Pathogenic Significance of Interleukin-6 (IL-6/BSF-2) in Castleman's Disease

By Kazuyuki Yoshizaki, Tadashi Matsuda, Norihiro Nishimoto, Taro Kuritani, Lee Taeho, Katsuyuki Aozasa, Tatsutoshi Nakahata, Hiroshi Kawai, Hiromi Tagoh, Toshihisa Komori, Susumu Kishimoto, Toshio Hirano, and Tadamitsu Kishimoto

Castleman's disease is a syndrome consisting of giant lymph node hyperplasia with plasma cell infiltration, fever, anemia, hypergammaglobulinemia, and an increase in the plasma level of acute phase proteins. It has been reported that clinical abnormalities disappear after the resection of the affected lymph nodes, suggesting that products of lymph nodes may cause such clinical abnormalities. Interleukin-6 (IL-6) is a cytokine inducing B-cell differentiation to immunoglobulin-producing cells and regulating biosynthesis of acute phase proteins. This report demonstrates that the germinal centers of hyperplastic lymph nodes of patients with Castleman's disease produce large quantities of IL-6 without any significant production of other cytokines. In a patient with a solitary hyperplastic lymph node, clinical improvement and decrease in serum IL-6 were observed following surgical removal of the involved lymph node. There was a correlation between serum IL-6 level, lymph node hyperplasia, hypergammaglobulinemia, increased level of acute phase proteins, and clinical abnormalities. The findings in this report indicate that the generation of IL-6 by B cells in germinal centers of hyperplastic lymph nodes of Castleman's disease may be the key element responsible for the variety of clinical symptoms in this disease.

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Table 1. Clinical and Laboratory Findings Before and After Resection of a Hyperplastic Lymph Node

<table>
<thead>
<tr>
<th>Patient</th>
<th>Before and After Surgery</th>
<th>Affected Lymph Nodes</th>
<th>Clinical Symptoms</th>
<th>Hemoglobin (g/dL)</th>
<th>ESR (mm/hr)</th>
<th>PLT (g/L)</th>
<th>γ-globulin (%)</th>
<th>Immunoglobulins</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (14, F)</td>
<td>Before Solitary (+)</td>
<td>9.1</td>
<td>8.9</td>
<td>42.0</td>
<td>4,350</td>
<td>468</td>
<td>332</td>
<td>12</td>
</tr>
<tr>
<td>After (2 wk)</td>
<td>No (-)</td>
<td>11.6</td>
<td>22</td>
<td>7.7</td>
<td>25.4</td>
<td>2,471</td>
<td>190</td>
<td>253</td>
</tr>
<tr>
<td>After (4 mo)</td>
<td>No (-)</td>
<td>12.9</td>
<td>6</td>
<td>7.1</td>
<td>19.4</td>
<td>1,813</td>
<td>165</td>
<td>246</td>
</tr>
<tr>
<td>P2 (52, F)</td>
<td>Before Multiple (+)</td>
<td>10.1</td>
<td>138</td>
<td>9.0</td>
<td>39.9</td>
<td>4,650</td>
<td>1,040</td>
<td>180</td>
</tr>
<tr>
<td>After (1 mo)</td>
<td>Multiple (+)</td>
<td>9.0</td>
<td>144</td>
<td>9.6</td>
<td>45.0</td>
<td>5,320</td>
<td>941</td>
<td>179</td>
</tr>
<tr>
<td>After (4 mo)</td>
<td>Multiple (+)</td>
<td>8.2</td>
<td>144</td>
<td>8.9</td>
<td>37.9</td>
<td>4,280</td>
<td>832</td>
<td>163</td>
</tr>
</tbody>
</table>

Abbreviations: Hb, hemoglobin; ESR, erythrocyte sedimentation rate; TP, total protein; γ-g, γ-globulins; CRP, c reactive protein; ND, not done.

Clinical symptoms of the two patients were described in the Materials and Methods section.
In this report, we demonstrate that IL-6 is produced by cells present in the germinal centers of the affected lymph nodes that show a mixture of hyaline vascular lesions and plasma cell lesions and there is a close relationship between the serum levels of IL-6 and the clinical features, suggesting that production of IL-6 from hyperplastic lymph nodes may be one of the pathogenic causes in Castleman’s disease.

MATERIALS AND METHODS

Patients. Patient P1, diagnosed as localized form of Castleman’s disease, was a 14-year-old girl with a 6-year history of general fatigue and arthralgia. She had hypergammaglobulinemia, an increased erythrocyte sedimentation rate (ESR), an elevated level of acute phase proteins, a slight increase of antiviral antibodies (serum titers of Epstein Barr virus capsid antigen (EB VCA) IgG, Epstein Barr virus nuclear antigen (EBNA) and cytomegalo IgG were x-40, x-80, and x-160, respectively) and the serum titer of antinuclear antibodies was x-20. Chest x-rays demonstrated a solitary mediastinal tumor. The clinical and laboratory abnormalities disappeared within 3 months following the surgical removal of the 6 x 4 cm mediastinal lymph node.

Patient P2, who had a multicentric form of Castleman’s disease,
was a 52-year-old woman with more than a 5-year history of generalized peripheral lymphadenopathy, subfever, and arthritis at limb joints; the patient also showed an elevation in ESR, polyclonal hypergammaglobulinemia, increased levels of acute phase proteins, a high titer of anti-Epstein Barr virus antibodies (VCA-IgG × 640, EBNA × 1,280) and presence of autoantibodies (antineutrin antibody x 2, anti-DNA 15 u/mL). Bone marrow examination showed panmyeloid hyperplasia with 4.2% plasma cells. Enlargement of multiple lymph nodes was detected along the abdominal aorta and iliac artery by the abdominal computed tomography (CT) and lymphangiography. Clinical and laboratory findings did not change after the surgical removal of one of the abdominal large hyperplastic lymph nodes. The other clinical and laboratory findings of the two patients are summarized in Table 1. Informed consent was obtained from all patients according to institutional policy.

**Histologic findings.** Histologic figures of the resected lymph nodes from these two patients were similar: many lymph follicles with hyperplastic secondary follicles and interfollicular lymphoid cells containing numerous plasma cells and a few immunoblasts. Proliferation of small vessels with thickened hyaline walls and plump endothe1ulums was prominent in the interfollicular spaces that radially penetrated into the secondary follicles, occasionally giving a hyaline appearance. The secondary follicles composed of germinal center cells occasionally showed epithelioid appearance. These secondary follicles were surrounded by small lymphoid cells frequently showing "onion-skin" layers. Russell’s bodies were occasionally found in the interfollicular space (Fig 1).

**Culture of lymph nodes.** Three small pieces of lymph nodes blocks (2 x 2 x 2 mm) were cultured in 2 mL of plain RPMI-1640 without any additional stimulant in Falcon dishes (no. 3001, Becton Dickinson, Oxnard, CA) for seven hours. The culture supernatants recovered was dialyzed against plain RPMI-1640, and sterilized by passing through a millipore filter (Millipore Co, Bedford, MA).

Recombinant IL-6 and antibodies specific to IL-6. Recombinant IL-6 (rIL-6) was produced in Escherichia coli and purified as described previously. 

Rabbit anti-IL-6 antibody was used as the standard for the measurement of IL-6 activity. Rabbit anti-IL-6 antibody used was the same as reported previously.

An IgG fraction of anti-IL-6 antibody was used in the neutralization experiments. Murine monoclonal antibodies (αBSF2-60, IgM, and αBSF2-166, IgG1) against human IL-6 were developed as described previously and used for the detection of IL-6-producing cells by immunohistochemistry.

**Measurement of IL-6 activities.** IL-6 activity in the culture supernatants was determined using Epstein-Barr virus (EBV)-transformed B-cell line, SKW6-CL-4 (CL-4) cells as described previously. Briefly, 1 x 10⁹ cells/200 μL/well were cultured in the presence of test samples for four days and the concentration of IgM produced by CL-4 cells was determined by enzyme-linked immunosorbent assay (ELISA). IL-6 activity in the serum was determined by the proliferation of IL-6-dependent murine hybridoma, MH60.BSF2. The cell growth of MH60.BSF2 was found to be absolutely dependent on IL-6 and none of the other factors examined, including IL-1, IL-2, IL-4, and IL-5, could induce cell growth. One x 10⁹ MH60.BSF2 cells/200 μL/well were cultured in the presence of test samples for 48 hours and ³H-thymidine (³H-TdR) uptake was assessed by a six-hour pulse with 0.5 μCi ³H-TdR before harvest. The IL-6 activity in the sample was expressed as an equivalent amount of rIL-6 required for the same biologic activity as that in the test sample.

**Measurement of other cytokines.** The concentrations of IL-1α, IL-1β, and IL-2 in the culture supernatants of the lymph nodes were measured by ELISA as described previously. The presence of IL-4 was examined by the Fce receptor-inducing activity as described previously. IL-5 activity was determined by Dr Takatsu (Kumamoto University, Kumamoto, Japan) using the BCL cell-proliferation assay as described previously. The concentrations of tumor necrosis factor-α (TNFα) and TNFβ were determined by Dr Kurimoto (Hayashibara Biochemical Inst, Okayama, Japan) using ELISA as described previously.

**Immunohistochemical analysis.** The tissues from the lesions were snap frozen at −150°C, sections were cut by Ames Cryostat II (Miles Laboratories, Elkhart, IN), and fixed in cold acetone (100%) for 10 minutes before staining. Immunohistochemical studies of intracytoplasmic IL-6 were performed using monoclonal mouse antihuman IL-6 antibodies (αBSF2-60, or αBSF2-166 at the concentration of 40 μg/mL) together with Stain-kit (no. 95-1001, Becton Dickinson). Polyclonal mouse of IgG or IgM adsorbed with both human liver powder and immunoglobulins was used as the negative control. T cells, B cells, macrophages, and follicular dendritic cells were stained with monoclonal anti-Leu-4 (CD3), anti-Leu-14 (CD22), anti-IgD, anti-Leu-M5 (Becton Dickinson) and anti-DRC1 (DAKO, Santa Barbara, CA) antibodies, respectively. Counter staining was performed with methyl-green or hematoxylin.

**RESULTS**

*Production of IL-6 by the affected lymph nodes of patients with Castleman’s disease.* In order to examine whether lymph node cells of the patients produce IL-6 in vitro, small pieces of enlarged lymph nodes obtained from patients P1 and P2 were cultured for seven hours in plain medium. The serial sections obtained from hyperplastic lymph nodes from the two patients P1 and P2 were stained with six kinds of monoclonal antibodies or with HE. Figures presented are from patient P2 (A, B, C, and D) and patient P1 (E, F, G, and H). The same fields were stained with (A) H & E, (B) anti-IL-6 antibody (αBSF2-60), (C) anti-Leu4 antibody (Pan T), and (D) anti-Leu14 antibody (Pan B) in patient P2. In patient P1, the same fields were stained with (E) anti-IL-6 antibodies, (F) anti-DRC1 antibody (dendritic cells), (G) anti-IgD antibody (mature resting B cells), and (H) anti-LeuM5 antibody (macrophages).
RPMI-1640 medium without any stimulant. IL-6 activity in the culture supernatant was examined using an IL-6 responsive B-cell line, CL-4 cells. As shown in Fig 2A, the culture supernatants of the lymph nodes derived from both patients induced IgM-production in CL-4 cells in a dose-dependent manner (see the right upper figure) and amounts of IL-6 in the culture supernatants of patients P1 and P2 were estimated to be equivalent to 69.2 ng/mL and 1.16 ng/mL, respectively. Culture supernatants of visceral lymph nodes obtained from patients with obstructive jaundice and pancreatic cysts showed the equivalent of 0.02 ng/mL and 0.04 ng/mL of IL-6 activity, respectively. The IL-6 activity in the culture supernatants was neutralized by anti-IL-6 antibody as shown in Fig 2B. The presence of other cytokines, IL-1α, IL-1β, IL-2, IL-4, IL-5, TNFα, and TNFβ in the supernatants was also examined. As shown in Table 2, none of the cytokines except IL-1α and IL-1β in P1 were detectable. The amounts of IL-1α and IL-1β in P1 were much less than that of IL-6. No cytokines except for trace amounts of IL-6 could be detected in the culture supernatants of controls N1 and N2 (Fig 2A).

Production of IL-6 by cells in the germinal center of the affected lymph node. In order to examine which cells in the affected lymph nodes produce IL-6, immunohistochemical analysis was performed using monoclonal mouse anti-IL-6 antibody, αBSF2-60 (IgM) or αBSF2-166 (IgG1) against lymph nodes obtained from both patients P1 and P2. Figure 3A shows one of the lymph follicles with a hyperplastic germinal center and the interfollicular region with vascular proliferation from patient P2 (hematoxylin—eosin stain). The same region on serial section was stained with αBSF2-60. As shown in Fig 3B, the cells in the germinal center were stained positively with αBSF2-60. Using the monoclonal anti-IL-6 antibody, αBSF2-166, essentially the same result was obtained (data not shown). The specificity of the staining was confirmed, since the germinal center was not stained with normal mouse IgM and IgG. Furthermore, the staining with anti-IL-6 was inhibited in the presence of 100 μg/mL of rIL-6 but not rIL-1β or bovine serum albumin (BSA) (data not shown). In order to determine the cell type positively stained with anti-IL-6 antibody, serial sections were stained with anti-pan T monoclonal antibody (Leu-4) or anti-pan B monoclonal antibody (Leu-14). As shown, T cells were mainly seen in interfollicular area and rarely in the germinal center (Fig 3C), whereas B cells were found in the follicular region including the germinal center (Fig 3D) and these B cells were mainly blastoid. Essentially the same staining pattern with anti-IL-6 was observed in the lymph node obtained from patient P1 (Fig 3E). In order to examine whether blastoid B cells in the germinal center produce IL-6, the germinal center of the affected lymph node from patient P1 was further stained with antidendritic cells, anti-IgD, or antimacrophage antibodies. As shown in Fig 3F, anti-DRC1 could also stain the germinal center. However, the relative staining patterns between anti-IL-6 and anti-DRC1 were different; the staining with anti-DRC1 was much wider and spread over the mantle zone of lymph follicles, while the cells stained with anti-IL-6 were localized in the germinal center.

Anti-IgD antibody stained the mantle zone but not the germinal center of lymph follicles (Fig 3G), and anti-Leu-M5-positive cells were observed scattered through the germinal center (Fig 3H). The histochemical findings indicate that the IgD-negative B cells may be the source of IL-6 production. In fact, a fraction of the B cells isolated from the affected lymph nodes were stained with anti-IL-6 (data not shown).

The germinal centers of normal lymph nodes obtained from patients with cholelithiasis and pancreatic cyst at the operation were not stained with anti-IL-6 antibody (data not shown), indicating that the production of IL-6 by cells of the germinal center may be abnormal.

Effect of surgical removal of the affected lymph nodes on the clinical manifestation and the serum level of IL-6. As shown in Table 1, clinical abnormalities as well as laboratory findings diminished after the resection of the affected mediastinal lymph node in patient P1. However, patient P2 continued to show abnormal clinical features after the excision of one of the hyperplastic lymph nodes. In order to investigate whether the change of these clinical features was related to the serum level of IL-6, the IL-6 activity in the sera obtained from the patients before or after the operation was determined using the IL-6-dependent murine hybridoma, MH60.BSF2. Two weeks after the operation, the elevated IL-6 activity in the serum of patient P1 decreased from equivalent of 110 pg/mL to 30 pg/mL (Fig 4A). In contrast, the elevated serum IL-6 level of patient P2 with multiple-affected lymph nodes was unchanged (equivalent to 70 pg/mL and 68 pg/mL before and 4 months after the operation, respectively) (Fig 4A). The IL-6 activity in the sera of the two patients could be neutralized with rabbit anti-IL-6 antibodies (Fig 4B), indicating that the active molecule in the serum was IL-6. The findings imply that the disappearance of the clinical and laboratory abnormalities, including hypergammaglobulinemia and the increased level of acute phase proteins, correlates with a decrease in the level of IL-6 in the serum.

DISCUSSION

In this study we demonstrate that the cells (most likely blastoid B cells) present in the germinal center of the enlarged lymph nodes produce IL-6 and there is a close relationship between the serum levels of IL-6 and the clinical symptoms in patients with Castleman's disease.

Patient P1, who had a solitary enlarged lymph node in the mediastinum, showed a major improvement of the clinical features after the resection of the lymph node. The levels of immunoglobulin as well as acute phase proteins in serum were reduced to the normal range 4 months following surgery. The serum levels of IL-6 decreased before the improvement in the clinical and laboratory findings. In contrast, patient P2, who had multiple affected lymph nodes, did not show any improvement in the clinical symptoms with a continuing elevated serum level of IL-6 after the surgical removal of a major enlarged lymph node in the abdomen. The data indicate a correlation between the serum level of IL-6 and the involved lymph nodes. Furthermore, the clinical
manifestations were closely related to the serum level of IL-6.

Many previous studies on Castleman's disease suggested a strong link between the enlarged lymph nodes and the clinical features, but the mechanism of the linkage was entirely unknown. IL-6 is an interleukin produced by a variety of cells including T cells, macrophages, and B cells with multiple biologic functions. IL-6 induces the final maturation of activated B cells to Ig-producing cells and was demonstrated to be one of the essential factors for B cells to differentiate to Ig-producing cells. IL-6 also acts on plasmacytoma cells to induce proliferation. Furthermore, it has been demonstrated that IL-6 is a major hepatocyte stimulating factor; it induces a variety of acute phase proteins in rat and human hepatoma cell lines as well as human primary hepatocyte culture. Moreover, it was demonstrated that IL-6 acted in vivo: (1) IL-6 administration enhanced antibody formation in mice; (2) IL-6 induced a rapid gene expression of a set of acute phase proteins in rats and there is a correlation between the serum levels of acute phase proteins and IL-6. Several other cytokines, such as IL-1 and TNF, are also responsible for the induction of inflammation, and IL-1 and TNF can induce IL-6 production in various tissues. IL-4 and IL-5 are involved in the induction of antibody production in B cells. However, these cytokines were not detectable in the lymph node culture supernatants of Castleman's disease. Therefore, constitutive expression of the IL-6 gene in the affected lymph node of Castleman's disease could explain various clinical features such as lymph node swelling with plasma cell infiltration, hypergammaglobulinemia, and increased serum levels of acute phase proteins. This is further supported by the previous findings. Patients with cardiac myxoma show similar clinical abnormalities observed in Castleman's diseases. These clinical abnormalities were found to disappear after the resection of cardiac myxomas, which were found to constitutively produce IL-6. Large amounts of IL-6 were also found in synovial fluids of rheumatoid arthritis (RA) patients, suggesting that hyperproduction of IL-6 may contribute to polyclonal B-cell activation and increased levels of serum acute phase proteins in RA.

IL-6 production could be occasionally observed in the germinals of hyperplastic lymphoid follicles obtained from several other chronic inflammatory lymph nodes. IL-6 production can be induced by IL-1 or TNF generated through the inflammatory process. Therefore, IL-6 production observed in the reactive lymph nodes could be due to a consequence of a consecutive inflammatory process. Whereas, in the case of Castleman's disease, the deregulated IL-6 gene expression is observed only in the affected lymph nodes without any inflammatory signs, such as production of other inflammatory cytokines, IL-1 and TNF and enlargement of the regional lymph nodes. Therefore, the deregulated IL-6 gene expression in Castleman's disease is considered to be the primary event that could be related to the etiologic of this disease.

It should be mentioned that in some cases of Castleman's disease, monoclonal plasmacytomas develop. This is particularly interesting, because IL-6 is involved in the oncogenesis of plasmacytoma/myeloma: (1) Pristane-induced plasmacytomas generated exclusively from the granuloma tissues that was found to produce large amounts of IL-6; (2) IL-6 is a potent growth factor of mouse hybridoma/plasmacytoma; and (3) IL-6 is an autocrine growth factor for human multiple myeloma. Furthermore, the polyclonal plasmacytosis is frequently observed in the pristane-induced granuloma. Therefore, all evidence may indicate that deregulated continuous IL-6 production induces polyclonal plasmacytosis, eventually leading to the generation of plasmacytoma/myeloma with the additional expression of other oncogenes.
Future investigations on the mechanism that induces the deregulated IL-6 gene expression will clarify the pathogenesis of Castleman's disease.

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