular areas of CD lymph nodes.\textsuperscript{1} This may reflect the stimulating effect of IL-6 on angiogenesis.\textsuperscript{41} IL-6-producing cells were also in close contact with the plasma cells, which are another target of IL-6.\textsuperscript{42-44} A contiguity between blood vessels, plasma cells, and IL-6-producing cells in interfollicular areas was thus a characteristic feature of all CD lymph nodes.

Thus, the abnormal expression of cytokine genes in the multicentric forms of CD is qualitative rather than quantitative, involves IL-1\textbeta and IL-1\textalpha as well as IL-6, and occurs mainly in nonplasma cells of the interfollicular compartment. In the localized forms, the selective IL-6 gene overexpression,\textsuperscript{45} when present, is associated with systemic symptoms and may reflect dysregulated IL-6 production by follicular dendritic cells, so far unobserved in other conditions. In all forms of CD, a common and specific feature is the production of cytokines (and particularly IL-6) in close contact with proliferating blood vessels and activated lymphocytes of the B lineage.

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