Hypercytokinemia in Familial Hemophagocytic Lymphohistiocytosis

By Jan-Inge Henter, Göran Elinder, Olle Söder, Mona Hansson, Birger Andersson, and Ulf Andersson

Familial hemophagocytic lymphohistiocytosis (FHL) is a frequently missed and almost uniformly fatal childhood disorder. It is characterized by fever, hepatosplenomegaly, cytopenia, coagulopathy, and hypertriglyceridemia. The pathogenesis of FHL is not known but the above clinical and laboratory findings are compatible with reported in vitro and in vivo effects of several inflammatory cytokines. We measured circulating interferon-γ (IFN-γ), tumor necrosis factor/cachectin (TNF), and interleukin-6 (IL-6) in nine children with FHL. During active disease, elevated IFN-γ was detected in seven of seven children, TNF in six of six, and IL-6 in two of seven children studied. Thus, important inflammatory cytokines are augmented in active FHL and may contribute to the pathogenesis of the disease. Soluble CD8 was also increased in seven of seven children, which suggests a pathophysiological importance of cytokotic T lymphocytes. Because FHL appears to be associated with a systemic hypercytokinemia, our results also indicate that studies of FHL may contribute to the understanding of cytokine effects in vivo. Moreover, FHL is a hereditary disorder, suggesting that the hypercytokinemia is caused by a genetic defect in cytokine regulation.

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MATERIALS AND METHODS

Patients. The study group consisted of nine children with FHL, five boys and four girls, between 2 months and 7 years old. They all had a clinical picture compatible with FHL and a lymphohistiocytic infiltration with hemophagocytosis in reticuloendothelial organs (Table 1). Two children had no pancytopenia at diagnosis, probably because of early recognition (no. 2) or previous steroid therapy (no. 8). All had siblings with FHL and/or parental consanguinity except one (no. 7) who was the first biologic child in the family.

Sampling of serum was performed during active disease as well as during remission. We defined the disease as active if the child was febrile (≥38°C, not caused by infection) and had splenomegaly or an increased degree of hemophagocytosis in a histopathological specimen. Remission was defined as a lack of all signs of disease during at least 1 month, whether treatment was administered or not. The samples, which were stored frozen before analysis, did not always suffice for the study of all cytokines at each sampling occasion. The controls were 10 children (eight boys and two girls) between 2 months and 3 years old, who were studied with informed parental consent at follow-up more than 5 weeks after a bacterial infection.

TNF assay. TNF was assayed with an enzyme-linked immunosorbent assay (ELISA) developed in our laboratory. Protein A purified rabbit IgG antihuman TNF (anti-huTNF), produced in our laboratory, was diluted in a bicarbonate buffer, pH 9.6, to a concentration of 7 μg/mL for coating 96-well immunoplates (Nunc, Roskilde, Denmark) with 50 μL of samples to be tested as well as serial dilutions of TNF standard were allowed to incubate for 1 hour at room temperature. Each sample was assayed in duplicate. Bound TNF was detected after sequential incubation of biotinylated rabbit anti-TNF (5 μg/mL) and streptavidin-biotinylated horseradish peroxidase (HRP) complex (Amersham, UK) diluted to 1:1,000. A limit, defined as the lowest concentration of TNF with absorbance 0.01% Tween 20, nonspecific binding of proteins to the plastic was blocked by 1 hour of incubation with 100 μL PBS containing 0.5% gelatin, whereafter 50 μL of samples to be tested as well as serial dilutions of TNF standard were allowed to incubate for 1 hour at 4°C. After subsequent washing with phosphate-buffered saline (PBS) with 0.01% Tween 20, nonspecific binding of proteins to the plastic was blocked with a 1 hour of incubation with 100 μL of PBS containing 0.5% gelatin, whereafter 50 μL of samples to be tested as well as serial dilutions of TNF standard were allowed to incubate for 1 hour at room temperature. Each sample was assayed in duplicate. Bound TNF was detected after sequential incubation of biotinylated rabbit anti-TNF (5 μg/mL) and streptavidin-biotinylated horseradish peroxidase (HRP) complex (Amersham, UK) diluted to 1:1,000. As a substrate, orthophenyldiamine (OPD; Dakopatts, Glostrup, Denmark) was used and absorbance was measured at 492 nm in a Titertec Multiscan (Flow Laboratories, Irvine, UK). The detection limit, defined as the lowest concentration of TNF with absorbance significantly different from the negative controls, was 5 to 10 U/mL (80 to 160 pg/mL) and no cross-reactivity with recombinant huIL-1, IL-2, IFN-α or IFN-γ was observed.

IL-6 assay. IL-6 was determined with ELISA reagents (Quanti
tikine; Research and Diagnostic Systems, Minneapolis, MN). The reaction was measured with a chemoluminescence system (Luminoscan, Labsystems Inc, Tyresö, Sweden) and the accuracy of the
assay system was determined by a bioassay using an IL-6-dependent murine hybridoma cell line (B45, supplied by Dr F. Melchers, Basel Institute of Immunology, Switzerland). The Quantikine ELISA used a polystyrene microtiter plate coated with a murine monoclonal antibody (MoAb) to human IL-6. Standard IL-6 preparations and serum samples diluted 1:10 were added to the plate in 200 µL assay diluent. After incubation and washing, 200 µL of a second polyclonal rabbit HRP-labeled antibody was added. After incubation and washing, an HRP substrate, tetramethylbenzidine (TMBZ; Sigma Chemical Co, St Louis, MO), was added. Twenty minutes later the plate was placed into the luminometer, in which 150 µL of 0.5 mmol/L NaLuminol, prepared from Luminol (Sigma), recrystallized three times, and supplemented with hydrogen peroxide, was added to each well. After 20 minutes an emitted light flash was measured with the photomultiplier having a spectral response of 350 to 680 nm. The signal was measured at peak mode in 1.0 second total time. Light signals above background values were detected and plotted linearly as they related in a log-log scale over the interval of 0.1 to 10.000 pg of IL-6. Standard IL-6 dilutions and selected negative and positive sera. After 4 days the number of living cells in the wells were determined with a colorimetric hexosaminidase method. "The coefficient at variation was between 5% and 10% with the highest variation in the lower part of the curve. For routine diagnostic purposes as well as for the tests performed in this study, single determinations were used. In the bioassay, 2,000 B45 cells were added to microtiter wells and allowed to grow in Dulbecco’s modified Eagle’s medium (DMEY) supplemented with 10% fetal calf serum. Controls with medium only were compared with IL-6 standard curve, as determined by parallel testing of a strongly positive sepsis serum (27.000 pg/mL) in the B45 bioassay. The lower detection limit was 12.5 pg/mL.

**IFN-γ assay.** The IFN-γ content was determined by a commercially available radioimmunoassay (Centocor, Malvern, PA). Polystyrene beads coated with mouse MoAbs against huIFN-γ were incubated with the specimen (0.2 mL). After washing, radiolabeled (122I) anti-IFN-γ was added. Bound radioactivity was determined in a gammacounter and the IFN-γ concentrations were calculated from standard curves. The limit of sensitivity of the assay was 0.1 U/mL.

**sCD8 assay.** Serum levels of sCD8 were determined with a sandwich immunoassay (Cellfree T8; T Cell Sciences Inc, Cambridge, MA). To microtiter plate wells, coated with an MoAb (C9) directed against one epitope of the CD8 molecule, 10 µL of serum sample diluted in 90 µL of sample diluent containing protein in a buffered solution was added. Samples giving greater than 2,000 U/mL with this procedure were further diluted 1:5 or 1:10 and retested. After incubation for 90 minutes at 37°C the wells were washed three times with PBS-containing surfactant (Tween 20). One hundred microliters of HRP-conjugated MoAb directed against a different epitope of CD8 was added and, after further incubation for 90 minutes and washing, the substrate TMBZ was added. The reaction was measured with the Luminoscan equipment as described for IL-6. The lower detection limit in this study was 5 U/mL.

**Statistical analysis.** The nonparametric Mann-Whitney test was used for the statistical comparisons of TNF, IL-6, IFN-γ, and sCD8 in active FHL versus controls. In children with multiple samples taken during active disease, the mean value for each child was used. For the purpose of analysis, with cytokine concentrations below the lower limit of sensitivity of the assays a value halfway between zero and the lower limit of detection was used. Probability (P) values were calculated on the basis of two-tailed tests.

**RESULTS**

All inflammatory cytokines studied, ie, IFN-γ, TNF, and IL-6, were increased in the peripheral blood of children.
Table 2. Inflammatory Cytokines and sCD8 in FHL

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ</th>
<th>TNF</th>
<th>IL-6</th>
<th>sCD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cut-off level</td>
<td>0.1 U/mL*</td>
<td>5 U/mL*</td>
<td>58 pg/mL†</td>
<td>614 U/mL†</td>
</tr>
<tr>
<td>FHL active</td>
<td>7/7</td>
<td>6/6</td>
<td>2/6</td>
<td>7/7</td>
</tr>
<tr>
<td>FHL remission</td>
<td>0/5</td>
<td>ND</td>
<td>0/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Controls</td>
<td>0/9</td>
<td>3/10</td>
<td>1/9</td>
<td>0/9</td>
</tr>
</tbody>
</table>

Expressed as patients with serum concentrations above the cut-off level/all children studied.

Abbreviation: ND, not done.

*The detection limit of the assay.
†Mean value ± 2 SD of controls.

with active FHL, as compared with controls (Table 2, Figs 1 through 3). The elevation was reversible (Table 2). Most prominent was the increase of IFN-γ. The content of sCD8 was also markedly augmented during active disease.

All specimens taken during active FHL (n = 8) were positive for IFN-γ, whereas all samples taken during FHL in remission and from controls were negative (P < .01) (Fig 1). Two specimens with more than 50 U/mL (approximately 60 to 80 U/mL) were both taken during severe disease and the IFN-γ levels seemed to reflect well the severity of the disease. The T-lymphocyte product sCD8 was also markedly augmented during active FHL, as compared with controls (P < .01) (Fig 4). The sCD8 levels were moderately, but significantly, elevated (P < .01) also during remission.

TNF was also elevated in children with active FHL, as compared with controls (P < .05) (Fig 2). Detectable levels were recorded in all the children studied (n = 6). The maximum value recorded was 72 U/mL but mostly the increase was moderate (10 to 15 U/mL). The serum content of IL-6 was also increased, albeit only moderately, during active FHL, as compared with controls (P < .05) (Fig 3).

During remission, the serum amount of IL-6 was reduced, as compared with controls (P < .01), and not detectable (<12.5 pg/mL) in four of five FHL children studied. The cerebrospinal fluid (CSF) of FHL patients was examined with regard to IFN-γ in six children. Two patients had detectable levels (no. 3 at onset and no. 7 at relapse) with 23 and 0.5 U/mL, respectively. Both specimens were taken during active cerebromeningeal disease. TNF was not
detected in CSF during active FHL (n = 4 children). Markedly elevated levels of sCD8 (>600 U/mL) during active FHL were found in two patients (no. 3 at onset and no. 7 at a relapse) with 4,700 and 750 U/mL, respectively.

**DISCUSSION**

Activated lymphocytes and mononuclear phagocytes constitute a rich source of several potent cytokines. Some of these have been collectively termed inflammatory cytokines because they are produced in high concentrations at inflammatory sites and exert a number of common effects related to inflammation. The present study shows that patients with active FHL have elevated serum levels of the inflammatory cytokines TNF, IL-6, and, particularly, IFN-γ. The findings indicate that these cytokines may have an important pathophysiologic role in this disorder, especially as the clinical and laboratory findings in FHL are in accord with many of their reported biologic effects (Table 3). Moreover, we know of no other human disorder with a more prominent noninfectious mononuclear inflammatory reaction. Studies of FHL may therefore also contribute to the understanding of the effects of cytokines in vivo.

The striking resemblance between biologic changes induced by inflammatory cytokines and the clinical and laboratory findings in FHL is summarized in Table 3. The cytokines interact in a network and may exert their effects both directly and indirectly, ie, by regulating other cytokines. Indeed, remarkable is the similarity between findings in patients with FHL and side effects of TNF in clinical studies, as summarized by Spriggs et al. Fever is prominent in FHL and the cytokines studied may all induce fever. The marked hypertriglyceridemia in FHL is associated with reduced lipoprotein lipase (LPL) activity. Lipoprotein lipase suppression after challenge with lipopolysaccharide was a key to the purification of TNF (cachectin) by Beutler and Cerami. Wasting is a well-known effect of TNF and cachexia with muscular atrophy is also present in FHL (Henter, unpublished observation). Even less well understood features of FHL such as hypofibrinogenemia and decreased natural killer (NK)-cell activity may be explained by cytokines, because TNF has a procoagulant activity and has been associated with hypofibrinogenemia but also suppresses NK-cell activity. Moreover, TNF and IL-6 are principally produced by activated macrophages, which are numerous in FHL. Lymphocytes, the source of IFN-γ, are even more frequent in active phases of this lymphohistiocytic proliferative disorder.

The serum levels of IFN-γ correlated well with the clinical condition because IFN-γ was increased in all specimens of active FHL but below detection level in healthy controls, during FHL in remission, and during bacterial meningitis. Also, the levels of sCD8 were elevated in active FHL and correlated well with the clinical condition, indicating that the CD8 lymphocytes are activated in FHL and that these cells may serve as a source of IFN-γ. These findings are in accord with the tremendous elevation of soluble IL-2 receptor (sIL-2R) in the serum of FHL patients, which has been reported recently and also indicates a prominent T-lymphocyte activation during active FHL. Moreover, neopterin, a macrophage product principally released by IFN-γ has been shown to be markedly released in serum as well as spinal fluid in FHL children. The elevation of sCD8 seen during remission may reflect a continuous but subclinical activity of the disease and, because sCD8 is a sensitive marker for activation of the T-cell system, our results suggest that sCD8 may be a useful marker of disease activity in FHL. The increase in TNF, found in all children studied, was mostly only moderate. Augmented levels were also found in three control samples, but similar findings have previously

**Table 3. Typical Findings in FHL Compared With Reported Biologic Effects of Inflammatory Cytokines**

<table>
<thead>
<tr>
<th>Findings in FHL</th>
<th>Effects of Inflammatory Cytokines</th>
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<tbody>
<tr>
<td>Fever</td>
<td>Induction of fever</td>
</tr>
<tr>
<td>Cytopenia</td>
<td>Anemia, neutropenia, thrombocytopenia</td>
</tr>
<tr>
<td>Serum transaminase elevation</td>
<td>Serum transaminase elevation</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>Hypertriglyceridemia</td>
</tr>
<tr>
<td>Lipoprotein lipase suppression</td>
<td>Lipoprotein lipase suppression</td>
</tr>
<tr>
<td>Hypofibrinogenemia</td>
<td>Hypofibrinogenemia, procoagulant activity</td>
</tr>
<tr>
<td>Neurologic symptoms</td>
<td>Neurologic symptoms</td>
</tr>
<tr>
<td>Low NK-cell activity</td>
<td>Suppression of NK-cell activity</td>
</tr>
<tr>
<td>Lymphohistiocytic accumulation in RES</td>
<td>Lymphohistiocytic infiltration, secreted by macrophages and lymphocytes</td>
</tr>
<tr>
<td>Hemophagocytosis by activated macrophages</td>
<td>Macrophage activation</td>
</tr>
</tbody>
</table>

**Fig 4. Serum sCD8 levels in patients with FHL (horizontal lines indicate median levels).**
been made also by others. A possible explanation for the elevation of this monokine in the systemic circulation of FHL patients not being even more apparent may be that it serves as an autocrine and paracrine factor rather than a circulating hormone. Therefore, circulating TNF might represent leakage from the site of production in the affected tissues. IL-6 was only moderately elevated in FHL as compared with controls, whereas the levels during remission were mostly undetectable and even lower than the controls (Fig 3), but the clinical importance of these alterations is difficult to evaluate. Using a previously described methodology, we failed to detect intracellular TNF, IL-6, and IFN-γ in lymphocytes and macrophages of the peripheral blood in two patients. It can be speculated that the cytokine-producing cells leave the circulation when they have been activated.

The increase of the lymphocyte products IFN-γ and sIL-2R in FHL is apparent and excessive in comparison with the findings in another, more well-known pediatric inflammatory disorder, Kawasaki’s disease (KD). In a recent study of children with KD, serum levels of IFN-γ and sIL-2R did not exceed 2.5 U/mL and 10^10 U/mL, respectively. The median level of IFN-γ in FHL was greater than 30 times higher than in the children with KD.

REFERENCES

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