**MAJOR ARTICLE**

Borrelia-specific Interferon-γ and Interleukin-4 Secretion in Cerebrospinal Fluid and Blood during Lyme Borreliosis in Humans: Association with Clinical Outcome

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The Borrelia-specific interferon (IFN)–γ and interleukin (IL)–4 responses of 113 patients and control subjects were analyzed using the sensitive enzyme-linked immunospot method. Cerebrospinal fluid (CSF) and blood samples were obtained, during the course of disease, from patients with chronic or nonchronic neuroborreliosis (NB) and from control subjects without NB. Blood samples were obtained from patients with Lyme skin manifestations and from healthy blood donors. Early increased secretion of Borrelia-specific IFN-γ (P < .05) and subsequent up-regulation of IL-4 (P < .05) were detected in the CSF cells of patients with nonchronic NB. In contrast, persistent Borrelia-specific IFN-γ responses were observed in the CSF cells of patients with chronic NB (P < .05). In patients with erythema migrans, increased IFN-γ (P < .001) was observed in blood samples obtained early during the course of disease, whereas increased IL-4 (P < .05) was observed after clearance. On the contrary, patients with acrodermatitis chronica atrophicans had Borrelia-specific IFN-γ (P < .001), but not IL-4, detected in blood samples. The present data suggest that an initial IFN-γ response, followed by up-regulation of IL-4, is associated with nonchronic manifestations, whereas a persistent IFN-γ response may lead to chronic Lyme borreliosis.

The tickborne disease Lyme borreliosis is a multistage infection with several manifestations. It is caused by the gram-negative spirochete Borrelia burgdorferi and may affect the skin, joints, heart, and nervous system. Stage I disease is a local infection of the skin; it often presents the circular skin rash known as “erythema migrans” (EM), which usually appears 1–4 weeks after the tick bite occurs [1]. Stage II disease is an early disseminated infection associated with neuroborreliosis (NB), Borrelia arthritis, or carditis. Neurological symptoms, which typically include subacute meningitis, facial palsy, and radiculitis, usually appear weeks to months after the primary infection occurs [2]. If chronic symptoms develop, the disease has entered stage III, which is associated with long-lasting symptoms (often defined as symptoms that persist for > 6 months) [3, 4] related to the nervous system (chronic NB), joints (chronic Lyme arthritis), or skin (acrodermatitis chronica atrophicans [ACA]). Typical late-stage neurological manifestations include radiculitis, facial palsy, and chronic encephalomyelitis, and they are associated with such symptoms as paresthesia, headache, general pain, cognitive dysfunction, subtle psychiatric symptoms, and fatigue [1, 4–6].

The immune response in humans with Lyme borreliosis is characterized by a type 1–like cytokine response with production of interferon (IFN)–γ, but no interleukin (IL)–4, either within the central nervous system (CNS), for patients with NB [7], or in the...
in seruamous months; OND, other neurological diseases. Borrelia-NB had IFN-β-relapsis. We therefore examined the be associated with a beneficial outcome for human Lyme borreliosis. These observations, we hypothesized that an early cytokine responses in association with clinical outcome. On the basis of the disease, when one is trying to understand the roles of these of studying cytokine responses during the different stages of regulation of IL-4 [15]. These results also stress the importance to be associated with an early IFN-γ response, followed by up-regulation of IL-4, would be associated with a beneficial outcome for human Lyme borreliosis. We therefore examined the Borrelia-specific type 1 (IFN-γ) and type 2 (IL-4) responses [16] during the different stages of human Lyme borreliosis, to investigate the association between cytokine patterns and clinical outcome, in terms of chronic versus nonchronic disease.

**Table 1. Patients and diagnostic groups.**

<table>
<thead>
<tr>
<th>Diagnostic subgroup</th>
<th>n</th>
<th>F:M</th>
<th>Age, median (range), years</th>
<th>Known</th>
<th>Neurological symptoms</th>
<th>In serum</th>
<th>Intrathecal production</th>
<th>CSF-MNC pleocytosis</th>
<th>No. of samples, by disease interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB</td>
<td>39</td>
<td>18:21</td>
<td>51 (22–80)</td>
<td>16</td>
<td>9</td>
<td>39</td>
<td>23/29</td>
<td>37</td>
<td>16</td>
</tr>
<tr>
<td>Nonchronic</td>
<td>12</td>
<td>4:8</td>
<td>50 (27–67)</td>
<td>7</td>
<td>1</td>
<td>12</td>
<td>7/8</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Chronic</td>
<td>27</td>
<td>14:13</td>
<td>53 (22–80)</td>
<td>9</td>
<td>8</td>
<td>27</td>
<td>16/21</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Non-NB</td>
<td>32</td>
<td>15:17</td>
<td>60 (23–78)</td>
<td>3</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OND</td>
<td>13</td>
<td>5:8</td>
<td>41 (23–74)</td>
<td>3</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CSF controls</td>
<td>19</td>
<td>10:9</td>
<td>68 (44–78)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EM</td>
<td>12</td>
<td>5:7</td>
<td>54 (30–78)</td>
<td>8</td>
<td>12</td>
<td>0</td>
<td>1/5</td>
<td>4/5</td>
<td>ND</td>
</tr>
<tr>
<td>ACA</td>
<td>5</td>
<td>2:3</td>
<td>65 (32–73)</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>0/2</td>
</tr>
<tr>
<td>Healthy blood donors</td>
<td>23</td>
<td>15:8</td>
<td>40 (22–65)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>111</td>
<td>55:56</td>
<td>50 (22–80)</td>
<td>28</td>
<td>21</td>
<td>54</td>
<td>29</td>
<td>18</td>
<td>37</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of patients, unless indicated otherwise. ACA, acrodermatitis chronica atrophicans; chronic NB, NB with duration of ≥6 months; CSF, cerebrospinal fluid; CSF-MNC, mononuclear cells in CSF; EM, erythema migrans; NB, neuroborreliosis; ND, not done; nonchronic NB, NB with duration of <6 months; OND, other neurological diseases.

- If not all patients were analyzed, the no. of patients analyzed is shown after the virgule (/). Antibody analyses were performed by ELISA.
- *Borrelia*-specific intrathecal antibody production refers to the *Borrelia*-specific CSF antibody index, according to Hansen and Lebech [17]. All patients with NB had *Borrelia*-specific intrathecal antibody production of IgG and/or IgM class.
- Mononuclear pleocytosis in CSF was defined by ≥5.0 × 10^6 mononuclear cells/L.
- Disease interval 1 was 0–3 months, interval 2 was between 3 and 12 months, and interval 3 was >12 months.
- CSF control subjects included patients undergoing elective orthopedic surgery who had no known history of *Borrelia* infection.

**PATIENTS, MATERIALS, AND METHODS**

**Patients and control subjects.** Patients and control subjects were recruited from the southeastern region of Sweden during 1996–2001. A total of 111 subjects were included in the study. CSF and blood samples were obtained from 39 patients with NB, from 13 subjects with other neurological diseases (OND), and from 19 patients who were undergoing elective orthopedic surgery and who were serving as CSF control subjects. In addition, blood samples were obtained from 12 patients with EM, 5 patients with ACA, and 23 healthy blood donors.

The patients were grouped into 5 diagnostic groups (table 1). Patients had NB diagnosed on the basis of clinically relevant neurological symptoms and demonstration of *Borrelia*-specific intrathecal antibody production, according to the criteria outlined by Hansen and Lebech in 1991 [17]. All patients for whom data from the acute phase were available (n = 34) showed mononuclear pleocytosis in CSF (number of CSF mononuclear cells, ≥5.0 × 10^6 cells/L). Of the remaining 5 patients, 3 had increasing levels of *Borrelia* intrathecal antibodies, an increased intrathecal IgG synthesis index, and/or selective oligoclonal bands in the CSF, supporting the presence of ongoing infection and/or inflammation in the CNS. Typical neurological symp-
toms included neck and back pain, facial palsy, radiculitis, muscle pain, headache, and fatigue (table 2).

Twelve of the patients with NB recovered within 6 months (median, 2 months; range, 3 weeks to 5 months) after the onset of neurological symptoms and were therefore considered to have “nonchronic NB”. For the remaining 27 patients, the course of disease included neurological symptoms that persisted for >6 months; these patients were considered to have “chronic NB” [3]. The patients with chronic NB all had persistent neurological symptoms at follow-up, which was done 8 months to 6 years after the onset of symptoms (median, 12 months).

The group of subjects without NB (hereafter known as the “non-NB control group” ; n = 32) consisted of patients with OND (n = 13) (table 3) and CSF control subjects (n = 19). All these control patients were completely negative for Borrelia-specific antibodies, both intrathecally in CSF and in serum. Moreover, the CSF control subjects were included only if they showed no history of Borrelia infection, no neurological symptoms, and no pleocytosis in CSF. The non-NB control group served as control subjects for the patients with NB.

The 12 patients with EM had a solitary EM lesion, and they did not develop any neurological symptoms during the follow-up period, which lasted for ≥12 months. Diagnosis of EM was made on the basis of clinical findings [2]. For the 5 patients with ACA, diagnosis was based on clinical findings and the presence of high levels of Borrelia-specific IgG in serum. Two patients with ACA had hyperesthesia in association with their skin lesion, but they had no CSF findings that indicated NB (table 1). The healthy blood donors had no known tick bite, no history or present signs of Borrelia infection, no neurological symptoms, and no pleocytosis in CSF. This group served as control subjects for the patients with skin manifestations.

All patients with Borrelia infection were treated with antibiotics. Patients with NB were treated with tetracycline (n = 14), ceftriaxone (n = 11), cefotaxime (n = 2), successive treatments of ceftriaxone and tetracycline (n = 7), or other combinations (n = 6). Patients with EM were treated with tetracycline (n = 7) or penicillin (n = 5), and patients with ACA were treated with tetracycline (n = 4) or penicillin (n = 1). The samples from disease interval 1 were obtained before the start of antibiotic treatment, with the exception of 3 samples that were obtained either during (n = 2) or 1 month after (n = 1) administration of tetracycline. The 3 samples were obtained from patients with chronic NB.

The patients with NB, EM, or ACA were followed for different periods and had varying numbers of samples taken during and after the course of the disease (up to 4 years of follow-up). These samples were classified on the basis of 3 different disease intervals, according to the time after onset of neurological symptoms (for patients with NB), the appearance of EM (for patients with EM), and the appearance of ACA (for patients with ACA). Disease interval 1 included samples obtained during the first 3 months of disease, interval 2 included samples obtained between 3 and 12 months, and interval 3 included samples obtained after >1 year. These disease intervals do not take into consideration the exact time after the primary infection occurred. EM usually appears 1–4 weeks after a tick bite is sustained [1], whereas the early neurological symptoms appear within weeks or months after infection [2]. ACA may develop years after the primary infection occurs. A patient was never represented by >1 sample in each disease interval. When >1 sample was available from a patient during a certain interval, only the first sample from that interval was included. Blood samples from >1 disease interval were available for 10 of the patients with NB, 7 of the patients with EM, and 1 of the patients with ACA. CSF samples from >1 interval were available from 2 patients with NB. The control subjects (the non-NB control group and healthy blood donors) contributed a single sample.

Preparation of mononuclear cells from the blood and CSF.

Blood (heparinized) and CSF samples were obtained from patients with suspected NB, patients with OND, and CSF control subjects, and heparinized blood was obtained from patients with EM, patients with ACA, and healthy blood donors. Peripheral blood mononuclear cells (PBMCs) were separated, by gradient centrifugation, on Lymphoprep (Medinor AB), according to the method of Bøyum [18], as described elsewhere [7]. Cells were reconstituted in tissue culture medium and were counted by the

| Table 2. Neurological signs and symptoms in patients with neuroborreliosis (NB). |

<table>
<thead>
<tr>
<th>Neurological finding</th>
<th>Nonchronic (n = 12)</th>
<th>Chronic (n = 27)</th>
<th>All (n = 39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neck and/or back pain</td>
<td>5</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Facial palsy</td>
<td>1</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Headache</td>
<td>5</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Fatigue</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Muscle pain</td>
<td>3</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Radiculitis</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Paresthesia</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Hyperesthesia</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Vertigo</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Balance and hearing disturbances</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Nausea</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Neck stiffness</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Depression</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Meningitis</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Rhizopathy</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*a NB with disease duration <6 months.  
*b NB with disease duration ≥6 months.
Ip90 was cultured to a concentration of cells/L. Borrelia garinii, which mainly contained OspA and OspB, was prepared from tissue culture medium. An outer surface protein (osp)–enriched fraction (OF), was obtained by centrifugation at 200 g. The supernatant was incubated with 2% octyl glucopyranoside (Calbiochem; Novablochem) at 37 °C, after incubation with 15,000 CSF lymphocytes were added per well, depending on the number of cells available. It has previously been reported that the frequency of spots is not altered by decreasing cell counts, even when there are low numbers of cells per well. Because of limited cell numbers, 400–92,000 CSF lymphocytes (median, 5000 CSF lymphocytes) were added per well, followed by gentle resuspension in tissue culture medium.

**Preparation of outer surface protein fraction Borrelia antigen.** An outer surface protein (osp)–enriched fraction (OF), which mainly contained OspA and OspB, was prepared from Borrelia garinii strain Ip90, as described elsewhere [19, 20]. In summary, Ip90 was cultured to a concentration of 10^11 cells/L in BSK II medium and was harvested in late log phase. After the cells were washed, they were resuspended and were kept on ice for 15 min before undergoing incubation with 2% octyl β-d-glucopyranoside (Calbiochem; Novablochem) at 37 °C for 2 h, followed by centrifugation. The supernatant was incubated at 56 °C, and the precipitate was washed and solubilized with 1% sodium lauryl sarcosinate. After incubation for 1 h at 37 °C and for 15 h at room temperature, and after centrifugation, the supernatant was filtrated on 0.45-μm nitrocellulose membrane and was dialyzed for 48 h against 25% methanol. The precipitate was dissolved in MilliQ water and was pulsed with ultrasound and was dialyzed for 48 h at 37 °C. To confirm the presence of OspA and OspB in the protein fraction, the respective murine monoclonal antibodies (HS332 and H6831 [obtained from Alan G. Barbour and Denee D. Thomas, University of Texas Health Science Center, San Antonio]) were assayed against the OF fraction and recombinant OspA by use of immunoblot analysis [21]. OF stimulation of PBMCs and CSF mononuclear cells was shown to discriminate patients with NB from patients with OND and healthy control subjects, by use of the ELISPOT assay [7, 22].

**Use of ELISPOT for analysis of cells producing IL-4 and IFN-γ.** The ELISPOT assay, which was originally described by Czerkinsky et al. [23], was used, in a slightly modified version, for analysis for Borrelia (OF)–stimulated and –unstimulated cells secreting IL-4 and IFN-γ, as described elsewhere [7, 22]. The plates were coated either with mouse anti–human IL-4 monoclonal antibody (MAb) IL4-I (82.4) or mouse anti–human IFN-γ MAb 1-D1K (Mabtech), 15 μg/mL, in sterile PBS. The PBMCs were analyzed at 100,000 lymphocytes/well. Because of limited cell numbers, 400–92,000 CSF lymphocytes (median, 5000 CSF lymphocytes) were added per well, depending on the number of cells available. It has previously been reported that the frequency of spots is not altered by decreasing cell counts, even when there are low numbers of cells per well [24]. The cells were stimulated with OF at the previously optimized concentration of 10 μg/mL. Unstimulated cells were analyzed to achieve the spontaneous secretion. Phytohemagglutinin (Sigma Chemical) was used as a positive control at the final concentration of 20 μg/mL. As a negative control, tissue culture medium only was added. For PBMC wells, cell cultures were run in triplicate, whereas, for CSF cells, IFN-γ cultures were run in triplicate, and, for IL-4, only single wells were analyzed, depending on cell supply. The cells were cultured, undisturbed, for 48 h at 37 °C, in an atmosphere of 5% CO₂ with 95% humidity. Development was achieved by successive incubation steps with 1 μg/mL biotinylated detector mouse anti–human IL-4 MAb [IL4-II (12.1)] or IFN-γ MAb [7-B6-1]
Figure 1. No. of *Borrelia*-specific cytokine-secreting mononuclear cells/100,000 lymphocytes in all patients with neuroborreliosis (NB). A, *Borrelia*-specific secretion of interferon (IFN)-γ in cerebrospinal fluid (CSF) was increased in all 3 disease intervals in subjects with NB, compared with control subjects without NB (non-NB controls). B, *Borrelia*-specific secretion of interleukin (IL)-4 in the CSF increased significantly between intervals 1 and 2. C, *Borrelia*-specific secretion of IFN-γ in blood was increased in interval 3 in patients with NB, compared with non-NB controls. D, *Borrelia*-specific secretion of IL-4 in blood showed a significant increase between intervals 2 and 3. The Kruskal-Wallis test was used for multiple comparisons, and the Mann-Whitney *U* test was used as a post hoc test. *Borrelia*-specific secretion was determined by subtracting the no. of spots in the unstimulated wells from the no. of spots in the wells stimulated with the *Borrelia* outer surface protein–enriched fraction. Disease interval 1 was 0–3 months, interval 2 was 3–12 months, and interval 3 was >12 months. Shown in the box plots are the median (line), interquartile range (box), 95th percentile (whiskers), outliers (open circles), and extreme values (asterisks).

(Mabtech AB), alkaline phosphatase–conjugated streptavidin in PBS-tween, and, finally, 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium in development buffer (AP conjugate substrate kit; BioRad).

The spots were counted under a dissection microscope. One spot denoted one cytokine-secreting cell.

Data handling and statistics. The mean of triplicates or duplicates was used in the analysis of cytokine results. "*Borrelia*-stimulated secretion" referred to the number of spots in *Borrelia* OF–stimulated wells, whereas the number of spots detected in wells with cells and medium was referred to as "unstimulated secretion." To determine "*Borrelia*-specific secretion," the unstimulated secretion was subtracted from the *Borrelia*-stimulated secretion. For CSF cells, data were recalculated to the number of spots/100,000 lymphocytes. *Borrelia*-specific IFN-γ predominance was assigned when the number of IFN-γ spots was greater than the number of IL-4 spots, whereas IL-4 predominance was assigned when the number of IL-4 spots was greater than the number of IFN-γ spots.

SPSS for Windows (version 10.0; SPSS) was used for statistical evaluation. For comparison of counts of cytokine-secreting cells between multiple groups or intervals, the Kruskal-Wallis test was used as a pretest, and the Mann-Whitney *U* test was used as a post hoc test. Wilcoxon signed rank test was used for paired analyses. Fisher’s exact test was used for comparison of intervals, with regard to the proportions of patients with IFN-γ or IL-4 predominance. *P* < .05 was considered to be statistically significant.
Figure 2. No. of Borrelia-specific cytokine-secreting mononuclear cells/100,000 CSF lymphocytes in patients with neuroborreliosis (NB), in association with clinical outcome. A, Borrelia-specific secretion of interferon (IFN)–γ in cerebrospinal fluid (CSF) was increased in disease interval 1 in patients with nonchronic NB, compared with subjects without NB (non-NB controls). B, Borrelia-specific secretion of interleukin (IL)–4 in CSF was increased in disease interval 2 in patients with nonchronic NB, compared with non-NB controls, and showed a significant increase between intervals 1 and 2. C, Borrelia-specific secretion of IFN-γ in the CSF of patients with chronic NB was increased in all 3 intervals, compared with that in the CSF of non-NB controls. D, There were no significant increases in Borrelia-specific secretion of IL-4 in the CSF of patients with chronic NB. The Kruskal-Wallis test was used for multiple comparisons, and the Mann-Whitney U test was used as a post hoc test. Borrelia-specific secretion was determined by subtracting the no. of spots in the unstimulated wells from the no. of spots in the wells stimulated with the Borrelia outer surface protein–enriched fraction. Disease interval 1 was 0–3 months, interval 2 was 3–12 months, and interval 3 was >12 months. Shown in the box plots are the median (line), interquartile range (box), 95th percentile (whiskers), outliers (open circles), and extreme values (asterisks). Chronic NB, disease duration ≥6 months; nonchronic NB, disease duration <6 months.

The present study was approved by the ethics committee of the Faculty of Health Sciences at Linköping University (Linköping, Sweden). Informed consent was obtained from each of the patients included in the study. The experimentation guidelines for clinical research outlined by the Department of Molecular and Clinical Medicine at Linköping University were followed in the conduct of the present study.

RESULTS

NB. In patients with NB, Borrelia-specific IFN-γ, but not IL-4, was increased in CSF samples during all 3 disease intervals ($P < .001$, $P < .0001$, and $P < .05$, respectively), compared with the non-NB control group (figure 1A and 1B). In blood samples, Borrelia-specific IFN-γ was increased in disease interval 3 ($P < .05$; figure 1C). There were no significant changes in IFN-γ in the CSF or blood during the course of NB. However, Borrelia-specific IL-4 showed a significant increase between intervals 1 and 2 in CSF samples ($P < .05$; figure 1B) and between intervals 2 and 3 in blood ($P < .01$; figure 1D).

Clinical outcome of NB. In comparisons of patients with chronic NB with patients with nonchronic NB, there were no significant differences regarding Borrelia-specific cytokine secretion in the different disease intervals (data not shown). However, in disease interval 2, a trend was observed regarding Borrelia-specific IL-4 secretion in CSF; such secretion tended to
be higher in patients with nonchronic NB than in patients with chronic NB (P = .05). Compared with the non-NB control group, patients who had chronic NB showed significantly increased *Borrelia*-specific secretion of IFN-γ in CSF (figure 2C) in all 3 disease intervals (P < .01, P < .001, and P < .05, respectively)—that is, they showed a prolonged IFN-γ response in the CNS. Conversely, *Borrelia*-specific IL-4 was not increased in the CSF samples obtained from patients who had chronic NB, compared with those obtained from the non-NB control group (figure 2D). On the other hand, *Borrelia*-specific IFN-γ was significantly increased only in disease interval 1 in patients with nonchronic NB (figure 2A), compared with the non-NB control group (P < .05). Furthermore, in disease interval 2, IL-4 was significantly increased (P < .05) in patients with nonchronic NB, compared with the non-NB control group (figure 2B). In addition, between disease intervals 1 and 2, there was a significant increase in *Borrelia*-specific IL-4 (P < .05) in the CSF of patients with nonchronic NB (figure 2B), whereas, in patients with chronic NB, there were no significant changes observed between the intervals (figure 2D). In disease interval 3, there were no samples available for patients with nonchronic NB, and only 1 CSF sample from a patient with chronic NB was available for IL-4 analysis.

**EM.** In comparisons of *Borrelia*-specific cytokine secretion in blood samples from patients with EM with blood samples from healthy blood donors, there was a significant increase in IFN-γ (P < .001), but not IL-4, in disease interval 1. In disease interval 3, IL-4 (P < .05), but not IFN-γ, was increased (figure 3A and 3B). Furthermore, in comparisons of the number of patients with *Borrelia*-specific IFN-γ and IL-4 predominance in the 3 disease intervals, there was a significant difference (P < .01) noted between intervals 1 and 3 (figure 4); IFN-γ dominance was observed in 8 of 8 patients in interval 1, and IL-4 dominance was observed in 6 of 8 patients in interval 3. In comparisons of the number of *Borrelia*-specific IFN-γ–secreting cells with the number of *Borrelia*-specific IL-4–secreting cells, the amount of IFN-γ was significantly higher than the amount of IL-4 in disease interval 1; however, no significant difference was noted either in interval 2 or in interval 3. In comparisons of the 3 disease intervals for each cytokine, no significant changes were observed.

**ACA.** The 5 patients with ACA were included in the study while their disease was in a late stage—namely, in disease interval 2 (n = 3) or 3 (n = 2). Although multiple samples were obtained from all of these patients, only 1 patient had samples obtained during both interval 2 and interval 3. This made it difficult to process the data as described for the other diagnostic groups. However, considering the data that were available, IFN-γ was found to predominate over IL-4 in 10 of the 12 samples obtained (i.e., the later-stage samples). The 2 samples with IL-4 predominance were obtained during intervals 2 and 3, respectively, and they originated from 2 different patients. In addition, analysis of the first sample obtained from each patient revealed that the group with ACA had significantly increased *Borrelia*-specific IFN-γ (P < .001), but not IL-4, compared with healthy blood donors (figure 5A and 5B). Furthermore, in com-
Figure 4. No. of patients with erythema migrans (EM) who showed Borrelia-specific interferon (IFN)-γ dominance (□) or interleukin (IL)-4 dominance (■) in blood-derived mononuclear cells in the 3 disease intervals. Fisher’s exact test was used for comparisons of disease intervals. Borrelia-specific secretion was determined by subtracting the no. of spots in the unstimulated wells from the no. of spots in the wells stimulated with the Borrelia outer surface protein–enriched fraction. Disease interval 1 was 0–3 months, interval 2 was 3–12 months, and interval 3 was >12 months.

Compared with Borrelia-stimulated secretion with Borrelia-unstimulated secretion in these samples, IFN-γ was increased (P < 0.05), but IL-4 was not (data not shown). Thus, longitudinal and, especially, early samples were missing, an IFN-γ-predominant response could be noted in late-stage samples obtained from patients with ACA.

DISCUSSION

In the present study, we have reported observations of an initial Borrelia-specific IFN-γ response and a subsequent up-regulation of Borrelia-specific IL-4 among nonchronic manifestations of Lyme borreliosis—that is, in the CSF samples (n = 3) of patients with nonchronic NB and in the blood samples (n = 8) of patients with EM. For chronic manifestations of the disease, however, persistent production of Borrelia-specific IFN-γ was seen, both in CSF samples from patients with chronic NB and in late-stage blood samples from patients with ACA, the chronic skin manifestation of Lyme borreliosis. These data support the findings of previous studies of Lyme borreliosis, which demonstrated a type 1 response [7, 8, 25] but also bring about new information, because previous studies had not taken into consideration changes that occurred during the course of the disease or related their findings to the clinical outcome of the infection. To date, there have been no published reports of up-regulated type 2 responses in cases of human Lyme borreliosis.

IFN-γ, a powerful proinflammatory cytokine, is the main mediator of the effector mechanisms of a type 1 response. It activates macrophages and stimulates their phagocytosis, intracellular killing of microbes, antigen presentation to T cells, and secretion of proinflammatory cytokines and chemokines [26]. During the type 1 response, B cells are stimulated to produce opsonizing and complement-activating antibodies [27, 28]. The type 1 immune response with high production of IFN-γ has been suggested to be the optimal response to all infections caused by intracellular or phagocytable microbes [29]. Thus, by clearing these pathogens, the type 1 response would diminish further antigenic stimulation, allowing a switch to a type 2 response by up-regulation of IL-4 and thereby resulting in reestablishment of homeostasis and limiting the risk for proinflammatory-mediated tissue destruction. However, if the type 1 response fails to completely clear the infection, persistent antigenic stimulation may induce chronic type 1 responses, leading to host damage. In Lyme borreliosis, such persistent antigenic stimulation has been suggested to be mediated, for example, by inactive cystic forms of B. burgdorferi [30]. Of interest, type 1 responses to OspA and, to a lesser degree, to OspB, the 2 major components of OF, were shown to correlate directly with the severity and duration of Lyme arthritis [31]. A relative lack of IL-4 (type 2) responses may predispose for development of chronic disease. This suggested model fits well with our results, which, although they are based on few observations, consistently showed findings that are in line with the model. Thus, IFN-γ, detected in the initial stage of chronic, as well as nonchronic, NB and in EM, could be involved in eliminating the infecting spirochete, whereas, if IFN-γ is persistently up-regulated, as observed in our patients with chronic NB and ACA, it might cause harm. On the other hand, the role for IL-4, detected later in our patients with nonchronic NB and in our patients with EM, might be to down-regulate the harmful effects of IFN-γ and, thereby, limit inflammation and long-lasting symptoms in these groups.

It cannot be excluded that, in cases in which the spirochetes are eliminated by antibiotics during treatment, IL-4 is up-regulated as a response to this treatment-induced clearance. Still, this shift is not seen after treatment of patients with chronic NB.

The reason for the continuing type 1 response in the CNS of patients with chronic NB is unclear. Apart from active or latent infection, as mentioned above, other possible explana-
Figure 5. No. of Borrelia-specific cytokine-secreting mononuclear cells/100,000 peripheral blood lymphocytes (PBLs) in blood samples from patients with acrodermatitis chronica artropicans (ACA). A, Borrelia-specific secretion of interferon (IFN)–γ was increased in the first sample obtained from patients with ACA, compared with samples obtained from healthy blood donors. B, Borrelia-specific secretion of interleukin (IL)–4 was not significantly increased. The Mann-Whitney U test was used for comparisons between groups. Borrelia-specific secretion was determined by subtracting the no. of spots in the unstimulated wells from the no. of spots in the wells stimulated with Borrelia outer surface protein–enriched fraction. Shown in the box plots are the median (line), interquartile range (box), 95th percentile (whiskers), outliers (open circles), and extreme values (asterisks).

The findings of the present study are interesting in relation to our previous demonstration of both IFN-γ– and IL-4–producing Borrelia-specific CSF cells in children with early-stage NB [40]. Although chronic NB does occur in children, it does not occur as frequently as it does in adults, and the course of childhood NB is relatively benign, compared with the course of NB in adults [5]. The children’s predisposition to respond with IL-4, in addition to IFN-γ, possibly contributes to their benign disease. Furthermore, we previously noted that patients who did recover from NB tended to have higher Borrelia-specific production of IL-4 in CSF than did patients with chronic NB [7]. Taken together, our data suggest an association between an initial type 1 response with subsequent up-regulation of IL-4 and clinical recovery of Lyme borreliosis.

We previously have reported increased levels of tumor necrosis factor (TNF)–α in the CSF of patients with nonchronic NB, but not in the CSF of patients with chronic NB [41]. The elevated levels were only seen in the early stage of the disease, which indicates the need for an early powerful inflammatory response. The up-regulation of IL-4 in patients with nonchronic NB, as reported in the present study, might play a role in the down-regulation of this early proinflammatory TNF-α response.

Several studies of experimental animal models of Lyme borreliosis have explored the type 1/type 2 immune balance in this disease, aiming to reveal which type of immune response is beneficial for efficient elimination of disease. The findings of these studies were complex. On one hand, a protective role for IFN-γ was demonstrated: CD4+ T helper 1 cells were shown to facilitate regression of murine Lyme carditis [11]. Furthermore, treatment with IFN-γ, either alone or in combination with TNF-α and IL-2, at the time of tick-mediated inoculation of mice with B. burgdorferi, rendered 67% or 95% protection, respectively, against the infection [42]. Another study concluded that neither IFN-γ nor IL-4 is absolutely necessary for resistance to Borrelia arthritis in mice, even if IL-4 seems to have a beneficial role [14]. However, other studies have found IL-4 to be correlated with disease resistance [13] or ability to eliminate infection [44] and have found IFN-γ to be associated with more pronounced symptoms [45, 46]. Surprisingly few studies of animals have analyzed how the immune response develops during the course of disease. However, in 1997,
Kang et al. [15] followed the immune response during the course of murine Lyme arthritis and were able to show that disease-resistant mice initially had a powerful IFN-γ response, which subsequently was down-regulated by an increase in IL-4, whereas susceptible mice mainly had a late IFN-γ response. Thus, this shows that cytokine levels should be evaluated during the course of the disease. Furthermore, the cytokine pattern obtained is in full agreement with our findings from studies of humans.

All of our patients with Lyme borreliosis received antibiotic treatment during the acute stage of the disease, although some of them were included in the study after the start of treatment. Some of the patients with NB also received multiple successive treatments. Little is known about the effects of antibiotic treatment on the immune response in vivo in humans. However, reports based on results from experimental animal models or in vitro experiments suggest widely differing effects, depending on study design and on the substance and/or response studied. [47, 48]. For ceftriaxone, cefotaxime, and tetracycline, no in vitro experiments suggest widely differing effects, depending on results from experimental animal models or treatments. Little is known about the effects of antibiotic treatment during the acute stage of the disease, although some of them were included in the study after the start of treatment. Some of the patients with NB also received multiple successive treatments. This pattern of antibiotic treatment in vivo will be investigated. Considering the few observations presented here, the results should also be confirmed in future studies.

In conclusion, the present study suggests an initial Borrelia-specific IFN-γ response, followed by a subsequent up-regulation of IL-4 in the nonchronic manifestations of Lyme borreliosis, in contrast to a persistent IFN-γ response and a lack of IL-4 in the chronic manifestations of Lyme borreliosis. Whether these differences in immune responses are a result of cleared or persistent infection, respectively, or whether these cytokine patterns do, in fact, influence the clinical outcome of Lyme borreliosis remains to be investigated. Considering the few observations presented here, the results should also be confirmed in future studies.

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