Development of Hemophagocytic Lymphohistiocytosis in Triplets Infected with HHV-8

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Running title: HLH in triplets infected with HHV-8

Scientific heading: Clinical Observations

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Abstract word count: 115
Total text word count (Introduction, Results & Discussion): 1,200
Total references: 20
Total Figures: 2

Key words: Hemophagocytic lymphohistiocytosis, natural killer cells, triplet, perforin, human herpes virus-8, flow-cytometry

Funding: This work was supported by the Children’s Miracle Network (FDG) and by NIH grant AI22795 (CG).

Financial Disclosures: The authors have no financial interests to disclose.
Abstract

Hemophagocytic lymphohistiocytosis (HLH) is a rare disorder of immune dysregulation, characterized by end-organ damage from lymphocytic infiltration and macrophage activation. All known mutations associated with the HLH occur in genes critical in the perforin/granzyme pathway. Herein, we report HLH occurring in two female triplet infants who also had associated human herpesvirus type 8 (HHV-8) infections. The subjects had identical novel compound-heterozygous mutations in the Perforin alleles, resulting in undetectable perforin expression and NK cell cytotoxicity. Both infants also had evidence of infection with HHV-8. These reports are to our knowledge the first cases of HLH in triplets, and the first reported cases of HHV-8 infection associated with HLH in non-renal transplant and non-HIV infected subjects.
Introduction

HLH is a rare disease in which the immune system becomes overactive due to its inability to effectively clear infections, and/or regulate its response to infections.\textsuperscript{1-3} Despite aggressive chemotherapeutic interventions, patients with HLH have a high mortality rate due to end-organ damage. All of the known mutations associated with HLH are in genes involved in the granule exocytosis pathway (e.g., Perforin, SAP, Munc13-4, Rab27a, CHS-1, and others), implicating the critical role of this pathway in controlling immune responses to infections.\textsuperscript{4-7} We report two cases of HLH in HHV-8 infected triplets, both having novel compound heterozygous mutations in the \textit{Perforin} gene and absent NK cell function. To our knowledge, this is the first report of triplets presenting with HLH, and are the first non-HIV and non-renal transplant subjects that have developed HLH in association with HHV-8 infection.
**Materials and Methods**

*Subject Samples.* Subject samples and informed consents were collected in accordance with protocols approved by the University of Iowa Hospitals and Clinics Human Studies Committee and Internal Review Board. Informed consents and procedures on all subjects were performed in accordance with institutional ethical standards and with the Helsinki Declaration of 1975, as revised in 2000.

*Light Microscopy.* Bone marrow aspirations were stained with standard May-Grunwald/Giemsa stain (Sigma). Light microscopy pictures were taken with a Nikon microscope (original magnification for all light microscopy pictures, 400X).

*Flow Cytometry Analysis.* Peripheral blood mononuclear cells (PBMC) were stained for surface and intracellular markers as previously described. Briefly, whole blood was stained with surface antibodies (CD4, CD8 and CD56; Becton Dickinson), then fixed, permeabilized (Cytofix/Cytoperm; Pharmingen), and stained with primary-conjugated anti-granzyme A (CB9; Becton Dickinson-Pharmingen), anti-granzyme B (GB12; Caltag), and anti-perforin (G9; Becton Dickinson-Pharmingen) antibodies. Samples were analyzed on a FACSCalibur (Becton Dickinson).

*Flow-Based Killing Assay.* A flow-based killing assay was performed as previously described. Briefly, K562 target cells were labeled with 125 nM of 5-(and-6-)carboxyfluorescein diacetate succinimidyl ester (Molecular Probes), and then mixed and plated with isolated subject PBMCs
at a 50:1 effector:target ratio. Duplicate samples were incubated for 6 hours. 1 µg/mL of 7-
amino-actinomycin (Calbiochem) was added to each sample before analysis. Negative (targets
only) and positive (normal control) samples were performed in parallel.

**Perforin Mutational Analysis.** The Molecular Genetics Laboratory at Cincinnati’s Children’s
Hospital (Dr. Alexandra Filiopovich) analyzed genomic DNA samples from each subject for
Perforin gene mutations through PCR and direct sequencing.

**Nested PCR for HHV-8.** Nested PCR for HHV-8 was performed as previously described. Briefly,
DNA was isolated from each subject sample (DNeasy; Qiagen), then the HHV-8 specific
336 bp ORF-26 region (nucleotide position 890 to 1226) was amplified by nested PCR using an
outside primer pair (5’-ATCTATCCAAAGTGCACACTGC-3’ and 5’-
CTGGGAACCAAGGCTGATAGG-3’) and an inside primer pair (5’-
GATGATCCCTCTGACAACCT-3’ and 5’-GGATCCGTGTTGTCTACG-3’). The product of
the nested PCR was resolved on a 1.5% agarose gel. The BC-3 and Mewo cell lines were used
for positive and negative controls, respectively.
Results and Discussion

Case Report-A

A four-month-old multiethnic girl (Triplet A; sister to triplets B & C) was transferred to our institution for persistent fevers and pancytopenia. Birth history was remarkable for delivery at 29 weeks, and Group B streptococcus septicemia at four weeks of age. Family history was unremarkable, and the parents were not related. Physical examination upon presentation revealed hepatosplenomegaly. Peripheral blood examination revealed a white blood cell (WBC) count of 3,400 cells/µL, hemoglobin (Hgb) of 5.2 g/dL, platelets of 37,000/µL, with an absolute neutrophil count (ANC) of 210 cells/µL, absolute lymphocyte count (ALC) of 2,500 cells/µL, and an absolute monocyte count (AMC) of 370 cells/µL. Abnormal chemistries included a triglyceride level of 151 mg/dL (normal: <150 mg/dL), ferritin of 2,529 ng/dL (normal: 10-290 ng/dL), and fibrinogen level of 222 mg/dL (normal: 230-390 mg/dL). Cerebral spinal fluid (CSF) evaluation revealed 5 WBCs/mm³. Bone marrow evaluation revealed hemophagocytosis (Figure 1A, a-c), without evidence of leukemia. An extensive infectious disease assessment was notable for a positive polymerase chain reaction (PCR) test for human herpesvirus-8 (HHV-8) from peripheral blood, but not from her CSF sample (Figure 2B). A clinical diagnosis of HLH was made based on Histiocyte Society Criteria (HSC), and she was started on the HLH-94 protocol consisting of etoposide, dexamethasone, and cyclosporine A.\textsuperscript{12} Immunophenotyping revealed a normal NK cell (CD56\(^{+}\)8\(^{-}\)) percentage (5%; normal: 2-14% for age) (Figure 1B), though \textit{in vitro} NK cell functional analysis showed no significant killing of K562 target cells (Figure 1C and 1D).\textsuperscript{3,8} Normal granzyme A and B expression was demonstrated in both NK and CD8\(^{+}\) T cells (Figure 1B; data not shown). Flow cytometry revealed no detectable perforin...
antibody reactivity (Figure 1B). Genetic analysis demonstrated novel compound heterozygous mutations in the *Perforin* gene (Figure 2A). Evaluation of her parents demonstrated their carrier status for the same *Perforin* gene mutations (Figure 2A). Triplet A responded to HLH-94 therapy with normalization of WBC counts and chemistries, and decreased bone marrow hemophagocytosis.

**Case Report-B**

Genetic testing on Triplet B (sister to Triplets A & C) revealed that she carried the same *Perforin* gene mutations as Triplet A (Figure 2A). She remained asymptomatic with normal laboratory evaluations until six months of age, when she developed respiratory symptoms with persistent fevers. Physical examination revealed an irritable infant with hepatomegaly. Peripheral blood examination showed a WBC count of 4,500 cell/µL, Hgb of 10.3 g/dL, platelets of 72,000/µL, an ANC of 1,300 cells/µL, an ALC of 2,600 cells/µL, and an AMC of 90 cells/µL. Abnormal chemistries included a ferritin of 2,105 ng/dL, prothrombin time of 13 seconds (normal: 9-12 seconds), alanine aminotransferase of 423 IU/L (normal: 3-30 IU/L), gammaglutamyl transpeptidase of 200 IU/L (normal: 1-39 IU/L), total bilirubin of 1.5 mg/dL (normal: 0.2-1 mg/dL), and a fibrinogen of 175 mg/dL. CSF evaluation showed a total of 1 WBC/mm³. Bone marrow evaluation revealed hemophagocytosis (Figure 1A, d-f). Virology assessment revealed a positive PCR test for HHV-8 DNA from her CSF (Figure 2B). Peripheral blood analysis revealed no circulating NK cells, and few granzyme A or granzyme B expressing cells (≤1%; Figure 1B). Similar to Triplet A, she had no detectable perforin antibody reactivity and minimal NK cell killing (Figure 1B and 1D). The clinical diagnosis of HLH was made, and she was started on HLH-94 therapy with subsequent improvement in her blood counts and serum chemistries.
Case Report-C

Triplet C (sister to Triplets A & B) presented for medical evaluation upon recognition of her two siblings (Triplets A & B) having HLH. Physical examination was unremarkable. All laboratory tests were within normal limits. Genetic analysis showed that she was a carrier of a single mutated Perforin allele, similar to her father’s mutation (Figure 2A). She has required no further medical intervention.

These case reports are to our knowledge the first cases of HLH occurring in a set of triplets. There have been two previous reports of Perforin gene mutations in twins.13,14 Twins reported by Lipton et al. presented in the newborn period with symptoms consistent with HLH.14 Genetic analysis demonstrated a single missense mutation in the Perforin gene in only one of the twins and their mother. It was presumed that each twin inherited an additional genetic mutation in the Perforin gene from their father; however, this point was not documented.14 Busiello et al. reported on two fraternal twins having the same homozygous Perforin mutations.13 Despite both twins having the same Perforin mutations, only one reportedly developed HLH. The unaffected twin reportedly demonstrated normal NK cell activity.13

In our study, the affected triplets presented within 12-weeks of each other following a viral prodrome. HHV-8 infection was documented by PCR amplification in both subjects using a previously published technique.10 HHV-8 was noted in the peripheral blood of Triplet A, but not her CSF, while Triplet B only had HHV-8 detected in her CSF. These differences are probably related to the timing of sample collections from each of the two subjects. The source of the HHV-8 infection is difficult to discern; however, since a small percentage of healthy
American adults are HHV-8 seropositive, one possibility is transmission via a blood product while in the nursery. Although HLH has been previously associated with HHV-8 infection, all of these reports have been in HIV infected individuals with associated Kaposi’s sarcoma, or renal transplant patients.

Genetic analysis demonstrated that triplets A and B had the same compound heterozygous mutations in the *Perforin* gene. Neither subject demonstrated intracellular perforin expression by flow cytometry. Although certain perforin mutations may not allow for antibody reactivity, functional analysis of both subjects also demonstrated no significant NK cell cytotoxicity, indicating that the compound heterozygous mutations result in non-expressed and/or non-functional perforin. Similar to Busiello et al., there were also phenotypic differences between each affected triplet in our study, including time of onset of disease, as well as differences in the percentage of circulating NK cells, and cellular granzyme A and B expression. These results suggest that despite having the same genetic mutations, there are significant polygenetic influences in the development of HLH.

This report and others demonstrate the critical importance of the perforin/granzyme pathway in NK cell function and viral clearance. Moreover, it stresses the importance of this pathway in regulating immune responses to viral infections. We have recently reported that human T regulatory cells also utilize the perforin/granzyme pathway. We demonstrated that both adaptive and natural human T regulatory cells are able to ‘suppress’ autologous naïve T cell proliferation by killing them through the perforin/granzyme pathway. These reports support the important role that this pathway plays in the immune dysregulation found in HLH. Specifically, they demonstrate that the perforin/granzyme pathway is not only important in the initial immune response to viral infections, but that it is also a pathway used in regulating or
‘suppressing’ the immune response during the contraction phase via the CD4⁺ T regulatory compartment.
References


Acknowledgments: Cincinnati Children’s Research Foundation Molecular Genetics Laboratory (moleculargenetics@cchmc.org) performed all Perforin gene mutation analyses.
Figure Legends

**Figure 1.** Hemophagocytosis and lack of perforin detection and NK cell cytotoxicity in Case Reports-A and -B. A) Bone marrow evaluation of Twin A (Case Report-A; a-c) and Twin B (Case Report-B; d-f) showing hemophagocytosis of bone marrow elements (e.g., red blood cells and neutrophils) by histiocytes. All pictures are from May-Grunwald/Giemsa stained slides, and are shown at 400X magnification. B) Perforin and granzymes A and B antibody reactivity in CD56^+8^- NK cells from control (top panels), Triplet A (middle panels), and Triplet B (bottom panels). Percentage of positive cells is shown in each quadrant. C) Representative flow-based cytotoxicity results demonstrating NK cell-induced apoptosis (FSC<sub>lo</sub>/7-AAD<sub>hi</sub>) of MHC class I negative target cells (K562) in control, Triplet A, and Triplet B. Percentage of apoptotic target cells is shown in the R3 gate of each panel. D) Summary of NK cell killing, demonstrating the average percentage of apoptotic target cells detected in the R3 gate for each subject. Effector to target (E:T) ratio was 50:1 for all samples. Values shown are the mean ± S.D. **P<0.01, NS = not significant.

**Figure 2.** *Perforin* gene mutations found in the family cohort, and PCR analysis for HHV-8 DNA in Case Reports-A and –B. A) *Perforin* gene nucleotide mutations detected in the genomic DNA from each subject. Predicted protein product resulting from each *Perforin* gene mutation is described. B) PCR analysis of buffy coat and CSF samples from affected triplets for HHV-8. Specific PCR product for ORF-26 of HHV-8 is shown (336 bp product; arrow).<sup>9</sup> Lane 1: Triplet B – CSF sample, Lane 2: Triplet A-buffy coat sample, Lane 3: Mewo cell line (negative control), Lane 4: BC3 cell line (positive control), Lane 5: DNA bp ladder.
Figure 1
A) 

**Perforin** gene mutations found in the reported kindred.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Perforin Allele Mutations</th>
<th>Predicted Protein Product</th>
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| Triplet A | 1) 916 G>T  
2) 1190 1191 ins TG | 1) missense mutation  
2) premature stop codon and termination of transcription |
| Triplet B | 1) 916 G>T  
2) 1190 1191 ins TG | 1) missense mutation  
2) premature stop codon and termination of transcription |
| Triplet C | 1) 916 G>T  
2) none | 1) missense mutation  
2) normal protein |
| Mother | 1) none  
2) 1190 1191 ins TG | 1) normal protein  
2) premature stop codon and termination of transcription |
| Father | 1) 916 G>T  
2) none | 1) missense mutation  
2) normal protein |

B) 

**Figure 2**
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