Hyercytokinemina 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 hypertriglyceridaemia. The pathogenesis of FHL is not known but both the 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 six 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 cytotoxic 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.

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 histopathologic 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 immunoabsorbent assay (ELISA) developed in our laboratory. Protein A purified rabbit IgG anti-human TNF (anti-huTNF), produced in our laboratory, was diluted in a bicarbonate buffer, pH 9.0, to a concentration of 7 μg/mL for coating 96-well immunoassay plates (Nunc, Roskilde, Denmark) with 50 μL overnight 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 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 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 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 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 (Quantikine; 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 Quanti- 
kine 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, tetrame-
thylbenzidine (TMBZ; Sigma Chemical Co, St Louis, MO), was 
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thylbenzidine (TMBZ; Sigma Chemical Co, St Louis, MO), was 
added. The reaction was measured with the Luminoscan equip-
ment as described for IL-6. The lower detection limit in this study 
was 12.5 pg/mL.

IFN-γ assay. The IFN-γ content was determined by a commer-
cially available radioimmunoassay (Centocor, Malvern, PA). Poly-
styrene beads coated with mouse MoAbs against huIFN-γ were 
icubated with the specimen (0.2 mL). After washing, radiolabeled 
(125I) 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, Cam-
bridge, 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 
icubation for 90 minutes and washing, the substrate TMBZ was 
added. The reaction was measured with the Luminoscan equip-
ment 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
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 cerebrospinal disease. TNF was not

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**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.
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.6,7 (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.14 Fever is prominent in FHL and the cytokines studied may all induce fever.6,7,14 Of particular interest in this context is our recent finding that the marked hypertriglyceridemia in FHL is associated with reduced lipoprotein lipase (LPL) activity.15 Hypertriglyceridemia and LPL suppression after challenge with lipopolysaccharide was a key to the purification of TNF (cachectin) by Beutler and Cerami.8 Wasting is a well-known effect of TNF,2,14 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 activity14 and has been associated with hypofibrinogenemia but also suppresses NK-cell activity.17 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.1

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 recently18 and also indicates a prominent T-lymphocyte activation during active FHL.9,13 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.20,21 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>
</tr>
</thead>
<tbody>
<tr>
<td>Fever1,2</td>
<td>Induction of fever7,8,12,14</td>
</tr>
<tr>
<td>Cytopenia2,11</td>
<td>Anemia, neutropenia, thrombocyto-</td>
</tr>
<tr>
<td></td>
<td>pytopenia8,14</td>
</tr>
<tr>
<td>Serum transaminase elevation1,2,6</td>
<td>Serum transaminase elevation10</td>
</tr>
<tr>
<td>Hypertriglyceridemia10</td>
<td>Hypertriglyceridemia6</td>
</tr>
<tr>
<td>Lipoprotein lipase suppression10</td>
<td>Lipoprotein lipase suppression10</td>
</tr>
<tr>
<td>Hypofibrinogenemia1,2</td>
<td>Hypofibrinogenemia1,4 procoa-</td>
</tr>
<tr>
<td></td>
<td>gulant activity12,16</td>
</tr>
<tr>
<td>Neurologic symptoms1,2</td>
<td>Neurologic symptoms14</td>
</tr>
<tr>
<td>Low NK-cell activity</td>
<td>Suppression of NK-cell activi-</td>
</tr>
<tr>
<td>Hemophagocytosis by activated macrophages11</td>
<td>Macrophage activation1,12,13</td>
</tr>
<tr>
<td>Lymphohistiocytic accumulation in RES11</td>
<td>Lymphohistiocytic inflamma-</td>
</tr>
<tr>
<td></td>
<td>tion1,14 secreted by macro-</td>
</tr>
<tr>
<td></td>
<td>phages and lymphocytes6,12,13</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 × 10¹⁰ U/mL, respectively. The median level of IFN-γ in FHL was greater than 30 times higher than in the children with KD.

Of major importance in this context is the finding that prolonged exposure to a low recombinant huTNF concentration is more toxic than brief exposure to a concentration 100-fold higher. Because the cytokine production in FHL most probably is continuous as well as prolonged, the toxic effects can be marked despite moderate serum concentrations. As indicated by this study, none of the inflammatory cytokines is likely to operate alone; instead, many factors probably contribute to the pathogenesis of FHL. The etiology of FHL remains unknown, but we speculate that IFN-γ, in the light of its macrophage-activating capacity, stimulates the mononuclear phagocytic system and thus the release of monokines as TNF, which then serve as important disease promoters. Our results also suggest that IFN-γ is secreted, at least in part, by CD8⁺ lymphocytes. We speculate that FHL may be caused by a genetic disturbance in the regulation of cytokine activity inducing a hypercytokinemia.

REFERENCES

Hypercytokinemia in familial hemophagocytic lymphohistiocytosis

JI Henter, G Elinder, O Soder, M Hansson, B Andersson and U Andersson