Interleukin-6-Associated Laboratory Parameters and Immunohistochemistry in Symptomatic Stage A and B Nodular Sclerosing Hodgkin’s Disease in Children*

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ABSTRACT

Interleukin (IL)-6-associated laboratory parameters obtained at diagnosis on 17 children with histologically confirmed nodular sclerosing Hodgkin’s disease (NSHD) are reported. When these patients were grouped as either symptomatic stage A or B, they were found to be similar in extent of disease, age, and gender. However, statistically significant differences between these two groups were observed for the means of the following IL-6-associated laboratory parameters: hematocrit (p = 0.019), platelet count (p = 0.009), serum albumin (p = 0.001), and ferritin (p = 0.037) concentrations. Moreover, trend analysis of abnormalcy revealed an increasing frequency of anemia, thrombocytosis, hypoalbuminemia, and hyperferritinemia between stage A and B patients and, when available, febrile controls (p values = 0.0012, 0.0009, 0.0406, and 0.0011, respectively). Correspondingly, IL-6 immunohistochemistry performed on archival material from representative cases in each group showed greater overall reactivity in specimens from stage B patients. A variety of cells accounted for this positivity for IL-6 antigen including Reed-Sternberg cells and their variants, lacunar cells, dendritic interdigitating cells, endothelial cells, fibroblasts, and vascular smooth muscle cells. In summary, greater and more frequent abnormalities in IL-6-associated laboratory parameters and increased immunohistochemical reactivity for IL-6 antigen coincide with the presence of fever in helping to identify children with clinical stage B NSHD.

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Introduction

Fever, a cardinal sign of inflammation, is a major criterion in the clinical staging of patients with Hodgkin’s disease. Those with a history of either fever higher than 38°C for three consecutive days, or night sweats, or unexplained loss of more than 10 percent body weight over a six-month period are placed in symptomatic stage B. Patients without any of these constitutional symptoms are grouped as symptomatic stage A. Such grouping has prognostic implications with “B” generally portending a worse outcome. Although a number of clinical laboratory abnormalities that are commonly associated with inflammation have been described in patients with Hodgkin’s disease, no systematic attempt has been made to correlate these with symptomatic staging nor to study their pathogenesis.

Specific abnormalities present to varying degrees in Hodgkin’s disease and detected by laboratory testing include anemia, thrombocytosis, an acute phase plasma protein response with hypoalbuminemia and an increased C-reactive protein, and hyperferritinemia. Because each of these abnormalities can be produced in human subjects and/or test animals following the administration of recombinant human IL-6 and in view of the fact that IL-6 is a pyrogenic cytokine, it is hypothesized by us that excess IL-6 production may be responsible for the symptoms in most cases of stage B Hodgkin’s disease.

The purpose of this report is threefold: firstly, to compare and contrast corresponding IL-6-associated laboratory parameters in stage A versus B NSHD occurring during childhood; secondly, to provide the immunohistochemical counterpart of IL-6 in lesional tissue from stage A and B cases; and lastly, to implicate excess IL-6 production in the pathogenesis of the febrile subset of children with NSHD.

Materials and Methods

Study Population

Seventeen (17) pediatric patients with histologically confirmed NSHD comprise the study population which accrued at Geisinger Medical Center from 1984 through 1995. These patients consisted of four males and 13 females ranging in age from 11 to 16 years with a mean age of 14.2 years. Each patient had been classified as either symptomatic A or B in accordance with the Ann Arbor Staging Classification (i.e., on the basis of the absence or presence of constitutional symptoms such as fever, night sweats, or unexplained loss of more than 10 percent body weight in a six-month period). Similarly, staging of patients according to extent of disease into I, II, III, or IV had been carried out utilizing the Ann Arbor Staging Classification and with the aid of standard imaging and invasive biopsy techniques. Parenthetically, although there originally had been two additional patients both with stage IA NSHD, these were not included in our study because there were no IB counterparts in the series.

Febrile Control Population

In an attempt to control for the effects of acute inflammation, necrosis, and the febrile state per se on platelet counts and hematocrit values, a group of febrile patients with histologically confirmed acute appendicitis was assembled. Two males and six females ranging in age from 11 to 16 years with a mean age of 13.1 years constitute this control population.

Laboratory Analyses

Complete blood counts and quantification of serum albumin and ferritin were performed concurrently on specimens obtained at or near the time of admission for diagnostic workup. These analyses were accomplished using standard automated particle counters, an albumin/BCG methodology on vari-
ous automated clinical chemistry analyzers and a microparticle, enzyme immunoassay for ferritin.†

**Pediatric Reference Ranges**

Published expected ranges⁸,⁹,¹⁰ were chosen as a frame of reference for the aforementioned clinical laboratory parameters examined in these study populations. The specific set of ranges selected most closely approximated the age range and, when indicated, gender of the patients and coincided with the analytical methodologies used in our laboratory.

**Routine Histopathology**

Brightfield microscopy on representative hematoxylin-eosin stained sections from lesional tissue sampled at the time of initial admission established the diagnosis of NSHD in each case.

**Immunohistochemistry**

Archival, paraffin-embedded material was available for immunohistochemistry in five cases categorized as symptomatic stage A and five cases of symptomatic stage B. The immunohistochemical probe for IL-6 antigen consisted of a monoclonal antibody, 1936.14.‡ The murine monoclonal antibody designated 1936.14 is an IgG2b isotype purified by protein A chromatography from ascitic fluid following injection of the corresponding hybridoma line into BALB/c mice. This hybridoma was produced by conventional cell fusion techniques using biologically active purified human recombinant (*Escherichia coli* expressed) IL-6 as the immunogen and screening antigen. Utilizing a common bioassay for human IL-6, R&D Systems has determined that the 1936.14 can neutralize the biological activity of IL-6. Furthermore, the antibody can be used as the capturing antibody in two-site enzyme-linked immunosorbent assays, and the latter have been used to estimate natural human IL-6 concentrations in tissue culture supernatants and in serum and plasma. In this role, it has been tested for crossreactivity against a wide variety of interleukins, colony stimulating factors and growth factors. None has shown any crossreaction.

Although the site on IL-6 recognized by 1936.14 has not been exactly defined, it appears to be a conformationally dependent, rather than a linear epitope, in view of the fact that fully denatured IL-6 binds poorly, if at all. The utility of this antibody for immunohistochemical purposes has not been explored until now. Because of the latter, a neutralization (block-off) experiment was carried out using a known immunoreactive tissue substrate and IL-6 antibody following prior incubation in a solution containing equal, final concentrations of either recombinant human monocyte colony stimulating factor (rhMCSF) or rhIL-6 and anti-IL-6-antibody. On a molecular basis and after taking into account the bivalent nature of the antibody, the excess amount of antigen/IgG was calculated to be ≥100 fold. The rhIL-6, but not the rhMCSF, effectively neutralized the anti-IL-6-antibody affirming its specificity in immunohistochemistry.

Briefly, the general immunohistochemical procedure employed involves antigen enhancement of the deparaffinized tissue sections with Target Unmasking Fluid (TUF).§ This is followed by a “protein block” (normal serum from whatever species the secondary antibody is made); application of the primary antibody (anti-IL-6) at a concentration of 20 μg/mL; a rinse with phosphate buffered saline (PBS, pH 7.4); application of the secondary antibody; a second PBS rinse; reaction with the alkaline phosphatase label; a third PBS rinse and then development using the chromogen, HistoMark Red,¶ and finally, counter-

† Methodology and test kits by Abbott Laboratories, Abbott Park, IL.
‡ Obtained from R&D Systems, Inc., Minneapolis, MN.
§ Signet Laboratories, Inc., Dedham, MA.
¶ Kirkegaard and Perry Laboratories, Gaithersburg, MD.
staining with standard hematoxylin. A negative control consisted of running the same reactions minus the primary antibody (anti-IL-6).

**Statistical Analyses**\textsuperscript{11,12}

Comparisons of the means and standard errors of the means of corresponding analytes or test results between the study groups was accomplished using the Student's t-test, except in the case of ferritin where the Cochran and Cox approximation of the probability level for the approximate t-statistic for unequal variances was employed.

Frequency of abnormalcy for each of the laboratory parameters within a study group was determined using the following published guidelines: age-specific abnormal hematocrit values are <35\% for 6 to 12 years olds and, for 12 to 18 year old females and males, <36\% and <37\%, respectively; age-specific abnormal platelet counts are >450 \times 10^3/\mu L; age-specific abnormal albumin values are <3.8 g/dL for females and <4.0 g/dL for males; and age-specific abnormal ferritin values are >142 ng/mL. Trend analysis of abnormalcy in the corresponding laboratory parameters among and between the study groups was carried out utilizing the exact Cochran-Armitage trend test for abnormal hematocrit values and platelet counts and Fisher's exact test for abnormal albumin and ferritin values.

Finally, the data from the Hodgkin's patients used in the trend analyses of abnormalcy were subjected to analysis using stratified exact nonparametric tests with the stratification factors being none, age, gender, and extent of disease. P-values in this instance were determined by the exact Cochran-Armitage trend test for hematocrit values and platelet counts and by the exact Mann-Whitney test for albumin and ferritin. All p-values in this paper are two-tailed.

**Results**

Ten patients were grouped as symptomatic stage A and seven as stage B. All patients placed in symptomatic stage B presented with a history of fever. Four of them (KC, JC, DH, and JL) also gave a history of night sweats; and three (KC, JC, and CC) had experienced significant weight loss. The demographics of each patient in her (his) respective group are contained in tables I and II, respectively. These groups were similar in age range (11 to 16

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Gender</th>
<th>Extent of Disease</th>
<th>Hct. (%)</th>
<th>Plt. Count (x10^9/\mu L)</th>
<th>Alb. (g/dL)</th>
<th>Ferritin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1—JH</td>
<td>11</td>
<td>F</td>
<td>II</td>
<td>36.2</td>
<td>444</td>
<td>N.P.</td>
<td>N.P.</td>
</tr>
<tr>
<td>2—HZ</td>
<td>12</td>
<td>F</td>
<td>II</td>
<td>38.3</td>
<td>335</td>
<td>4.7</td>
<td>41</td>
</tr>
<tr>
<td>3—RL</td>
<td>15</td>
<td>F</td>
<td>II</td>
<td>37.7</td>
<td>223</td>
<td>4.6</td>
<td>54</td>
</tr>
<tr>
<td>4—MG</td>
<td>16</td>
<td>M</td>
<td>II</td>
<td>39.8</td>
<td>451</td>
<td>4.8</td>
<td>98</td>
</tr>
<tr>
<td>5—MY</td>
<td>16</td>
<td>F</td>
<td>II</td>
<td>28.9</td>
<td>283</td>
<td>4.0</td>
<td>83</td>
</tr>
<tr>
<td>6—RC</td>
<td>16</td>
<td>F</td>
<td>II</td>
<td>32.7</td>
<td>496</td>
<td>3.7</td>
<td>58</td>
</tr>
<tr>
<td>7—BL</td>
<td>13</td>
<td>F</td>
<td>III</td>
<td>31.9</td>
<td>698</td>
<td>4.1</td>
<td>135</td>
</tr>
<tr>
<td>8—CC</td>
<td>14</td>
<td>M</td>
<td>III</td>
<td>38.7</td>
<td>397</td>
<td>N.P.</td>
<td>126</td>
</tr>
<tr>
<td>9—NY</td>
<td>16</td>
<td>F</td>
<td>III</td>
<td>39.4</td>
<td>558</td>
<td>4.4</td>
<td>86</td>
</tr>
<tr>
<td>10—MW</td>
<td>15</td>
<td>F</td>
<td>IV</td>
<td>34.0</td>
<td>446</td>
<td>4.2</td>
<td>58</td>
</tr>
</tbody>
</table>

Demographics, Extent of Disease, and Laboratory Findings at Diagnosis in Symptomatic Stage B, Nodular Sclerosing Hodgkin’s Disease in Children

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Gender</th>
<th>Extent of Disease</th>
<th>Hct. (%)</th>
<th>Plt. Count (x10^9/μL)</th>
<th>Alb. (g/dL)</th>
<th>Ferritin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-KC</td>
<td>11</td>
<td>F</td>
<td>II</td>
<td>33.8</td>
<td>708</td>
<td>4.2</td>
<td>N.P.</td>
</tr>
<tr>
<td>2-JC</td>
<td>13</td>
<td>M</td>
<td>II</td>
<td>33.1</td>
<td>991</td>
<td>3.5</td>
<td>139</td>
</tr>
<tr>
<td>3-DH</td>
<td>13</td>
<td>M</td>
<td>II</td>
<td>30.9</td>
<td>724</td>
<td>3.2</td>
<td>251</td>
</tr>
<tr>
<td>4-TL</td>
<td>14</td>
<td>F</td>
<td>II</td>
<td>29.8</td>
<td>652</td>
<td>3.8</td>
<td>331</td>
</tr>
<tr>
<td>5-CC</td>
<td>16</td>
<td>F</td>
<td>II</td>
<td>35.6</td>
<td>254</td>
<td>3.2</td>
<td>13</td>
</tr>
<tr>
<td>6-JL</td>
<td>14</td>
<td>F</td>
<td>III</td>
<td>23.6</td>
<td>764</td>
<td>2.9</td>
<td>666</td>
</tr>
<tr>
<td>7-CZ</td>
<td>16</td>
<td>F</td>
<td>IV</td>
<td>29.3</td>
<td>771</td>
<td>3.3</td>
<td>659</td>
</tr>
</tbody>
</table>


Laboratory findings pertaining to the presence or absence of anemia, thrombocytosis, and the acute phase plasma protein response at the time of initial diagnostic work-up are also indicated for each patient in tables I and II. Statistical analysis of mean ± standard error of the mean for each of these laboratory parameters revealed significant differences (p < 0.05) between the febrile control group and Hodgkin’s stage A group for hematocrit and platelet counts. Between Hodgkin’s groups A and B, there were significant differences for hematocrit, platelet count, and serum albumin and ferritin concentrations, respectively. These data are summarized in table III.

Moreover, calculation of the frequency of decreased hematocrit values, increased platelet counts, reduced serum concentrations of albumin, and increased ferritin concentrations followed by statistical trend analysis of abnormality for each of these parameters showed an increasing frequency of anemia and thrombocytosis comparing febrile controls to Hodgkin’s stage A and then to Hodgkin’s stage B groups. Similarly, these analyses disclosed an increasing frequency of hypoalbuminemia and hyperferritinemia between Hodgkin’s stage A and B subgroups (table IV).

### TABLE III

Comparison of Means of Clinical Laboratory Parameters at Diagnosis in Febrile Controls and Symptomatic Stage A and B, Nodular Sclerosing Hodgkin’s Disease in Children

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Hct. (%)</th>
<th>Plt. Count (x10^9/μL)</th>
<th>Alb. (g/dL)</th>
<th>Ferritin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Febrile controls</td>
<td>40.7±1.1</td>
<td>275±29</td>
<td>N.P.</td>
<td>N.P.</td>
</tr>
<tr>
<td>Hodgkin’s stage A</td>
<td>35.6±1.2</td>
<td>433±43</td>
<td>4.3±0.1</td>
<td>82±11</td>
</tr>
<tr>
<td>Hodgkin’s stage B</td>
<td>30.9±1.5</td>
<td>695±84</td>
<td>3.4±0.2</td>
<td>363±99</td>
</tr>
</tbody>
</table>

P Values:

| FC vs. A | 0.0084 | 0.0111 | N.P. | N.P. |
| A vs. B  | 0.0194 | 0.0068 | 0.0012 | 0.0373 |


*All values expressed as mean ± standard error of the mean.

All variables tested with Student’s t are approximately normally distributed. All variances are similar except Hodgkin’s Stage A and Stage B for ferritin; in this case, the Cochran and Cox approximation of the probability level for the approximate t statistic for unequal variances is used.
TABLE IV

Trend Analysis of Abnormalcy in Laboratory Parameters Among Febrile Controls and Symptomatic Stage A and B, Nodular Sclerosing Hodgkin’s Disease in Children

<table>
<thead>
<tr>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Febrile controls</td>
<td>Hct.</td>
<td>12%</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hodgkin’s stage A</td>
<td>Hct.</td>
<td>40%</td>
<td>40%</td>
<td>12%</td>
<td>0%</td>
</tr>
<tr>
<td>Hodgkin’s stage B</td>
<td>Hct.</td>
<td>100%</td>
<td>86%</td>
<td>71%</td>
<td>67%</td>
</tr>
<tr>
<td>P values</td>
<td></td>
<td>0.0012</td>
<td>0.0009</td>
<td>0.0406</td>
<td>0.0011</td>
</tr>
</tbody>
</table>


*All values expressed as percent of patient population outside the expected range with adjustments for gender and/or age (see details in Statistical Analyses under Materials and Methods).

bP values for abnormal hematocrit values and platelet counts determined by exact Cochran–Armitage trend test, and for abnormal albumin and ferritin values by Fisher’s exact test.

(All of the above trends in abnormalcy across study groups remain statistically significant when the analyses are adjusted for age, gender or extent of disease).

Assessment of immunoreactivity for IL-6 antigen by brightfield microscopy in the lesional tissues from five cases of symptomatic stage A (patients RL, MG, MY, RC, and MW in table I) and five cases of symptomatic stage B (patients KC, JC, CC, JL, and CZ in table II) collectively reveals: (1) variable positivity for IL-6 antigen in fibroblasts, endothelial cells, and smooth muscle cells of vessels; (2) occasional positive Reed-Sternberg cells and their variants or lacunar cells in both stage A and B cases but with overall greater reactivity in individual patients from the stage B subgroup (i.e., none to approximately 25% of Reed-Sternberg cells and lacunar cells in A versus approximately 10% to 50% in B); (3) marked positivity in dendritic interdigitating cells in several of the cases with “B” symptoms (JC and JL); and (4) no immunoreactivity in the negative control (minus the anti-IL-6 antibody) and in the lesional tissue following neutralization of the anti-IL-6 antibody with recombinant human IL-6. The salient immunohistochemical features are illustrated in figure 1. Extensive necrosis in the biopsy obtained from patient CC (table II) was associated with intravascular thrombi and hampered the interpretation of IL-6 immunoreactivity, although some positivity was noted in fibroblasts, endothelial cells, and Reed-Sternberg cells.

Discussion

The findings in this study indicate that anemia, thrombocytosis, and the acute phase reaction are more pronounced and more frequent in febrile symptomatic stage B NSHD in children as compared with stage A. Moreover, this constellation of findings in symptomatic stage B NSHD may have pathogenetic and prognostic implications.

The specific laboratory abnormalities observed in this study, including decreased hematocrit and serum albumin concentrations, and increased platelet counts and serum ferritin concentrations, can be induced with recombinant human IL-6 administration in humans and primates or other experimental animals; and, therefore, may be regarded as IL-6-associated parameters. The contention that these are IL-6-associated parameters is reinforced by a report that intravenously administered anti-IL-6 antibody increased the hemoglobin and albumin concentrations and decreased the platelet count in a patient with Castleman’s disease. (This patient’s disease was accompanied by elevated serum IL-6 concentration and complicated by anemia, hypoalbuminemia and thrombocytosis.)

Therefore, the more pronounced and more frequent anemia, thrombocytosis, hypoalbuminemia, and hyperferritinemia in our series of febrile stage B patients suggest a state of exaggerated IL-6 production in this subset of NSHD. Furthermore, when coupled with our observation of an overall increase in IL-6
FIGURE 1. Frame A depicting strong immunoreactivity for IL-6 antigen (HistoMark Red chromogen) in the cytoplasm of dendritic interdigitating cells; Frame B showing absence of detectable IL-6 antigen in corresponding section following prior neutralization of anti-IL-6 antibody with rhIL-6; Frame C illustrating cytoplasmic positivity for IL-6 antigen in Reed-Sternberg (arrow RS) and dendritic interdigitating cells; and Frame D with binuclear lacunar cell (arrow) containing IL-6 antigen (original magnification x312, A or B; original magnification x788, C or D).
immunoreactivity in such cases and the known pyrogenicity of IL-6\textsuperscript{4,7} support our hypothesis that excessive or protracted IL-6 production may play a major role in the pathogenesis of the constitutional symptoms in most cases of stage B NSHD.

The prognostic implications of an elevated serum IL-6 concentration at the time of diagnosis in patients with Hodgkin’s disease are controversial. One study concluded that the rates of complete remissions and freedom from treatment failure were not different in those patients with Hodgkin’s disease whose sera had detectable levels (12 to 32 pg/mL) of IL-6 versus those with undetectable levels.\textsuperscript{14} A later study suggested that a serum IL-6 concentration of \( \geq 20 \) pg/mL presages a worse outcome with a shorter median survival.\textsuperscript{15} The discordant findings and interpretations regarding serum IL-6 concentrations in this disease may be related, at least in part, to fluctuations in IL-6 production by the lesional tissues and its relatively short half-life in serum (i.e., \( t_{1/2} \approx 1.5 \) hours).\textsuperscript{16}

Because changes in IL-6-associated laboratory parameters take days to several weeks to induce and a similar period of time to normalize or decrease during rhIL-6 and anti-rhIL-6 antibody administrations, respectively,\textsuperscript{5,13} such parameters may more closely reflect a state of protracted IL-6 production and IL-6 excesses than a singular serum IL-6 concentration. Therefore, using IL-6-associated laboratory parameters may be a more accurate means of testing the prognostic implications of excessive IL-6 production in Hodgkin’s disease.

In summary, it has been demonstrated that greater and more frequent abnormalities in IL-6-associated laboratory parameters and IL-6 immunohistochemical positivity correlate with febrile stage B NSHD in children. In view of this, it is suggested that excessive production of IL-6 may play a role in the pathogenesis of symptomatic stage B disease. A larger study specifically designed to test the predictive value of IL-6 associated laboratory parameters on the outcome in NSHD may be warranted.

Acknowledgment

Thanks are extended to Glen Kauwell for his help with the immunohistochemical studies.

References