Large granular lymphocyte proliferation and revertant mosaicism: two rare events in a Wiskott-Aldrich syndrome patient

We report on a 6 year old patient with an unusual clinical presentation of WAS and oligoclonal proliferation of TCRγδ⁺ large granular lymphocytes (LGL). Flow cytometry demonstrated two distinct populations of lymphocytes with strongly decreased (WASP⁻) or normal expression levels of WASP (WASP⁺), respectively. Molecular analysis confirmed a splice site mutation in intron 2 of the WASP gene in the WASP- cells but not in WASP⁺ cells. LGL cells were WASP⁺, suggesting that two independent rare events, somatic revertant mosaicism and LGL expansion, have occurred in a child with WAS. Our report points to diagnostic difficulties in the presence of partial WASP reversions and LGL.

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Introduction

Expansion of large granular lymphocytes (LGLs) can be observed in a wide spectrum of different clinicopathological scenarios, ranging from benign polyclonal expansion to aggressive clonal disease. LGL lymphoproliferation is associated with viral infections, autoimmune diseases, malignancies, immunodeficiencies and immunosuppression after organ transplantation. The clonal form of LGL expansion, termed LGL leukemia, commonly occurs in adult patients beyond 55 years of age and is a rarity in children.[1] Wiskott Aldrich syndrome (WAS) is a rare primary immunodeficiency disorder characterized by immunodeficiency, eczema, autoimmune phenomena and susceptibility to malignant lymphoma. *WASP*, the defective gene in this disorder, is a key regulator of actin polymerization.[2] Recently, several cases of somatic revertants have been reported leading to somatic mosaicism for *WASP* in T or NK cells.[3-6] We here report on a 6 year old child with oligoclonal expansion of LGLs and revertant mosaicism for *WASP* in T cells.

Design and Methods

Patient and controls. The material from the patient, his mother, and healthy donors was obtained with informed assent/consent in accordance with the Declaration of Helsinki.

Flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient separation. For immunophenotypic analysis, cells were stained using the following antibodies: anti-CD3-FITC and –APC, anti-CD8-APC, anti-HLA-DR-PE-Cy7 (all from BD Biosciences, Heidelberg, Germany), anti-CD4-PE-Cy7, anti-CD16-FITC, anti-CD56-PE-Cy5, anti-CD57-FITC (all from Beckman Coulter, Krefeld, Germany), and anti-TCRγδ-PE (Caltag/Invitrogen, Karlsruhe, Germany). Intracytoplasmic WASP staining in PBMCs was performed using anti-WASP antibody (clone B-9; Santa Cruz Biotechnology, Heidelberg, Germany). Identification of leukocyte subsets was performed using the following antibodies: anti-CD3-APC, anti-CD4-PE-Cy7, anti-CD8-PE, anti-CD19-PE-Cy7 (Beckman Coulter), anti-CD56-PE, and anti-CD14-APC (Serotec, Düsseldorf, Germany). *WASP* expression analysis in thrombocytes was performed on was performed in anti-CD41-PE (BD Biosciences) counterstained platelets from thrombocyte-rich plasma. Flow cytometric analysis was performed using a FACSCanto flow cytometer. Subsequent data analysis was performed using FlowJo software.

Mutation analysis. Genomic DNA was extracted from hair, nail, urine and mouth epithelial cells obtained from the patient or from patient PBMCs after flow cytometric sorting on a FACSAria cytometer. PCR reactions using appropriate primers were employed to amplify all 12 exons of the *WASP* gene including intron-exon boundaries.

Clonality analysis. DNA was extracted from bone marrow aspirate and PBMCs, specific primer pairs were used to amplify the different V_ and V_ gene fragments by PCR, and the products were analysed using denaturing gel electrophoresis.

CMV reactivity of PBMCs. PBMCs were stimulated using HLA-matched CMV peptide (NLV) linked tetramers, as described previously.[7]

Chimerism analysis. Chimerism analysis was performed using microsatellite markers as described previously.[8]

Results and discussion. The patient is a 6 year old boy who was referred to us at the age of 4 years with a history of chronic respiratory tract infections, pulmonary and skin abscesses with *H. influenzae*. He had suffered from a neonatal CMV infection and intermittent thrombocytopenia (ranging from 5 to 74/nl; mean platelet volume 6.5 to 9.0 fl), leading to a presumptive diagnosis of ITP. Immune assessment revealed normal levels of immunoglobulin levels but decreased levels of specific antibodies against *H. influenzae* and *S. pneumoniae* (0.86 and 14.28 mg/l, respectively). An episode of acute cerebral hemorrhage occurred at the age of 4.5 years. At this time, his platelet count was 3/nl. Splenectomy was performed and resulted in increased thrombocyte counts of 150-170/nL. Since then, one episode of thrombocytopenia with anti-thrombocyte antibodies has occurred which was treated successfully using corticosteroids and IVIG. Recurrent infections with *H. influenzae* prompted immunophenotypic analysis of PBMCs. An abnormal population comprising around 35% of lymphocytes was identified. These cells were marked by a characteristic immunophenotype ($CD3^{low}TCR\gamma\delta^{low}$), distinct from normal TCRγδ-positive T lymphocytes (Figure 1a). A peripheral blood smear from the patient showed increased numbers of LGLs with typical morphology (Figure 1b). Further analysis characterized the LGL cells in this patient as $CD3^+CD4\text{-}CD8^+CD16^+CD56^-CD57^+HLA\text{-}DR^+$, consistent with a T-LGL phenotype (Figure 1c). Molecular analysis of the TCRγδ chain revealed the presence of 2 different clones in peripheral blood, in agreement with an oligoclonal nature of the LGL cell expansion (data not shown). Chromosomal analysis and bone marrow examination did not show any abnormalities. The working diagnosis of T-LGL expansion was supported by highly elevated levels of TNF-α and IL-1_ in resting and stimulated T-cells, as shown by ELISA and RT-PCR (data not shown). Although the clinical findings, including humoral immunodeficiency and thrombocytopenia, might be compatible with a diagnosis of LGL expansion, we also considered WAS as a differential diagnosis and stained PBMCs for WASP. Interestingly, flow cytometry showed the presence of $WASP^+$ and $WASP^-$ cells among PBMCs. Sequence analysis of sorted $WASP^+$ and WASP- lymphocyte subpopulations revealed a previously described splice site mutation in intron 2 (IVS2-1 g>a) of the *WASP* gene[9] in the WASP- cells but wildtype sequence in $WASP^+$ lymphocytes (Figure 2a), confirming hematopoietic mosaicism in this patient. Leukocyte subset-specific analysis demonstrated that mosaicism was restricted to $CD3^+CD4^+$ lymphocytes and $CD3^+CD8^+$ lymphocytes, where around 65% and 25% of

Figure 1. Expansion of large granular lymphocytes. (A) Flow cytometry of LGLs (CD3lowTCRγδlow T cells). (B) Peripheral blood smear showing two LGLs with characteristic morphology. (C) Flow cytometric assessment of cell surface markers on LGLs.

cells were WASP$^+$, respectively. In contrast, no WASP+ cells were found among B lymphocytes, NK cells or monocytes (Figure 2b). In light of the patient's history of severe bleeding diathesis, we hypothesized that no reversion would be seen in platelets. In fact, WASP was absent in patient thrombocytes, while detectable levels of WASP were found in thrombocytes from both a healthy donor and the patient's mother, as shown by flow cytometric analysis (Figure 2c). The patient's mother was found not to be a carrier for WAS (*data not shown*). Maternal engraftment of WASP$^+$ cells was excluded by hematopoietic chimerism analysis (*data not shown*). To determine whether the *de novo WASP* mutation had occurred oocytes or in early embryogenesis, we analyzed DNA material from extrahaematopoietic tissues including mouth epithelium, hair, nail and urothelial cells and found the same mutation in the *WASP* gene as in the WASP$^-$ cell fraction of PBMCs. Taken together, our results suggest that the patient carries a *de novo* mutation in the *WASP* gene with *in vivo* reversion in T lymphocytes. In an attempt to further elucidate the association between WAS and LGL cell expansion, we speculated that the LGLs might be WASP$^+$ and thus have proliferative advantage in a WASP-deficient host, as described previously for gene-corrected T lymphocytes.[4,6,10] However, the entire population of LGL cells carried the known mutation in the *WASP* gene (*data not shown*). A cryoconserved bone marrow sample from the age of 13 months was used to analyze whether the occurrence of LGLs may have preceded the hematopoietic mosaicism or vice versa, but our analysis showed that neither WASP revertant cells nor LGLs could be detected (data not shown). Finally, we hypothesized that the severe, systemic CMV infection at the age of 13 months may have triggered LGL expansion, as described previously for CMV[11] or other viral infections.[12] Stimulation of PBMCs with CMV-specific tetramers showed that a fraction of 0.5% of CD8$^+$ cells were reactive to the tetramers, in line with a previous exposure to CMV. Wiskott-Aldrich syndrome is a rare, complex primary immunodeficiency disorder with an estimated frequency of 1:250,000.[2] More recently, a limited number of cases of somatic mosaicism have been described for Wiskott-Aldrich syndrome, where an *in vivo* reversion led to correction of a significant proportion of patient T and/or NK cells.[3-6] The notion that reversion of the genetic defect in a few cells leads to partial restoration of the clinical phenotype and an accumulation of WASP-expressing cells lends strong support to the hypothesis that WASP expression confers a selective advantage for cells of the lymphoid compartment. Similar to the studies cited above, we found significant numbers of genetically reverted leukocytes exclusively amongst lymphocytes. Nonetheless, these findings underline a potential selective advantage of WASP-positive cells over WASP-negative cells at least for cells of the lymphoid compartment and have important implications for the potential success of gene therapy trials for WAS.[13] Expansion of LGLs can be seen in a variety of clinicopathological scenarios, yet the underlying mechanisms remain largely unknown. It has been suggested that chronic stimulation of CD8$^+$ T lymphocytes with an autoantigen or viral antigens may be initial stimuli leading to expansion of LGLs.[1] Viral infections including CMV, EBV and HTLV have been implicated in the pathogenesis of LGL expansion.[1,11] Studies have also underlined the association of LGL leukemia with autoimmune disorders including rheumatoid arthritis,[1] and more recently, proliferation of LGLs has been linked to immunodeficiency disorders such as Hyper-IgM-syndrome[14] or common variable immunodeficiency.[15] Although the precise mechanism responsible for LGL expansion in this child remains unclear, one could attrib-

Figure 2. Revertant mosaicism for WASP. (A) Genomic sequencing results on sorted WASP+ and WASP- cell populations. (B) FACS analysis of peripheral blood mononuclear cells. Histograms represent intracytoplasmic WASP staining (filled lines) compared to isotype staining (open lines) in gated subpopulations. (C) FACS analysis of thrombocytes. Thrombocytes were identified by FSC SSC properties and counterstained with anti-CD41 antibody. Histograms represent intracytoplasmic WASP staining (filled lines) compared to isotype staining (open lines).

ute this to a preceding systemic CMV infection during early childhood. The observation that infections with CMV can lead to preferential expansion of TCRγδ T cells in immunocompromised patients would be in agreement with this hypothesis.[16] Alternatively, the immunodysregulation in WAS may constitute a predisposing factor for the development of LGL lymphoproliferation, similar to the situation in other patients suffering from primary immunodeficiencies[14] or autoimmune disorders.[1] Taken together, we report for the first time an association between WAS and the expansion of large granular lymphocytes. Furthermore, this case adds to the growing knowledge on *in vivo* reversion events in Wiskott-Aldrich syndrome.

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