Genetic subtypes of familial hemophagocytic lymphohistiocytosis: correlations with clinical features and cytotoxic T lymphocyte/natural killer cell functions

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Mutations of the perforin (PRF1) and MUNC13-4 genes distinguish 2 forms of familial hemophagocytic lymphohistiocytosis (FHL2 and FHL3, respectively), but the clinical and biologic correlates of these genotypes remain in question. We studied the presenting features and cytotoxic T lymphocyte/natural killer (CTL/NK) cell functions of 35 patients for their relationship to distinct FHL subtypes. FHL2 (n = 11) had an earlier onset than either FHL3 (n = 8) or the non-FHL2/FHL3 subtype lacking a PRF1 or MUNC13-4 mutation (n = 16). Deficient NK cell activity persisted after chemotherapy in all cases of FHL2, whereas some patients with FHL3 or the non-FHL2/FHL3 subtype showed partial recovery of this activity during remission. Alloantigen-specific CTL-mediated cytotoxicity was deficient in FHL2 patients with PRF1 nonsense mutations, was very low in FHL3 patients, but was only moderately reduced in FHL2 patients with PRF1 missense mutations. These findings correlated well with Western blot analyses showing an absence of perforin in FHL2 cases with PRF1 nonsense mutations and of MUNC13-4 in FHL3 cases, whereas in FHL2 cases with PRF1 missense mutations, mature perforin was present in low amounts. These results suggest an association between the type of genetic mutation in FHL cases and the magnitude of CTL cytolytic activity and age at onset. (Blood. 2005;105:3442-3448) © 2005 by The American Society of Hematology

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is characterized by fever and hepatosplenomegaly associated with pancytopenia, hypertriglyceridemia, and hypofibrinogenemia. Histologically, infiltrations of lymphocytes and histiocytes with hemophagocytic activity are seen in the reticuloendothelial system, bone marrow, and central nervous system (CNS). HLH can be classified as either primary or secondary. The primary form, also known as familial hemophagocytic lymphohistiocytosis (FHL), is inherited as an autosomal recessive disorder that usually arises during infancy.

Although generally attributed to uncontrolled T-cell activation leading to the excessive production of inflammatory cytokines and abnormal macrophage activation, the pathogenesis of HLH remains controversial. Thus, identifying the molecular genetic defects that underlie HLH has been a major concern for many years. Recently, mutations of the perforin (PRF1; MIM170280) and MUNC13-4 genes were found in patients with FHL. Several reports indicated that the mutation in PRF1 occurs in about 20% to 30% of patients with FHL, thus defining the FHL2 molecular subtype. In Japan, MUNC13-4 mutations were identified in 6 of 16 families with non-FHL2 subtypes, suggesting an FHL3 subtype that may account for as many as 20% to 25% of all FHL cases. Attempts to identify molecular genetic defects in the 45% to 50% of FHL cases that lack both PRF1 and MUNC13-4 mutations are under way.

Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) both rely on the Fas-FasL system and the secretion of lytic granules containing granzyme and perforin to kill virus-infected and malignant cells. Perforin is synthesized as an inactive glycosylated precursor that is subsequently cleaved at the C-terminus to yield an active mature form. Perforin protein in the form of a 534-amino acid polypeptide is constitutively expressed by NK cells and CTLs. In effector-target cell interactions, intracellular perforin is secreted by a mechanism of regulated exocytosis. The secreted perforin then becomes integrated into the target-cell membrane, followed by polymerization that generates poly-perforin pores in the presence of Ca2+ in the plasma membrane. This pore formation leads to the destruction of cells by osmotic lysis and by allowing entry of apoptosis-inducing granzymes. The perforin gene, PRF1, encodes transmembrane, endothelial growth factor–like, and C2 functional domains. The C2 domain binds to phospholipids in a Ca2+-dependent manner, and proteolytic cleavage of the carboxy-terminal domain uncovers the C2 domain, allowing perforin to bind to the membrane. Thus, perforin is produced as a precursor form, and posttranslational processing by proteolysis and glycosylation is
required for its maturation.15 Less is known about MUNC13-4, a homolog of MUNC13-1 that functions as a priming factor for neurotransmitter release. Because of its critical role in regulating the exocytosis of lytic granules in NK cells and CTLs, MUNC13-4 is expected to play a similar role in the exocytosis of perforin-containing vesicles.19 The onset of FHL typically occurs within the first year of life in 70% to 80% of cases,20,21 examples of late-onset cases,20 as well as a case in a teenager with genetic defects in PRF1 in the absence of clinical symptoms,21 have also been described. NK cell function was impaired in the majority of these cases, including those considered to be late onset.20,22 These observations suggest that the defining characteristics of FHL may vary with both the type of genetic defect and the function of NK cells or CTLs.23,24 We therefore analyzed the relationships among the clinical features, genetic defects, and CTL/NK cell functions of FHL patients with different molecular subtypes of this disease.

Patients, materials, and methods

Patients

A total of 67 Japanese patients with HLH (34 boys and 33 girls) were registered in the study from 1994 to 2003, 10 of whom were excluded because (i) the diagnosis was not compatible with FHL by criteria of the Histiocyte Society,3 (ii) a blood sample was not obtained, or (iii) permission for the analysis was not given by the parents. Thus, 57 patients met the diagnostic criteria for FHL and had documented informed consent, fulfilling the principal requirements for eligibility. Informed consent was provided according to the Declaration of Helsinki. Thirty-five were tested for both PRF1 and MUNC13-4 mutations and were the focus of the presenting analysis. The study was approved by the institutional review boards at Kyushu University, Saga University, Ehime University, and Kyoto Prefectural University. Twenty-one patients received chemotherapy according to the HLH94 protocol, which specifies a combination of dexamethasone and etoposide as induction therapy, followed by dexamethasone and etoposide as maintenance therapy.25 The onset of FHL typically occurs within the first year of life in 70% to 80% of cases,20,21 examples of late-onset cases,20 as well as a case in a teenager with genetic defects in PRF1 in the absence of clinical symptoms,21 have also been described. NK cell function was impaired in the majority of these cases, including those considered to be late onset.20,22 These observations suggest that the defining characteristics of FHL may vary with both the type of genetic defect and the function of NK cells or CTLs.23,24

Flow cytometric and genetic analyses

To analyze perforin expression, we obtained peripheral blood mononuclear cells (PBMCs) before or during treatment and performed flow cytometric analysis as described in our previous reports.11,13 Intracellular perforin was considered deficient when less than 1.0% of CD3+/CD8+ or CD56+ cells expressed this antigen. When perforin expression was negative, we extracted genomic DNA and used it for sequencing analysis of PRF1 by a previously described procedure with selected primers.11,13 Polymerase chain reaction (PCR) products were subcloned and sequenced on the ABI PRISM 377 Sequence Detection System (PE-Applied Biosystems, Foster City, CA).

Assay for NK cell activity

The NK cell activity of PBMCs was measured at diagnosis, during remission (between 2 and 6 months after diagnosis at completion of induction chemotherapy), and after HSCT by incubating cells with K562 targets for 4 hours with an effector-target (E/T) cell ratio of 20:1.26 Target cells were also added to wells containing medium alone and to wells containing 1% Triton X-100 to determine the spontaneous and maximal levels of 51Cr release, respectively. After 4 hours, 0.1 mL of supernatant was collected from each well. The percentage of specific 51Cr release was calculated as (cpm experimental release – cpm spontaneous release)/(cpm maximal release – cpm spontaneous release) × 100, where cpm indicates counts per minute. At an E/T ratio of 20:1, normal values based on findings in 50 healthy children ranged from 18% to 40%, with 5% or less denoting a deficiency and 6% to 17% a moderate decrease.26 NK cells were not routinely quantified; however, only specimens with PBM effector cell counts above 10⁶ per tube were included in the analysis.

Generation of alloantigen-specific CTL lines and analysis of CTL cytotoxicity

Alloantigen-specific CD8+ CTL lines were generated as previously described.27,28 Briefly, PBMCs were obtained from FHL2 and FHL3 patients, their healthy parents, and unrelated healthy controls. These cells were cocultured with a mitomycin C (MMC)–treated B-lymphoblastoid cell line (B-LCL) established from an HLA-mismatched individual. Magnetized polystyrene beads coated with an anti-CD8 monoclonal antibody (DYNAL, Oslo, Norway) were used to isolate CD8+ T lymphocytes from PBMCs that had been stimulated with allogeneic B-LCLs for 6 days. CD8+ T lymphocytes, cultured in medium with interleukin-2 (Genzyme, Boston, MA), were stimulated with MMC-treated allogeneic B-LCLs 3 times at 1-week intervals; subsequently, these lymphocytes served as CD8+ alloantigen-specific CTL lines. Alloantigen-specific CTL clones were generated from bulk CTL lines by a limiting dilution method as previously described.27,28 The alloantigen specificity of CTL lines was determined by interferon-γ (IFN-γ) production in response to stimulation with allogeneic B-LCLs as described previously.27,28 Briefly, 1 × 10⁶ T lymphocytes were cocultured with or without 5 × 10⁴ MMC-treated B-LCLs in 0.2 mL of RPMI 1640 medium supplemented with 10% fetal calf serum in a flat-bottomed microtiter well. In some experiments, an anti–HLA class I monoclonal antibody (w6/32) was added to wells at an optimal concentration. After 72 hours, the supernatant was collected from each well and assayed for the production of IFN-γ with an enzyme-linked immunosorbent assay (ELISA; ENDOGEN, Woburn, MA). Cytotoxic activity was determined by a 5-hour 51Cr release assay as described earlier.7 To evaluate the role of perforin in CTL-mediated cytotoxicity, we pretreated effector cells with an inhibitor of the perforin-based cytotoxic pathway, concanamycin A (CMA; Wako Pure Chemical Industries, Osaka, Japan), at a concentration of 100 nM for 2 hours before incubation with target cells.

Western blot analysis

T-cell lines established from FHL2 and FHL3 patients and a healthy control were used for Western blot analysis. Cell lysates were prepared by 1% Triton X-100 extraction. The extracts (20 μg per lane each) were analyzed by the Western blot method with antiperforin (Lab Vision, Fremont, CA) and anti–MUNC13-4 rabbit polyclonal antibodies.19 Horseradish peroxidase–labeled anti–rabbit immunoglobulin G (IgG) polyclonal antibody was used as the secondary antibody with detection by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Statistical analysis

Differences in the distribution of categorical variables (eg, age at onset, family history, CNS involvement, NK cell activity at diagnosis, NK cell activity during remission, chemotherapy, and outcome) were analyzed with the Fisher exact test. When any significant variability (P < .05) was observed among the 3 subgroups (FHL2, FHL3, and non-FHL2/FHL3), additional analyses were done with the Bonferroni test to identify significant differences between discrete subgroup pairs. The Student t test was used to assess CTL lytic activity in 4 patient subgroups (FHL2 nonsense, FHL2 missense, FHL3, and healthy controls) at an E/T ratio of 10:1.
**Results**

Comparison of genotype and phenotype in 3 different molecularly defined subgroups

Since more than 80% of all FHL patients in Japan were registered by our study group over the 10-year accrual period, the findings of this analysis are believed to reflect the actual epidemiology of FHL in Japan. Of the 57 patients who met all eligibility criteria, 11 had PRF1 mutations and lacked perforin expression by flow cytometry, whereas 46 were positive for perforin expression (Figure 1). Thus, the frequency of the FHL2 subtype in Japan can be estimated as 19% of all FHL cases. Twenty-four of the 46 patients with perforin expression were examined for MUNC13-4 mutations and 8 (6 of whom were described in a previous report\(^1\)) had a positive result, suggesting that the FHL3 subtype accounts for approximately one fourth of the FHL cases in Japan. The 16 patients with neither PRF1 nor MUNC13-4 mutation were classified as having non-FHL2/FHL3 disease.

Selected characteristics of patients with PRF1 mutations (FHL2), MUNC13-4 mutations (FHL3), or neither of these defects (non-FHL2/FHL3) are summarized in Table 1. The Fisher exact test demonstrated a significant difference in age at diagnosis among the 3 subgroups (Table 1). None of the other features examined (family history, CNS involvement, NK cell activity at diagnosis, type of chemotherapy, or outcome) showed any important variability. By the Bonferroni test, patients with the FHL2 subtype had an earlier age at disease onset (\(P < .01\) for comparisons with both FHL3 and non-FHL2/FHL3).

Twenty-one patients were treated entirely on the HLH94 protocol whereas the remaining 14 received alternative therapy, including steroids, cyclosporine A, etoposide, and other cytotoxic drugs. There was partial recovery of deficient NK cell activity in the FHL3 and non-FHL2/FHL3 subgroups after induction chemotherapy or during remission compared with none in the FHL2 subgroup (\(P < .05\) for FHL3; \(P < .1\) for non-FHL2/FHL3). This improvement was not related to the type of chemotherapy administered. Full recovery of NK cell activity was noted in all 6 patients tested after HSCT, encompassing the FHL2, FHL3, and non-FHL2/FHL3 subgroups. Of the 22 patients who underwent allogeneic HSCT, 17 have survived for 10 months to 11 years (median, 4 years): 5 FHL2 patients, 4 FHL3 patients, and 8 non-FHL2/FHL3 patients. Thus, allogeneic HSCT was effective therapy in a majority of patients who underwent this procedure, regardless of the molecular subtype. By contrast, 8 of the 13 patients treated without HSCT have died; the remaining 5 have survived for 22 months to 7 years (median, 2 years 8 months).

### Table 1. Characteristics of patients with the FHL2, FHL3, and non-FHL2/FHL3 subtypes of familial hemophagocytic lymphohistiocytosis

<table>
<thead>
<tr>
<th>Category</th>
<th>No. patients with FHL2, (n = 11)</th>
<th>No. patients with FHL3, (n = 8)</th>
<th>No. patients with non-FHL2/FHL3, (n = 16)</th>
<th>(P)</th>
</tr>
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<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
<td></td>
<td>.007</td>
</tr>
<tr>
<td>0-3 mo</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>4-11 mo</td>
<td>0</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>1-14 y</td>
<td>3</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Family history</td>
<td></td>
<td></td>
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<td>.513</td>
</tr>
<tr>
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<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>3</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>CNS involvement</td>
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<td></td>
<td></td>
<td>.713</td>
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<tr>
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<td>7</td>
<td>5</td>
<td>10</td>
<td></td>
</tr>
<tr>
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<td>4</td>
<td>3</td>
<td>3</td>
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<td>2</td>
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</tr>
<tr>
<td>NK cell activity‡</td>
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<td></td>
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<td>.247</td>
</tr>
<tr>
<td>0%-5%</td>
<td>9</td>
<td>3</td>
<td>7</td>
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<td>6%-17%</td>
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<td>18%-40%</td>
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<td></td>
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<td>2</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
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<td>.199</td>
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<td>5</td>
<td>7</td>
<td>9</td>
<td></td>
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<tr>
<td>Various regimens</td>
<td>6</td>
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<td>Outcome</td>
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<td>.292</td>
</tr>
<tr>
<td>Alive with HSCT</td>
<td>5</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Alive without HSCT</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Dead with HSCT</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dead without HSCT</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

CNS indicates central nervous system; NK, natural killer; nonsense, nonsense mutation including frameshift; missense, missense mutation; donor site, splice-donor site; acceptor site, splice-acceptor site; and HSCT, allogeneic hematopoietic stem cell transplantation.

*The Fisher exact test was used to determine the global \(P\) value for all 3 subgroups. By the Bonferroni test, patients with FHL2 had an earlier age at onset by comparison with either of the remaining subgroups (\(P < .01\)).

‡Defined by neurologic symptoms and/or pleocytosis in cerebrospinal fluid.

\(\dagger\)NK cell activity measured at diagnosis and defined as deficient (≤ 5%), moderately decreased (6%-17%), or normal (18%-40%).

IFN-γ production and cytotoxic activity of T lymphocytes in patients with the FHL2 and FHL3 subtypes

Alloantigen-specific CD8\(^+\) CTL lines were generated for all patients with available PBMCs (6 FHL2 and 2 FHL3). The antigen specificities of T-cell lines were initially examined by measuring IFN-γ production. As shown in Figure 2, all CD8\(^+\) T-cell lines generated by stimulation with allogeneic B-LCLs (KIN-LCLs) produced large amounts of IFN-γ in response to stimulation with these lymphoblastoid cells but not with TAK-LCLs, which shared no HLA antigens with the KIN-LCLs. The production of IFN-γ by
Figure 2. IFN-γ production by alloantigen-specific CD8⁺ T-cell lines in response to stimulation with allogeneic B-LCLs. CD8⁺ T-cell lines were generated from the PBMCs of the patients with FHL and healthy controls by stimulation with allogeneic B-LCLs (KIN-LCLs). Responder cells were cocultured with or without KIN-LCLs or TAK-LCLs, which shared no HLA antigens with KIN-LCLs, in the presence or absence of anti–HLA class I monoclonal antibody for 3 days. IFN-γ production was determined by ELISA; the results are mean values (± SD) from triplicate experiments. HLA types of B-LCLs were as follows: KIN-LCL, HLA-A01/30, B13/17, Cw6/9, DRB1*0701; TAK-LCL, HLA-A24/26, B62/6, Cw4/9, DRB1*0405/0901. NS indicates nonsense mutation; MS, missense mutation; and UPN, unique patient number.

Data on the cytotoxic activity of CD8⁺ alloantigen-specific bulk T-cell lines generated from FHL2 and FHL3 patients, their healthy parents, and 2 healthy controls are shown in Figure 3. CTLs generated from the FHL2 patients with PRF1 nonsense mutations (NSs; n = 2) were entirely deficient in antigen-specific cytotoxicity (percent cytotoxicity at E/T ratio of 10:1, mean 3.7 ± 1.1 SD), whereas those from the FHL3 patients (n = 2) showed very low but still detectable levels of this activity (mean 23.5 ± 2.1 SD). The cytotoxicity of CTLs generated from the patients with PRF1 nonsense mutations (MSs; n = 4) was high (mean 44.0 ± 4.2 SD) compared with those of CTLs generated from FHL3 patients and patients with PRF1 nonsense mutations but was clearly low compared with values for healthy controls and healthy parents with heterozygous mutations (mean 76.1 ± 4.7 SD). Although the patient sample in this comparison was small, statistical analysis indicated a significant difference in cytotoxicity between the FHL2 (NS)/FHL3 and FHL2 (MS) subgroups and between the FHL2 (MS) and the combined parent/healthy control groups (P < .001 for each comparison). Results similar to those in Figure 3 were obtained for alloantigen-specific CTL clones generated from the FHL patients and healthy controls (data not shown). The cytotoxicity of the CD8⁺ CTL lines generated against allogeneic cells appeared to be mediated via the perforin-dependent pathway, since treatment of CTLs with CMA, a potent inhibitor of the perforin-mediated granule exocytosis cytolytic pathway, resulted in nearly complete inhibition of T-cell–mediated cytotoxicity.

Western blot analysis for perforin and MUNC13-4 in the FHL2 and FHL3 subtypes

To account for the different patterns of CTL activity shown in Figure 3, we analyzed perforin expression in FHL patients whose CTL lines were established by Western blot analyses under reducing conditions (Figure 4A). Perforin in T cells from a healthy control and 2 FHL3 patients migrated at an apparent molecular mass of approximately 65 to 70 kDa. By contrast, the gene product associated with the PRF1 nonsense mutation 1090.91delCT, present in 3 patients (UPN17, UPN24, and UPN42; Table 2), migrated at approximately 55 kDa. The gene product associated with the 207delC mutation (UPN24) was predicted to be an 84–amino acid peptide and therefore was too small to be seen. Gene products resulting from PRF1 missense mutations 1228C>T (UPN42) and 1349C>T (UPN53 and UPN36) migrated at molecular masses similar to that of a healthy control, although the intensities of the bands appeared weaker compared with the control (Figure 4A). These results suggest that the mutant perforins were expressed in reduced amounts as full-length proteins with point mutations of R410W and T450M, respectively. Since a single band was also detected for UPN26 (a compound heterozygote with 949G>A and 1A>G; Table 2) at a position similar to the control, at least one of these mutants may be expressed as a full-length protein with a point mutation.

Figure 4. Perforin expression by Western blot analysis. Analysis is shown under reducing (A) or nonreducing (B) conditions in FHL patients. T cells from healthy control (C), FHL2 patients, and FHL3 patients were grown as described in “Generation of alloantigen-specific CTL lines and analysis of CTL cytotoxicity.” Cell extracts of these cells (20 µg each) were analyzed by Western blot with anti-perforin antibody. The data shown are representative results of 3 independent experiments. NS indicates nonsense mutation; and MS, missense mutation. The numbers above the lanes correspond to the UPNs of patients listed in Table 2.
To identify the active mature form of perforin generated from the precursor of this protein, we performed Western blot analysis under nonreducing conditions (Figure 4B), as in a previous report. Although the precursor forms of perforin (Figure 4B, upper bands) were detected in patients with PRF1 missense mutations, FHL3 patients, and a healthy control, the active mature protein (Figure 4B, the lowest band) was markedly reduced in the patients with PRF1 missense mutations, indicating that the proteolytic cleavage of perforin was inhibited. Under these conditions, the nonsense mutations (1090.91delCT and 207delC) did not yield a detectable band.

As shown in Figure 5, a single MUNC13-4 band of 120 kDa (expected size) was detected in a healthy control and all FHL2 patients but was missing in both of the FHL3 patients studied. Since the antibody used for the Western blot analysis was raised against the N-terminus of MUNC13-4 (1-262),19 it is likely that the MUNC13-4 protein, even its N-terminal portion, was not expressed in the T cells of either of these 2 FHL3 patients.

**Discussion**

This study compared the frequencies, clinical features, genotypes, and CTL/NK cell functions of the FHL2 and FHL3 subtypes of familial hemophagocytic lymphohistiocytosis. Since more than 80% of all FHL cases in Japan were analyzed, the proportions of patients with PRF1 or MUNC13-4 mutations are likely to reflect the true epidemiology of this disorder. The FHL3 subtype appears more common than the FHL2 subtype (25% vs 19%), although this difference may vary depending on ethnic background and other factors.9-13,29 Precise calculation of the frequency of each FHL subtype in countries outside of Japan is clearly needed.

FHL is sometimes difficult to diagnose when the patient is a first child or lacks an affected sibling. However, several clinical findings can support this diagnosis, including an age of less than 2 years, CNS involvement, and especially defective NK cell activity,5 which has been observed in a majority of patients.30-32 Comparison of NK cell activity among the FHL2, FHL3, and non-FHL2/FHL3 subtypes at diagnosis did not reveal any significant differences, as most of the evaluable cases were deficient or low in this activity. However, none of the FHL2 patients showed any recovery of NK cell activity after chemotherapy, in contrast to the FHL3 and non-FHL2/FHL3 subgroups. There was no apparent relationship between the recovery of functional NK cells and the type of chemotherapy received by these patients. Our findings agree with a recent report by Schneider et al,24 who classified patients into 4 types according to the level of NK cell activity. All cases with PRF1 homozygous nonsense mutations lacked any evidence of NK cell activity, regardless of the type of stimulation or the length of culture.24 Thus, PRF1 mutations appear to play an important role in the persistence of deficient NK cell activity in FHL.32 Whether the transience of this deficiency in the FHL3 subtype is associated with MUNC13-4 or still unidentified mutations, or perhaps a combination of the 2, remains to be determined. The partial recovery of NK cell activity may be associated with an increased number of NK cells or their release from the external suppressive effects of chemotherapy.

Perforin deficiency in FHL2 causes severe immune defects through defective NK and CTL activities.6,30,31 A lack of perforin expression in CD8+ T cells and/or NK cells by flow cytometric analysis generally correlates with mutated PRF1 sequences.10,13 However, Molleran Lee et al32 recently reported cases of late-onset FHL with PRF1 mutations but positive expression of perforin by flow cytometry. We identified 3 novel missense mutations of PRF1 in this study: 949G>A, 1228C>T, and 1349C>T (Table 2), and each of the affected patients, except UPN36, showed late-onset clinical manifestations. In contrast to a previous report,11 perforin expression by CD8+ or CD56+ cells in our study was also negative in all patients with PRF1 missense mutations. Feldmann et al32 characterized the genotype and phenotype of FHL2 patients; clinical and biologic analyses did not distinguish between patients

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**Table 2. Relation of genotype to the cytotoxic functions of NK cells and CTLs in patients with the FHL2 or FHL3 subtypes**

<table>
<thead>
<tr>
<th>Subtype and UPN</th>
<th>Age/sex</th>
<th>Mutations</th>
<th>NK activity at diagnosis, % (% after treatment)</th>
<th>CTLs, %*</th>
<th>Western blot (active protein)</th>
<th>Outcome</th>
<th>HSCT</th>
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</thead>
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<tr>
<td><strong>FHL2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>17</td>
<td>2 mo/F</td>
<td>1090.91delCT/1090.91delCT</td>
<td>2 (2)</td>
<td>3.6</td>
<td>Absent (p)</td>
<td>Dead</td>
<td>Y</td>
</tr>
<tr>
<td>24</td>
<td>1 mo/F</td>
<td>1090.91delCT/207delC</td>
<td>1 (2)</td>
<td>3.9</td>
<td>Absent (p)</td>
<td>Alive</td>
<td>Y</td>
</tr>
<tr>
<td>42</td>
<td>12 y/F</td>
<td>1090.91delCT/1228C &gt; T (mis)</td>
<td>2 (2)</td>
<td>41.2</td>
<td>Reduced (p)</td>
<td>Alive</td>
<td>N</td>
</tr>
<tr>
<td>53</td>
<td>7 y/F</td>
<td>1349C &gt; T (mis)/1349C &gt; T (mis)</td>
<td>0 (0)</td>
<td>46.5</td>
<td>Reduced (p)</td>
<td>Alive</td>
<td>Y</td>
</tr>
<tr>
<td>26</td>
<td>11 y/F</td>
<td>949G &gt; A (mis)/1A &gt; G (mis)</td>
<td>2 (2)</td>
<td>40.1</td>
<td>Reduced (p)</td>
<td>Alive</td>
<td>Y</td>
</tr>
<tr>
<td>36†</td>
<td>1 mo/F</td>
<td>1349C &gt; T (mis)/1349C &gt; T (mis)</td>
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<td>40.9</td>
<td>Reduced (p)</td>
<td>Alive</td>
<td>Y</td>
</tr>
<tr>
<td><strong>FHL3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>6 mo/M</td>
<td>754–1G &gt; C/754–1G &gt; C</td>
<td>10 (1)</td>
<td>23.1</td>
<td>Absent (m)</td>
<td>Alive</td>
<td>N</td>
</tr>
<tr>
<td>23</td>
<td>5 mo/M</td>
<td>1596–1G &gt; C/754–1G &gt; C</td>
<td>19 (12)</td>
<td>23.8</td>
<td>Absent (m)</td>
<td>Dead</td>
<td>Y</td>
</tr>
</tbody>
</table>

Mutations in both alleles are listed in the table.

NK indicates natural killer; CTLs, cytotoxic T lymphocytes; p, perforin; mis, missense mutation; m, MUNC13-4 protein; Y, yes; N, no; and UPN, unique patient number.

†FHL was diagnosed at 1 month of age, with relapse occurring 5 years later.
with PRF1 nonsense or missense mutations, although age at diagnosis was delayed in patients in the missense mutation group. It was also reported that several patients with late-onset disease had missense mutations of PRF1.31,34

In the FHL2 patients with PRF1 missense mutations, we detected a mutant form of perforin whose molecular mass was similar to that of the wild-type protein by Western blot analysis under reducing conditions; however, as shown in Figure 4B, there was no mature form of the perforin proteins in these cases. McCormick et al35 generated the 3-dimensional structure of the T435M PRF1 mutant using comparative molecular modeling techniques applied to data from a single FHL patient. Their results predicted that the ability of the protein to bind Ca2+, and hence its cytolytic function, would be strongly compromised. Katano et al36 demonstrated the accumulation of an uncleaved precursor form of perforin in a patient with a PRF1 missense mutation. Using Western blot analysis under nonreducing conditions we showed that the active mature form of perforin, the only form localized in lytic granules,15 was absent in PRF1 nonsense mutations and definitely reduced in all patients with PRF1 missense mutations. These findings suggest that the missense mutations of PRF1 lead to conformational changes in the perforin molecule that inhibit proteolytic cleavage of perforin precursors.

In our study, the cytotoxic function of CTLs in patients with PRF1 missense mutations remained relatively intact compared with that associated with nonsense mutations of this gene. By contrast, all patients with any type of PRF1 mutation showed a persistent deficiency in NK cell activity. Since the number and cytotoxic function of NK cells can vary depending on numerous factors, including the nature of the disease, infections, and type of treatment, measurements of NK cell activity in FHL2 cases may not accurately reflect the immune status of patients. We therefore established antigen-specific CTL lines from patients with the different subtypes of FHL and compared their cytotoxic activities. Since most of the patients with FHL are young children with an immunodeficient status, generating CTLs that are specific for viral or other recall antigens can be quite difficult; hence, we elected to generate alloantigen-specific CTL lines. The levels of cytotoxicity mediated by alloantigen-specific CTLs generated from patients with PRF1 nonsense or missense mutations or with MUNC13-4 mutations were clearly distinguishable from each other. Although many previous studies have relied on NK cells to examine cytolytic mediators in FHL, our findings suggest that functional analysis of antigen-specific CTLs may yield more useful insights into the immune competence of patients. Further studies with larger patient samples are needed to clarify the benefits of CTL assays in each subtype of FHL.

MUNC13-4 is a member of the Munc13 gene family, which is required for neurotransmitter release in the small nematode Caenorhabditis elegans.37 Feldmann et al8 demonstrated that the MUNC13-4 protein colocalizes with cysteine proteases near the site of contact between T lymphocytes and their targets and that granule exocytosis is blocked at a post-RAB27A stage in CTLs from FHL3 patients. We recently demonstrated that MUNC13-4 is a direct target of guanosine triphosphate–RAB27.19 MUNC13-4 deficiency also results in defective cytolytic granule exocytosis despite polarization of the secretory granules and docking with the plasma membrane.8 When we compared the cytotoxic activity of CTLs with PRF1 or MUNC13-4 mutations, the latter showed a lower affinity for target cells than did CTLs with PRF1 missense mutations. The MUNC13-4 protein was not detected by Western blot analysis, indicating that its function was completely eliminated in FHL3 patients. The difference in the cytotoxic activity of CTLs may reflect the functions of the molecules responsible for FHL pathogenesis: while MUNC13-4 is a regulator of exocytosis in perforin-containing vesicles, perforin is the killing tool itself.

The only accepted curative therapy for FHL is allogeneic HSCT. Although the outcome of therapy did not differ between patients with the FHL2 or FHL3 subtype, 1 child with a PRF1 missense mutation and 4 in the non-FHL2/FHL3 subgroup have survived without HSCT. Thus, early recognition of informative genetic defects and the cytotoxic activity of CTLs in cases of FHL may identify patients who could be spared the toxic effects of intensive chemotherapy with stem cell rescue.

Recent molecular genetic findings have demonstrated the diversity of FHL pathogenesis, but very little is known about the clinical and biologic correlations of the genetic subtypes of this disease. Although we observed a significant difference in age at onset and NK cell recovery between the FHL2 and FHL3 variants, further distinctions could not be made with any certainty. Nonetheless, the results of CTL assays performed on T-cell lines from a limited number of patients suggested an association with clinical features, type of mutation, and the subsequent production of functional proteins in FHL patients. If confirmed by studies of additional cases, the use of CTL assays to monitor the immune status of FHL patients might aid in the selection of therapy for cases without a documented genetic defect.

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Appendix

The members of the Japan HLH Study Group were as follows. Yukiko Tsunematsu, MD, Department of Hematology, National Children’s Hospital, Tokyo, Japan; Shuki Mizutani, MD, Department of Pediatrics, Tokyo Medical and Dental University, Tokyo, Japan; Naoko Kinukawa, Department of Pediatrics, Showa University, Tokyo, Japan; Hirokazu Kanegae, MD, Department of Pediatrics, Toyama Medical and Pharmaceutical University, Toyama, Japan; Keizo Horibe, MD, Clinical Research Center, National Nagoya Hospital, Nagoya, Japan; Ikuyo Ueda, MD and Akira Morimoto, MD, Kyoto Prefectural University, Kyoto, Japan; Naoki Sakata, MD, Department of Pediatrics, Kinki University, Osaka, Japan; Shinshaku Imashuku, MD, Department of Pediatrics, Takasago Seibu Hospital, Hyogo, Japan; Masaki Yasukawa, MD, Department of First Internal Medicine, Ehime University, Ehime, Japan; Shoichi Ohta, MD, and Toshiro Harai, MD, Department of Pediatrics, Kyushu University, Fukuoka, Japan; Nobuhiro Kimura, MD, First Department of Internal Medicine, Fukuoka University, Fukuoka, Japan; and Eiichi Ishii, MD, Department of Pediatrics, Saga University, Saga, Japan.
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Genetic subtypes of familial hemophagocytic lymphohistiocytosis: correlations with clinical features and cytotoxic T lymphocyte/natural killer cell functions

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