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The 3rd Neutropenia Network Conference
Clinical and Laboratory Investigation
Cellular and Molecular Biology

Heraklion, Greece, September 26-27, 2008

Guest Editors
Helen A. Papadaki, Jan Palmblad

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# The 3rd Neutropenia Network Conference

**Clinical and Laboratory Investigation**  
**Cellular and Molecular Biology**

**Heraklion, Greece, September 26-27, 2008**

*Guest Editors*  
*Helen A. Papadaki, Jan Palmblad*

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The organizers would like to thank Genesis Pharma SA for its contribution
The molecular basis of cyclic neutropenia

D.C. Dale
Professor of Medicine, University of Washington, USA

Cyclic neutropenia is a unique hematological disorder characterized by recurrent severe neutropenia, mouth ulcers, infections and fever occurring at regular three week intervals. In many patients other blood cells also cycle with the same periodicity. The neutrophil cycle is due to periodicity in neutrophil formation, i.e., interruptions followed by surges of new cell formation associated with increased colony stimulating activity in the urine and plasma. Morphological studies of both the marrow and blood show a paucity of mature neutrophils during the neutropenic period with normalization during recovery. Even during the neutropenic phase, recognizable neutrophil precursors, i.e., myeloblasts and promonocytes are seen in marrow samples. In vitro cultures of marrow cells (colony assays) have shown that marrow progenitors from patients with cyclic neutropenia have an impaired response to the hematopoietic growth factors: granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3) and stem cell factor (SCF) and various combinations of these factors. Studies using annexin V and other methods show that cell formation is impaired by accelerated apoptosis of progenitors and ineffective production of neutrophils. The regular oscillations in blood counts are primarily attributable to the increased rate of apoptosis of neutrophil precursors which destabilizes the hematopoietic stem cell compartment.

Cyclic neutropenia is inherited as an autosomal dominant disorder. Most cases are attributable to mutation in the gene for neutrophil elastase or ELA-2, a protease normally found in the primary granules of neutrophils. Normally this enzyme is synthesized in the endoplasmic reticulum, transported through the Golgi and processed in the granules, although some may also be constitutively secreted. Expression of mutant ELA-2 initiates the unfolded protein response (UPR), leading to accelerated apoptosis of patients’ cells and cells transfected with the mutant gene. The magnitude of the UPR appears to correlate with the severity of neutropenia and may account for the phenotypic differences between cyclic and congenital neutropenia. The effectiveness of treatment with recombinant G-CSF may be directly related to its inhibition of apoptosis triggered by the UPR. There are many interesting and unanswered questions about cyclic neutropenia:

1. How many CBCs are needed to make the diagnosis of cyclic neutropenia?
2. Does cycling of the blood neutrophil count begin at birth? If not, when does it begin and how does it vary over a lifetime?
3. In families with multiple affected members having the same mutation, do they all cycle similarly? If not, why not?
4. Can cyclic neutropenia now be diagnosed by genetic tests-sequencing the ELA-2 gene?
5. What is the best treatment for cyclic neutropenia? If G-CSF is used, what is best dose and schedule?
6. Are cyclic neutropenia patients at risk of transformation to myelodysplasia and leukemia? If so, how should they be monitored? If not, what should they be told?

References

Severe congenital neutropenia (CN) is a heterogeneous group of inherited disorders. A number of defined genetic defects have been described recently. These investigations allow a refined genetic diagnosis of patients with congenital neutropenia. The identification of novel genetic defects sheds light on important pathways that control differentiation and homeostasis of neutrophil granulocytes. In addition, basic principles of cell biology and metabolic control of cellular differentiation are now being elucidated. In this presentation, an overview of diverse genetic subgroups of congenital neutropenia will be presented. Starting from the clinical presentation, the principles of genetic diagnosis will be covered. The biological role of various molecules implicated in the pathophysiology of congenital neutropenia will be discussed. Additional authors: Karl Welte, Cornelia Zeidler, Kaan Boztug.

SEVERE CONGENITAL NEUTROPENIA: MOLECULAR PATHOGENESIS AND GENETICS

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Severe congenital neutropenia (CN) is a heterogeneous bone marrow failure syndrome characterized by a maturation arrest of granulopoiesis at the level of the promyelocytes/myelocytes with no mature neutrophilic granulocytes in the peripheral blood. Even though G-CSF treatment results in increased neutrophil numbers in more than 90% of CN patients, G-CSF-dependent granulocytic differentiation is severely affected in these patients. Thus, the levels of biologically active G-CSF in serum as well as G-CSF receptor (G-CSFR) expression on myeloid cells are elevated in CN patients, but neutrophil counts increased only after daily injections of pharmacological doses of G-CSF (100–1000 times higher than physiological levels). There are two major subtypes of CN, as judged by inheritance: 60% of patients harbor autosomal dominant ELA2 mutations and in appr. 20% of patients autosomal recessive mutations in HAX1 gene have been identified. This genetic heterogeneity suggests that several pathologic mechanisms may lead to the same phenotype (bone marrow morphology, response to G-CSF, etc.) due to downregulation of common myeloid transcription factors. Recently, we found that lymphoid enhancer factor 1 (LEF-1) is a decisive transcription factor in induction of granulopoiesis, which directly up-regulates C/EBPa.1 Downregulation of LEF-1 and its target gene C/EBPa is a common pathomechanism of CN irrespective of mutation status. In addition to C/EBPa, C/EBPb is a granulocyte-specific transcription factor. However they play different roles in granulopoiesis: C/EBPa is responsible for “steady-state” granulopoiesis and C/EBPb is crucial for cytokine-induced “emergency” granulopoiesis.2 Since C/EBPb is expressed at the normal levels in CN, we hypothesize that in CN patients, treatment with G-CSF activates C/EBPb-dependent “emergency” pathway of granulopoiesis only leading to marginal maturation of neutrophils, whereas the “steady-state” regulation of neutrophil granulopoiesis by LEF-1 and C/EBPa does not function due to a lack of LEF-1. Previously, we have shown that G-CSF levels in plasma as well as G-CSFR expression on myeloid cells of CN patients are elevated, but not sufficient for “normal” granulocyte production in these patients. This supports our hypothesis that high dosages of G-CSF lead to activation of C/EBPb-triggered “emergency” granulopoiesis, independent of LEF-1/C/EBPa. CN is considered as a preleukemic syndrome and the cumulative incidence for leukemia is more than 25% after 20 years of observation. Since AML/MDS are not observed in cyclic (CyN) or idiopathic neutropenia patients treated with G-CSF, an underlying defect downstream of G-CSFR signaling rather than G-CSF therapy per se predisposes to malignant transformation in CN. Acquired G-CSFR mutations are detected in approximately 50% of CN patients who developed AML, suggesting that these mutations have a growth advantage over wild type cells when exposed to G-CSF in vivo due to activation of factors/signaling pathways, known to be involved in leukemogenesis.3 We found that G-CSF induced a strong phosphorylation of STAT5a in hematopoietic progenitors of CN patients, as compared to patients with another types of neutropenia and to healthy individuals. Interestingly, activated STAT5a binds directly to the promoter of LEF-1 gene and inhibits its expression by disturbing the LEF-1-autoregulatory loop in a dose-dependent manner.4 These intracellular events may contribute to the malignant transformation in CN and may help to understand the mechanisms of “normal” granulopoiesis and leukemogenesis in general.

References
Severe chronic neutropenia disorders represent a group of inherited bone marrow failure syndromes causing severe neutropenia and recurring severe at times life-threatening infections. These congenital disorders, some of which undergo malignant transformation, exhibit overlapping but distinct clinical and molecular phenotypes and include cyclic neutropenia, severe congenital neutropenia, WHIM syndrome or myelokathexis, Shwachman-Diamond syndrome, and Barth syndrome. Patients with cyclic neutropenia (CN) exhibit characteristic regular oscillations of blood neutrophils from zero to near normal levels with ~21-day periodicity.1,2 The molecular defect of CN is attributed to heterozygous mutations in the neutrophil elastase (NE or ELA2) gene encoding a serine protease expressed in myeloid progenitor cells.3,4 Severe congenital neutropenia (SCN) with consistently low neutrophil levels and a characteristic block of myeloid differentiation is mainly due to either heterozygous NE mutations (autosomal dominant form) or homozygous mutations in the HAX-1 gene (autosomal recessive Kostmann syndrome).5 SCN can also be due to heterozygous mutations in the WAS gene (X-linked SCN),6 mutations in GFI-1 (rare autosomal dominant SCN) or other genes.7,8 Myelokathexis or WHIM syndrome is characterized by a hypercellular bone marrow and severe neutropenia and lymphopenia in the peripheral circulation. The molecular defect of this autosomal dominant disorder is attributed mainly to heterozygous mutations in the CXCR4 gene, a membrane receptor for stromal-derived growth factor (SDF-1).9 Most of severe neutropenia patients with Shwachman-Diamond syndrome harbor mutations in the SBDS gene that cause impaired ribosomal function and FAS-hypersensitivity. Barth syndrome patients are characterized by cardio- and skeletal myopathy and severe neutropenia, which are due to the loss of function mutations in the tafazzin (TAZ) gene, an acyltransferase involved in remodelling of cardiolipin of the inner mitochondrial membrane.10,11 We and others reported that most of these congenital neutropenia syndromes are characterized by accelerated apoptosis of bone marrow-derived CD34+ stem cells and/or more differentiated myeloid cells.12,13 Furthermore, recent studies demonstrated that expression or knock-down of the above mentioned disease-specific mutant gene products, albeit employing various molecular mechanisms, results in accelerated apoptosis of human myeloid cells, impaired production of differentiated neutrophils, resulting in the lack of circulating neutrophils and subsequent severe neutropenia.14,15,16,17 Thus, accelerated apoptosis of human myeloid cells and impaired myeloid differentiation, triggered by different disease-specific gene mutations, is the common and unifying cellular mechanism underlying the pathogeneses of congenital neutropenia disorders and their malignant evolution. Current studies are focused on elucidation of disease-specific molecular mechanisms and screening/identification of potentially novel therapeutic agents capable of restoring normal phenotype.

References

FUNCTIONAL STUDIES ON THE SBDS PROTEIN IN RELATION TO NEUTROPHIL DEFECTS IN SHWACHMAN-DIAMOND SYNDROME

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Shwachman-Diamond Syndrome (SDS) is a rare inherited disease with a wide range of manifestations because of mutations in the SBDS gene. Defects of the hematopoietic system, exocrine pancreas dysfunction and short stature are most prominent features in SDS. To gain more understanding of the molecular properties of the ubiquitously expressed SBDS protein, we examined the intracellular localization and mobility of the protein. SBDS full-length protein is localized in both the nucleus and cytoplasm, where it is found to co-localize in part with various cytoskeletal elements which may contribute to cell cycle as well as directed cell motility. In contrast, SBDS mutant proteins localize more prominently to the nucleus. Life imaging studies has been useful to study the nucleo-cytoplasmic trafficking of SBDS and mutant proteins, supporting that normal SBDS is indeed stabilized by its localization in larger protein complexes. Our structure-function analysis provides new insight into the dynamics of the SBDS full-length protein and shows that mutant SBDS proteins are altered in their molecular properties, that may well contribute to the clinical features observed in SDS patients.

WHIM SYNDROME: A DISEASE OF CXCR4 HYPERFUNCTION

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WHIM syndrome is an autosomal dominant inheritance immune deficiency caused by a gain in function mutation in CXC chemokine receptor 4 (CXCR4). The name of the syndrome is an acronym derived from major features of the disorder that include, but are not limited to Warts, Hypogammaglobulinemia, recurrent bacterial Infections and Myelokathexis (apoptosis of mature myeloid cells in the marrow). Most, but not all, patients with WHIM syndrome are heterozygous carriers of mutations of CXCR4 characterized by partial truncations of the carboxyterminal segment of this receptor (reported kindreds include R334X, S339fsX342X, E343X, and G335X). CXCR4 is the main receptor for CXCL12 chemokine, also known as stromal cell-derived factor 1 (SDF1). CXCR4 is a G-protein-coupled receptor (GPCR) possessing the canonical seven transmembrane helical domains and cytoplasmic C-terminus elements essential for feedback down-regulation of receptor activity. We and others have shown that c-terminal truncation of CXCR4 results in retention of receptor activation function, but partial loss of the ability of the cell to down-regulate the receptor. The net result is an increase and prolongation of signaling (the gain in function) by this receptor in response to SDF1. We have shown that the cell physiology consequences of this WHIM-type mutant CXCR4 gain in function following SDF1 stimulus is enhanced/prolonged intracellular ionized calcium rise, reduction in endocytic internalization of receptor with more rapid recovery, and enhanced chemotactic migration. The clinical presentation of WHIM is variable though generally comes to attention and correct diagnosis because of severe problems with human papilloma virus (HPV) related warts, condylomata, and cervical dysplasia. These lesions often progress to carcinoma and can be a cause of early mortality in these patients. Patients may also suffer from recurrent bacterial pneumonias that can lead to chronic bronchiectasis. A variety of other types of bacterial infections have been reported. It is of note that WHIM patients do not appear to be at particular risk from virus infections other than HPV, suggesting a unique host defense defect in WHIM that is quite specific to HPV. Laboratory abnormalities in WHIM include low serum immunoglobulins and moderate to severe chronic neutropenia. Bone marrow examination paradoxically may show myeloid hyperplasia, but with excessive numbers of apoptotic neutrophils. Patients are managed with prophylactic antibiotics and chronic administration of G-CSF and intravenous gamma globulin. The mechanism by which hyperfunction of CXCR4 leads to excessive infection with HPV is not known, but it is of interest that CXCR4 is normally found on keratinocytes where it may in part regulate proliferation, and SDF1 is produced by stromal elements in the dermis. It is possible, though needs to be proven experimentally, that HPV infection uses the SFF1/CXCR4 axis in skin to enhance extent of infection. B lymphocytes express CXCR4, though it is not known how hyperfunction of CXCR4 leads to impairment of immunoglobulin production, and should be an area of study. We have performed a series of experiments in which we introduced retrovirus vector transduction excess WHIM-type mutant CXCR4 into human CD34 hematopoietic stem cells (HSC) and engrafted them into NOD/SCID mice which accept this human xenograft. The consequence of expression of the WHIM-type mutant CXCR4 in human HSC is an extraordinary 3- to 6-fold increase in engraftment in this xenograft model, demonstrating a potent and central role for CXCR4 in HSC trafficking. Alteration in trafficking of HSC in WHIM patients is predicted from this model, but such studies in WHIM patients have not been reported. Mature human neutrophils arising from the mutant CXCR4 transduced HSC in the xenograft appear to be retained in the marrow where they undergo apoptosis rather than being released normally to the circulation. However, WHIM-type CXCR4 transduced human HSC retained in tissue culture and differentiated into neutrophils in vitro show no excessive apoptosis. Our data suggest that changes in CXCR4 function or expression in neutrophils likely
plays an important role in release of neutrophils from the marrow. We hypothesize that WHIM neutrophils are retained in the patient marrow beyond the normal time for release into the circulation. There they progress to apoptosis after the normal lifespan, but within the marrow rather than in the circulation and peripheral tissues. This would explain the paradoxical myeloid hyperplasia with myelokathexis typical of WHIM syndrome. Given that many of the clinical problems affecting WHIM may be a consequence of hyperfunction of CXCR4, we propose that chronic treatment of WHIM patients with a potent inhibitor of CXCR4 function such as Plerixafor (MOBOZIL®; AMD3100) should be studied in an experimental clinical protocol.

**NEUTROPENIA ASSOCIATED TO PRIMARY IMMUNODEFICIENCIES**

A. Durandy

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Besides congenital and auto-immune neutropenia, a decrease in the absolute number of circulating neutrophils can be observed in several deficiencies affecting adaptive immunity, and especially in primary antibody deficiency disorders. It is a common and severe complication of the X-linked CD40L-deficiency, the common variable immunodeficiency (CVID) and the X-linked agammaglobulinemia (btk-defect).

**X-linked CD40L deficiency.** CD40L-deficiency is a combined T and B lymphocyte functional deficiency characterized by deleterious mutations in CD40L gene. CD40L is transiently expressed on activated T helper cells and its interaction with CD40, constitutively expressed on B lymphocytes and dendritic cells, is required for IgG, IgA and IgE production and efficient T cell responses. Defect in CD40L leads therefore to an humoral deficiency associated to a cellular deficiency. Intermittent or often chronic neutropenia is a common finding, occurring in over 60% of the patients, even in those receiving regular immunoglobulin substitution. Its physiopathological mechanism remains obscure. Study of myeloid differentiation in bone marrow indicates an arrest at the promyelocyte-mycelocyte stage. Immature and mature neutrophils express at their membrane CD40 molecules and the arrest of maturation in the myelocytic lineage could be related to defective activation of precursors. Alternatively, defective interaction between CD40L on activated T cells and CD40 on stromal cells could lead to impaired G-CSF production. An argument in favor of this hypothesis is the efficacy of G-CSF treatment in most of the patients. However, some cases are resistant to high dose G-CSF infusion and are only cured by bone-marrow transplantation. Similar chronic neutropenia is reported in the few patients affected by autosomal recessive CD40-deficiency, supporting the essential role of CD40L/CD40 in myelopoiesis. It has also been shown that CD40L/CD40 interaction is required for efficient neutrophil function: sCD40L is able to prime neutrophil oxidase activity through CD40 activation. A defect in either CD40L or CD40 molecules could therefore lead to susceptibility to severe bacterial infections not only through neutropenia but also through impaired neutrophil oxidase activity.

**Common variable immunodeficiency (CVID).** CVID is characterized by defective production of IgM, and IgG or IgA. Its molecular basis is known only for a few cases. Very likely, CVID could be a consequence of a B or a T cell abnormal function. 13% of patients present with acute or chronic severe neutropenia, that is not always related to infectious episodes and not prevented by immunoglobulin substitution. A defective T cell activation (leading to impaired CD40L expression) could underlie this condition.

**X-linked agammaglobulinemia.** This disease is characterized by an absence of B lymphocytes and immunoglobulins. Neutropenic episodes are reported (26%), but generally transient and associated to acute infection. Although neutropenia could be directly related to btk deficiency since btk is expressed in myeloid cells, it is rather attributed to toxic effects of infectious agent. Indeed, neutropenia is not seen in any btk-deficient patient receiving regular immunoglobulin substitution and is also found in few patients with autosomal recessive agammaglobulinemia (with normal btk).

Other antibody defects. Are more rarely associated to neutropenia (IgG subclasses deficiencies, specific antibody deficiency or Ataxia-Telangiectasia). In few other deficiencies affecting the adaptive immunity, neutropenia can be a main (or the unique) symptom.

**X-linked neutropenia in WASP-deficiency.** Although most mutations in WASP gene lead to a T cell immune deficiency associated with thrombocytopenia and eczema, named Wiskott-Aldrich syndrome, some mutations leading to constitutively active WASP protein are responsible for chronic severe neutropenia. Neutropenia in this defect is the only symptom. It is likely caused by defective precursor mitosis and cytokinesis, according to the role of WASP in actin polymerization and cytoskeleton formation.

**The autosomal dominant WHIM syndrome.** Due to monoallelic CXCR4 mutations, associates profuse Warts, hypogammaglobulinemia, immunodeficiency and myelokathexis. CXCR4, a chemokine receptor expressed by myeloid cells, interacts with the stromal derived chemokine CXCL12. In the absence of such interaction, myeloid cells fail to exit the bone-marrow, and undergo increased apoptosis, leading to severe congenital neutropenia.

**References**


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NEONATAL NEUTROPENIA: PATHOPHYSIOLOGY AND CLINICAL IMPLICATIONS

Y. Barak
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Most human newborns are presented in the first week of life with a 'physiological' neutropenia, up to 15,000/mm³ in the first 60 hours of life and 6000/mm³ thereafter. Neonatal Neutropenia (NN), defined as an absolute neutrophil count (ANC) of less than 1500/mm³ is therefore an alarming finding, which is, however, frequently observed. As many as 8% to 56% of all patients admitted to Neonatal Intensive Care Units (NICUs) have this problem. In many cases, neutropenia is transient and benign, but in many others it is severe, prolonged and is associated with life threatening sepsis. Most cases of NN are associated with either perinatal events such as maternal preeclampsia, or with neonatal infections, but also in otherwise healthy neonates. Causes of clinically well defined NN include: 1. rare genetic disorders associated with decreased number and function in these neonates by the prophylactic and the therapeutic use of human recombinant G-CSF (rhG-CSF) and GM-CSF (rhGM-CSF). Exhaustive review of 12 studies and two meta-analyses concerning trials of rhG-CSF and rhGM-CSF administration to 422 neutropenic and non neutropenic septic and non septic neonates throughout the last fifteen years, reveals the following results:

1. In almost all studies rhG/GM-CSF have been shown to significantly enhance neonatal neutrophil numbers, without displaying relevant side effects;
2. Prophylactic administration of G/GM-CSF to neutropenic non septic neonates did not prove to be clinically beneficial in preventing infections in most studies;
3. Meta-analysis of both non-randomized and randomized placebo controlled studies shows that rhG-CSF administration was associated with significantly lower mortality rates in neutropenic neonates with bacterial sepsis. This was mostly pronounced in septic VLBW infants.

In conclusion, existing clinical trials of rhG-CSF – and to a lesser extent rhGM-CSF – administration in neonates have demonstrate safety and a trend of efficacy of these cytokines in reversing neutropenia and in the therapy of neonatal sepsis. In order to prove this trend, further large randomized placebo controlled studies, involving mostly the target group of VLBW neutropenic and septic infants are warranted.

IS IT POSSIBLE TO DECREASE THE RISK OF LEUKEMIA AMONG PATIENTS WITH SEVERE CONGENITAL NEUTROPENIA DEPENDING OF HIGH OF GCSF DOSE? EXPERIENCE OF THE FRENCH SEVERE CHRONIC NEUTROPENIA REGISTER

J. Donadieu
Registre Français des neutropénies congénitales, Service d’Hématologie Pédiatrique, Hospital Trousseau Paris, Centre de référence des déficits Immunitaires Héréditaires, France, France

Background. Recently, two studies (Donadieu J, Hae-matologica, 2005; Rosenberg P, Blood, 2006) have shown that the risk of leukaemia among patients with SCN is statistically associated with high dose of GCSF given as prevention of infections. In these analyses, patients with SCN who received currently GCSF at above 10 – even 20 µg/kg/day – are a group of patients at high risk of secondary leukaemia.

Methods. The French SCN was established in 1994. It prospectively monitors the health status of patients with severe chronic neutropenia. Methods and inclusion criteria have been published elsewhere (Donadieu, Haematologica, 2005). Briefly, the register monitors both the exposition to GCSF (dose, rhythms of administration, duration) and the medical outcome (infections, MDS or leukaemia). We present here data analysed by December 1st 2007. 424 patients (8623 person-years) of which 350 patients with congenital neutropenia (157 patients with severe congenital neutropenia, 78 patients with cyclic neutropenia, 17 patients with glycogen storage disease Ib, 89 patients with Shwachman-Diamond syndrome, 9 patients with syndrome WHIM) and 74 patients with neutropenia adult (or idiopathic
The use of G-CSF had transformed the infectious status of patients receiving treatment with G-CSF to consider the possibility of a bone marrow transplant.

**G-CSF RECEPTOR MUTATIONS IN THE DEVELOPMENT OF MDS/AML IN PATIENTS WITH SEVERE CONGENITAL NEUTROPENIA**

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Mutations in the gene encoding G-CSFR (CSF3R) are found in severe congenital neutropenia (SCN) but are absent in other types of neutropenia. The most frequent mutations seen in SCN are acquired nonsense mutations in a critical glutamine-rich stretch, which result in a C-terminal truncation of the cytoplasmic domain of G-CSFR. Clones harboring such mutations are detected in approximately 30% of SCN patients. In some cases, affected myeloid cells arise from minority clones, originally making up only 1 to 2% of the myeloid progenitor cell compartment. However, clones with G-CSFR mutations become overt in ~80% of the SCN cases upon progression to MDS and AML, suggesting that G-CSFR truncations represent an important step in the expansion of the (pre-) leukemic clones. The role of these CSF3R mutations has been studied in knock-in mouse models. It has become clear that these mice exhibit G-CSFR-mediated Jak/STAT signaling and hyperproliferation of myeloid progenitors in response to G-CSF. However, they do not develop leukemia, even after continuous administration of G-CSF. This would argue against a strong leukemia-initiating role of CSF3R mutations, at least within the relatively short lifespan of mice. On the other hand, a recent study has demonstrated that G-CSF-induced reactive oxygen species production is substantially increased in bone marrow cells expressing truncated G-CSF receptors, which might be associated with increased DNA damage. This would provide a potential mechanism by which activation of truncated G-CSFR could result in an increased mutation rate in myeloid progenitor cells during G-CSF therapy, leading to enhanced clonal outgrowth owing to the hyperproliferative signaling function of these receptor mutants. The most direct approach to investigate genomic evolution of SCN/AML is to study sequential DNA samples from patients. We are currently analyzing samples from a patient who presented with a CSF3R mutation in 1992 and developed RAEB/AML in 2007. During these 15 years, the patient received continuous G-CSF treatment and the clone harboring the CSF3R mutation persisted throughout this entire period. Nucleotide sequencing and SNP array-based comparative genome hybridization have been performed and results of these analyses will be presented at this meeting. Multiple signaling abnormalities have been linked with G-CSF-R truncations, including defective receptor internalization and an increased and sustained STAT5 activation. Although the exact underlying molecular mechanisms remain to be elucidated, it was recently shown that STAT5 activation by the G-CSFR-d715 is directly implicated in its hyperproliferative signaling function. Another major defect that is characteristic of the truncated G-CSFR forms found in SCN is the loss of the specific docking site (Tyr729) for the suppressor of cytokine signaling protein SOCS3. We recently reported that lysosomal routing and degradation of the G-CSFR depends on recruitment of SOCS3 to the activated receptor. SOCS3 forms a cullin-based E3 ligase complex by recruitment of Elongin C and B as well as Cullin 2 via its SOCS box (a so called ECS E3 ligase). SOCS3-directed lysosomal routing and signaling downregulation of the G-CSFR was dependent on ubiquitination of lysine residues of the G-CSFR as well as on the presence of the SOCS box in SOCS3. Importantly, in vivo evidence for a specific role of the SOCS box in G-CSFR negative signaling recently emerged from studies in knock-in mice expressing a truncated SOCS3 protein lacking the C-terminal SOCS box. A key finding was that SOCS3 induced downregulation of STAT5 activity and lysosomal routing of the G-CSFR to a major extent depended on a juxtamembrane lysine residue at position 632 (Lys632), even though there are in total five conserved lysines present in the cytoplasmic domain of the G-CSFR. We found that G-CSFR deletion mutant d715 is severely hampered in ubiquitination, which corroborates the importance of ubiquitination for proper G-CSFR function. Lack of ubiquitination thus provides an additional mechanism for the dysfunction of this receptor mutant.
References


THE TRANSCRIPTIONAL PROGRAM OF NEUTROPHIL DIFFERENTIATION AND ITS REGULATORS

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Polymorphonuclear neutrophilic granulocytes (neutrophils, PMNs) constitute the most abundant population of white blood cells and are key effector cells of the innate immune system. They are short-lived cells and are continuously generated from HSCs in the BM by a process referred to as neutrophil differentiation. The hallmark of early neutrophil differentiation is a marked proliferation combined with the successive commitment of pluripotent HSCs via multipotent common myeloid progenitors (CMPs) and bipotent granulocyte macrophage progenitors (GMPs) toward unipotent progenitors restricted to the neutrophil lineage.1-3 Once progenitors are committed to the neutrophil lineage, they terminally differentiate and finally become mature neutrophils. The process of terminal neutrophil differentiation gives rise to a series of morphological distinct stages, which are readily identified by their characteristic nuclear shape and their content of granules. At the myeloblast/promyelocyte stages the cells still proliferate and generate azurophil granules (primary granules) with their constituent granule proteins (GPs). At the myelocyte/metamyelocyte stages, cell proliferation ceases and synthesis of azurophil GPs stops concomitant with the successive generation of specific and gelatinase granules (secondary & tertiary granules) and their constituent GPs. Finally, the synthesis of GPs ceases, and the cells acquire their full antimicrobial potential when maturation proceeds toward the stages of neutrophils with band shaped and segmented polymorphic nuclei.3-5

The process of neutrophil differentiation has been studied by microarray analysis of highly purified bone marrow populations representing successive stages of neutrophil differentiation. These studies demonstrated that neutrophil differentiation is a most complex biological process, which is governed by a massive change of gene expression involving at least one third of all currently annotated genes and all major functional gene categories.3-7

Analysis of early differentiation showed that commitment of HSCs via CMPs toward GMPs is governed by a transcriptional program, which progressively downregulates genes affiliated with multiple lineages.5 In contrast, analysis of terminal differentiation revealed that differentiation of promyelocytes (PMs) via myelocytes/meta-myelocytes (MYs) into bone marrow neutrophils (bM-PMNs) is directed by a transcriptional program, which promotes cell cycle exit and expression of differentiation-associated genes critical for immune response (such as GPs) before cells gain responsiveness toward inflammatory stimuli.4 Hence, terminal neutrophil differentiation is directed by a fail-safe program, which promotes completion of differentiation by avoiding premature responsiveness to activating stimuli that accompany infections. Differentiation of HSCs into mature blood cell lineages relies in part on growth factors and in part on transcription factors. While hematopoietic growth factors are crucial for cell survival and proliferation, they are not considered instructive for lineage-restricted differentiation. In contrast, several transcription factors have been identified that are indispensable for the establishment of transcriptional programs intrinsic for lineage-restricted differentiation. Such programs are, however, not established by single transcription factors alone, but rather by key regulators that operate in conjunction with other transcription factors and within an appropriate cellular context.4-10

Transcriptional activators and repressors involved in neutrophil differentiation include members of the CCAAT/enhancer-binding protein (C/EBP) family, the retinoic acid receptor-alpha (RARA), PU.1, growth factor independent 1 (Gfi-1), and the CAAT displacement protein (CDP).10,15 Among these transcription factors C/EBPα and C/EBPε have emerged as key regulators of neutrophil differentiation.12-14 C/EBPα is expressed at low levels in HSCs and myeloid progenitors – that is CMPs and GMPs – and maintained at higher levels during terminal neutrophil differentiation but downregulated during monocytic, megakaryocytic, and erythroid differentiation.13,14,15 C/EBPε acts both as a transcriptional activator of myeloid-specific genes (i.e. the early appearing azurophil GP myeloperoxidase (MPO)
and the granulocyte colony-stimulating factor receptor (G-CSFR) as well as an inhibitor of cellular proliferation by binding to E2Fs, which leads to repression of E2F-mediated transcription of cell cycle genes such as MYC.\textsuperscript{17,18}

Mice with conditional disruption of C/EBP\textsuperscript{ε} in the hematopoietic system exhibit a block at the CMP to GMP transition.\textsuperscript{19} As a result, these mice lack mature neutrophils and accumulate myeloid blasts in the BM, similar to what is observed in humans with acute myeloid leukemia (AML).\textsuperscript{20} Disruption of C/EBP\textsuperscript{ε} at the GMP stage, however, does not affect terminal neutrophil differentiation of GMPs toward mature neutrophils.\textsuperscript{15} In agreement with these murine studies, mutations of the CEBPA gene encoding dominant negative proteins have been identified in a significant number of AML patients with normal karyotype.\textsuperscript{21–22} Moreover, the leukemic fusion proteins identified in a significant number of AML patients with normal

\textit{References}


TELOMERE BIOLOGY AND BONE MARROW FAILURE

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Telomere repair first was linked to bone marrow failure by genetic linkage analysis of the constitutional syndrome dyskeratosis congenita. In X-linked pedigrees, the gene DKC1 is mutated, and one function of DKC1 protein is participation in the telomere repair complex. TERC, which encodes the RNA template, is mutant in some autosomal dominant dyskeratosis congenital and also, absent physical anomalies, presentation in early childhood, and an obvious family history, in some patients with apparently acquired aplastic anemia. TERT, the gene that encodes the reverse transcriptase telomerase, is mutated in other patients with acquired aplastic anemia. TERC and TERT mutations act by haploinsufficiency to decrease telomerase activity and result in accelerated telomere attrition, which can be accurately quantitated by flow cytometry of a patient’s leucocytes in comparison to age-matched controls. Detailed study of extended pedigrees shows variable penetrance, as individuals with mutations may have normal or near normal blood counts despite marrow hypocellularity and evidence of hematopoietic deficiency (by progenitor assays and CD34 cell enumeration) and stress (elevated levels of hematopoietic growth factors). Affected patients show patterns of oligoclonal T cell expansion identical to those observed in aplastic anemia in general, suggestive of a similar immune attack but on a hematopoietic compartment quantitatively reduced and qualitatively unable to repair and regenerate. As expected, telomere length powerfully predicts responsiveness to immunosuppressive therapy. Furthermore, inadvertent transplantation of stem cells from subclinically affected sibling donors has resulted in unfavorable outcomes in the recipient. In families, telomeres may be also be reduced in length in genetically normal offspring of affected individuals, an epigenetic consequence of the direct transmission of short telomeres in germline cells.

Abnormal telomere biology has implications beyond bone marrow failure. TERT mutations have been linked to idiopathic pulmonary fibrosis; smoking appears to be a common risk factor. We have associated mutations in telomere repair complex genes with cirrhosis in affected family members, which pathologically shows components of both fibrosis and inflammation; alcohol is frequent as an exposure, but onset of hepatic disease occurs early and can be severe and fatal. In “knock-out” animal models of telomere repair complex genes, telomere shortening leads to genomic instability, due to end-to-end chromosome fusions and non-reciprocal translocations; in combination with p53 gene mutations, cancers are produced. For primary human cells bearing TERT mutations, obtained from clinically normal family members, aneuploidy and translocations can be detected by fluorescent in situ hybridization and spectrakaryotyping after brief periods of in vitro culture. TERT mutations are present in patients with acute myeloid leukemia; the mutations are inherited, not acquired, and associate with complex and often specific chromosomal abnormalities.

Telomere shortening is physiologic and may underlie normal aging. However, telomere attrition is most marked at the extremes of life and is relatively static during adulthood. One explanation for this pattern is the positive effect of sex steroids on telomerase activity: both androgens and estrogens, acting through the estrogen receptor, increase TERT transcription and telomerase expression in primary hematopoietic cells (there are two estrogen receptor response elements in the TERT promoter). This activity may explain the mechanism by which androgens pharmacologically stimulate hematopoiesis in some patients with marrow failure.

NEUTROPENIA IN THE SETTING OF MELODYDYSPLASTIC SYNDROMES

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Myelodysplastic syndrome is a heterogeneous group of disorders, presenting mostly in older patients and characterized by blood cytopenias along with a propensity towards leukemic transformation. Neutropenia is a common presenting finding in patients with myelodysplastic syndromes and is correlated both with infectious complications and prognosis. In most cases this happens in the
setting of bi- or pancytopenia. Among cases evolving to MDS after an initial stage of monolineage cytopenia, severe neutropenia is probably less frequent than anemia or thrombocytopenia.

In cases of solitary neutropenia it is of utmost importance to take into consideration other possible diagnoses, including drug toxicity, various autoimmunity states, chronic idiopathic neutropenia and copper deficiency. The presence of characteristic dysplastic morphological abnormalities in myeloid cells is helpful in some ambivalent cases, along with chromosomal aberrations.

The pathogenesis of neutropenia in MDS is a matter of continuing investigation and usually attributed to increased marrow apoptosis, although peripheral destruction and decreased expression of G-SCF receptor in progenitor cells have also been implicated. Both clonal and "normal" neutrophils have been detected in peripheral blood of MDS patients, with clonality more evident in late stage cases (RAEB) and multilineage involvement (RCMD). The presence of neutrophils derived from more than one clone is evidence enough for the heterogeneity of mechanisms leading to neutropenia. Differential expression of genes, like p16, bmi-1, p53, bcl-2 etc. may be of importance in determining clone dominance and apoptosis of progenitor cells in MDS marrow and consequently of mature cells composition. Mutations of G-SCF receptor are present in cases of severe congenital neutropenia (SCN) and their role in the development of MDS/AML in a significant proportion of such patients, especially when treated with G-CSF, is strongly debated. A role for such mutations in the pathogenesis of de novo MDS cases has also been suggested.

Management of symptomatic neutropenia with G-CSF is sometimes necessary, although a more comprehensive treatment strategy for MDS is certainly more advisable, especially with newer and more "targeted" therapies. Issues of cost effectiveness, leukemia-transformation risk and quality of life evaluation are of importance and should be addressed in individual patients, before establishing G-CSF therapy.

**CHRONIC IDIOPATHIC NEUTROPENIA: AN ORPHAN DISEASE ACQUIRES AN IDENTITY**

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Chronic idiopathic neutropenia (CIN) is a neutrophil disorder characterized by the persistent and "unexplained" reduction in the number of absolute neutrophil counts (ANC) below the lower limit of the normal range for a given ethnic population. CIN has long been considered as an "orphan disease" with unknown pathogenesis. This is mainly due to the fact that "immune" (antibody-induced) and "nonimmune"-mediated cases are usually recorded under the term "idiopathic" with subsequent inconsistencies in bone marrow (BM) morphology, granulocytic progenitor culture studies and clinical characteristics of the patients. To clearly define CIN, we have introduced distinct diagnostic criteria for the disorder, representing mainly exclusion criteria: (a) ANCs lower than 1800/mL blood for Caucasians and lower than 1500/mL blood for individual of African origin more than three months; (b) no evidence for any underlying disease that might be associated with neutropenia following a detailed clinical, serologic and ultrasonic investigation; (c) no history of exposure to irradiation, use of chemical compounds or intake of drugs to which neutropenia might be ascribed; (d) normal BM karyotype; (e) negative serum leucocagglutination and immunofluorescence techniques for antineutrophil antibodies.

Patients fulfilling the above diagnostic criteria display hypoplastic and left-shifted granulocytic series in the BM, low frequency of CD34+/CD33+ granulocytic progenitor cells and impaired granulocytic colony formation in BM culture assays. These abnormalities have been mainly attributed to increased local production of TNFα, IFNγ and Fas-ligand that induce accelerated, Fas-mediated, apoptotic death of the granulocytic progenitor cells. Activated T-lymphocytes, expressing high levels of HLA-DR, CD25, CD38, CD69 and Fas, in the BM of CIN patients display an important role in the pathogenesis of the disease. It has been demonstrated that these cells are the main source of IFNγ and Fas-ligand in patients' BM microenvironment whereas in co-culture assays it was shown that patient T-lymphocytes significantly suppress the *in vitro* colony growth of autologous and normal BM granulocytic progenitor cells. Interestingly, flow cytometric analysis of TCR Vβ repertoire and CDR3 spectratyping has revealed a skewed profile almost in 100% of CD8+ cells in PB and BM, with oligoclonal pattern in most cases, and in a very lower proportion with monoclonal pattern. In contrast, the CD4+ cells display a polyclonal pattern. Importantly, the frequency of prominent clones is higher in BM than in PB suggesting the possible existence of a "cell target" in patients' BM.

According to the definition of autoimmune diseases as clinical syndromes caused by the activation of T cells or B cells, or both, in the absence of an ongoing infection or other discernible cause, we consider CIN as an autoimmune, T-cell mediated, disorder of haemopoiesis. The female predominance, the HLA-class II predisposition and the oligoclonal T-cell expansions corroborate this assumption. Regarding the pathophyslogic process, CIN seems to share common pathogenetic mechanisms with acquired BM failure syndromes and may represent the mild form of a spectrum of diseases characterized by T-cell- and cytokine-mediated suppression of haemopoiesis such as the large granular proliferative disorder (LGL-PD), aplastic anemia and myelodysplastic syndromes (MDS).

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THE SPECTRUM OF NEUTROPHIL ELASTASE MUTATIONS FOR THE DIAGNOSIS OF SEVERE CHRONIC NEUTROPENIA

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Severe chronic neutropenia disorders include autosomal dominant cyclic neutropenia (CN) and severe congenital neutropenia (SCN) with overlapping but clinically distinct phenotypes. CN is characterized by regular oscillations of circulating neutrophils from zero to near normal levels with approximately 21-day periodicity, whereas in SCN there are steady low levels of neutrophils in peripheral blood and a characteristic block of myeloid differentiation at the promyelocytic stage of differentiation in the bone marrow. In addition, SCN patients may evolve to develop myelodysplastic syndrome and acute myeloid leukemia (MDS/AML), whereas leukemic evolution in CN is an extremely rare event. Majority of SCN patients and nearly all CN patients have heterozygous mutations in the neutrophil elastase (NE or ELA-2) gene, encoding a serine protease localized in azurophil granules of myeloid cells. Neutrophil elastase contains 2 N-glycosylation sites and 4 disulfide bridges and it is expressed in CD34+ stem cells and more differentiated myeloid progenitor cells, reaching its highest peak at the promyelocytic stage of differentiation. The protease functions as a dimer or trimer and has broad substrate specificity. To date, we and other groups identified more than 50 NE mutations, which affect all five exons of the ELA-2 gene and result in substitution, truncation, deletion or insertions in the coding region of the mutant NE protease. Yet, the spectrum of NE mutations identified in CN patients is vastly different from that in SCN patients. Of note, there are no mutational hot-spots identified yet that can be classified as purely leukemogenic mutations. The heterozygous NE mutations appear to have a gain-of-function effect rather than being dominant-negative. This is supported by the absence of severe neutropenia phenotype in mice with homozygous deletion of NE, as well as by normal survival and differentiation of human myeloid cells with nearly complete inhibition of proteolytic activity of neutrophil elastase.

Molecular modelling and analyses of the tertiary structure of NE revealed that mutations observed in CN appear to affect mostly the active catalytic site of the protease, possibly altering its proteolytic activity or resistance to protease inhibitors. The SCN-specific NE mutations appear to affect different domains of the protease: 1) mutations in the regions around the N-glycosylation sites, which may result in altered subcellular localization of the mutant protease; 2) mutations in the side chain necessary for proper oligomerization of NE, and 3) mutations in the binding pocket of the protease that alter the substrate-specificity of the mutant protease. These mutations expose the mutant protease to a new range of substrates, premature degradation or block of function of which will contribute to observed accelerated apoptosis of myeloid progenitor cells in severe neutropenia. Indeed, recent reports confirmed that expression of CN-specific and SCN-specific NE mutants results in accelerated apoptosis of human myeloid cells. Importantly, the majority of SCN-specific mutations appear to leave intact the active site of NE, suggesting that small molecule inhibitors may be effective in blocking mutant NE-triggered apoptosis and subsequent severe neutropenia. In summary, more comprehensive studies of the spectrum of NE mutations and their structure-to-function implications are needed before the information on specific NE mutations can be used for the diagnosis of cyclic or severe congenital neutropenia.

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Neutrophil antibodies are of documented significance in a range of clinical conditions which can be either alloimmune in origin, e.g. neonatal alloimmune neutropenia (NAI), transfusion related acute lung injury (TRALI), transfusion related alloimmune neutropenia (TRAIN), following granulocyte transfusions or autoimmun e in origin, e.g. autoimmune neutropenia of infancy (ANI), chronic adult autoimmune neutropenia (AIN). The autoimmune neutropenias may be further subdivided into primary conditions in which the neutropenia is the main or sole presenting feature, and into secondary conditions in which autoimmunity neutropenia is the result of another disease process, e.g. rheumatoid arthritis or haematological proliferative disorders. In the post bone marrow transplant setting, both alloimmune or autoimmune neutrophil specific antibodies may occur and it is important to be able to determine which process is occurring in order to optimize patient management. Neutrophil specific antibodies can also mediate drug dependent neutropenias. Neutrophils are difficult cells with which to undertake serological investigations for a number of reasons:

- Neutrophils carry a large and variable number (~200-300K copies) of FeγRIIb receptors which non-specifically bind the Fc portion of IgG molecules.
- During isolation, neutrophils may aggregate non-reversibly or may up-regulate or shed the glycoproteins carrying the antigens of interest. The expression of certain surface antigens can also be upregulated in disease states and following iatrogenic treatments, e.g. GCSF administration.
- Neutrophil membranes contain both human neutrophil antigens (HNA) and HLA class I antigens and, as antibodies to HLA class I commonly occur in parous females and transfused patients, detecting and therefore identifying any HNA-specific antibodies can be difficult.

A consequence of these challenges, is that there are only a small number of laboratories worldwide which undertake neutrophil serology to a high standard. The current recommendation for the laboratory investigation for neutrophil antibodies from the International Granulocyte Immunology working group is to use two techniques; the indirect granulocyte immunofluorescence test (GIFT) and the granulocyte agglutination test (GAT) (or other validated technique for detecting HNA-3a antibodies) together with a panel of granulocytes typed for the major HNA. However, in order to distinguish between granulocyte-specific and HLA antibody binding additional tests are often required. A simple strategy to confirm that antibodies are granulocyte-specific is to perform the lymphocyte immunofluorescence test (LIFT) using cells from the same donor(s) as used in the GIFT. A GIFT positive, LIFT negative reaction pattern indicates the presence of granulocyte-specific antibodies. Use of the monoclonal antibody immobilisation of granulocyte antigens (MAIGA) assay enables identification of granulocyte specific antibodies in the presence of complex mixtures of antibodies reactive with the granulocyte membrane, e.g. multispecific HLA antibodies, when serological analysis using whole cells would give ambiguous results. The use of additional techniques for detecting HLA class I antibodies, e.g. lymphocytotoxicity, ELISA assays, recombinant antigen bead technology, provide further evidence to determine the nature of any granulocyte reactive antibodies. The GAT may be performed with a microscopic or flow cytometric endpoint with the latter providing an objective, semi-quantitative end-point of antibody binding. The GAT uses metabolically active granulocytes in a prolonged incubation of 2-6 hours with test serum. The end-point of the GAT is a microscopic assessment of granulocyte agglutination and is prone to observer error. The GAT is very sensitive in detecting HNA-3a antibodies but, in collaborative workshops, has been shown to be insensitive (relative to the GIFT) for other HNA specific antibodies. Consequently, the GAT does not readily lend itself for use in a modern pathology laboratory and alternative techniques to the GAT have been developed, e.g. the granulocyte chemiluminescence test (GCLT). The GCLT measures the interaction between neutrophils opsonized with patient serum and human monocytes, is semi-automated and has a semi-quantitative endpoint. The GCLT has a sensitivity comparable to the GIFT for a range of HNA antibodies (including HNA-3a) in titration studies. It also has the advantage that it provides an indicator of clinical significance of the antibodies detected because it has a biologically mediated end-point, i.e. monocyte activation. The panel of neutrophils used for antibody detection and identification should include cells typed for HNA-1a, -1b, -1c, -2a, -2a’, 3a’ and 3a(-) as a minimum, as these are recognized as being clinically significant in the conditions described above. The HNA-1a, -1b, -1c status of donors can be determined using the polymerase chain reaction with site specific primers (PCR-SSP). Molecular typing techniques have also been described for HNA-4a, -4bw and -5a and -5bw, thereby providing the potential to detect rare, but clinically significant, antibodies to these antigens. Determination of HNA-2a cannot be easily determined using molecular typing techniques and must be typed using human antisera or monoclonal antibodies. Similarly, molecular typing techniques for determining the HNA-3a status of neutrophils are not available and phenotyping using human sera is required. The detection and identification of neutrophil antibodies currently remains technically demanding and is the preserve of a
limited number of reference laboratories with the appropriate experience and expertise. However, the development of recombinant HNA and the ability to couple these to fluorescent microbeads, in a technique analogous to that already used for detecting HLA class I and class II antibodies, may have profound impact upon current investigation strategies.

FLOW CYTOMETRIC ANALYSIS OF BONE MARROW GRANULOCYTIC PROGENITOR/PRECURSOR CELLS FOR THE DIFFERENTIAL DIAGNOSIS OF ACQUIRED NEUTROPENIAS

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Acquired neutropenias are classified in three sub-groups; neutropenias of unknown cause (primary or idiopathic), neutropenias associated with an underlying disease (secondary), and neutropenias due to drug administration (drug-induced) (Table 1).

Table 1. Classification of acquired chronic neutropenias.

1. Primary autoimmune neutropenia
2. Chronic idiopathic neutropenia
3. Secondary neutropenias
   A. Haemopathies
      1. Primary benign haemopathies (i.e. aplastic anaemia)
      2. Nutritional deficiencies (i.e. B12, folate, copper)
      3. Malignant myelopathies (i.e. myelodysplastic syndromes)
   B. Autoimmune Diseases
      1. Thyroid disease (i.e. Grave’s disease, Hashimoto)
      2. Collagen vascular disorders (i.e. systemic lupus erythematosus, rheumatoid arthritis)
   C. Infection-related chronic neutropenias
      1. Viral infections
      2. Bacterial and parasitic infections
   D. Hypersplenism
      1. Congestive splenomegaly (i.e. cirrhosis)
      2. Haemopoietic/infiltrating splenomegaly (myelo-lympho-proliferative and metabolic disorders)
      3. Phagocytic splenomegaly (i.e. parasitic infections, chronic haemolytic syndromes)
      4. Immune-reactions (i.e. Felty’s syndrome)
   E. LGL-proliferative disease
4. Drug-induced chronic neutropenias

A first step in the diagnostic procedure of acquired neutropenias is the establishment of the “chronic” character of the disorder by performing cell blood counts every month for three consecutive months. After confirming the “chronic” character of the disorder, one has to complete patient’s investigation by a detailed medical history including drug administration and appropriate clinical and laboratory studies including flow cytometric analysis of peripheral blood lymphocytes to exclude the large granular lymphocyte proliferative disorder (LGL-PD), standard biochemistry and serum virology and bacterial antibody evaluation, spleen ultrasonography, bone marrow (BM) aspiration/biopsy and cytogenetics. An important step is the detection of serum anti-neutrophil antibodies using repetitively at least two methods, i.e. the granulocyte agglutination and immunofluorescence tests, to exclude the possibility of an antibody-mediated neutropenia (autoimmune neutropenia, AIN) either in the setting of other autoimmune disease (secondary AIN) or as a primary disorder (primary AIN). It is very important to differentially diagnose chronic idiopathic neutropenia (CIN) from myelodysplastic syndrome (MDS) cases presented with isolated neutropenia, since CIN patients display also dysplastic features in the BM. We propose the flow cytometric analysis of the sequential stages of BM granulocytic progenitor/precur sor cells as a useful method for the differential diagnosis of the above disease states, in addition to BM aspiration/biopsy and cytogenetics. Five-parameter, 3-color flow-cytometry using CD45 versus side scatter (SSC) is the basic scattergram for the definition of the granulocytic progenitor/precursor cell gate. The following populations can be then identified: the CD34+/CD33- cells corresponding to the granulocytic progenitors; the DR+ cells corresponding to myeloblasts; the CD13+/CD16- cells comprising mainly to promyelocytes; the CD11b+/CD16-, CD11b+/CD16dim and CD11b+/CD16bright cells comprising mainly the myelocytes, metamyelocytes, and bands plus neutrophils, respectively (Figure 1).

Figure 1. Flow cytometric analysis of bone marrow granulocytic progenitor and precursor cells. PMC, Promyelocytes; Neutro, Neutrophils; MMC, Metamyelocytes; MC, myelocytes.

The mode of CD10 expression, normally expressed in bands and neutrophils among cells of the granulocytic lineage, may also give interesting information. Finally, the proportion of myeloperoxidase (MPO) positive cells and the SSC properties (mean SSC value) of total granulocytic cells give information for overall granularity. Having analysed a large number of CIN and MDS patients and healthy controls we concluded that in accordance with the BM morphologic findings, CIN...
patients display a shift to the left of the granulocytic series with increased percentage of DR+ cells (myeloblasts), markedly increased proportion of CD11b+/CD16– cells (myelocytes) and decreased proportion of CD11b+/CD16+ (metamyelocytes) and CD11b+/CD16–/dim (bands and neutrophils) cells. In accordance with the low proportion of the mature cells is the low proportion of the CD10+ cells in CIN patients. The proportion of MPO+ cells and the mean SSC value are normal suggesting normal granularity of the granulocytic precursor cells in CIN. Patients with MDS display a marked shift to the left with increased proportion of CD34+/CD33+ granulocytic progenitor cells, compared to CIN patients, and low proportion of mature band and neutrophils. Characteristically, MDS patients display low proportion of MPO+ cells and low mean SSC value of the granulocytic cells indicative of their low granular content. MDS patients display also abnormal cell subpopulations such as the DR+/CD11b+ cells which do not normally exist in either CIN patients or healthy controls. Overall, we propose flow-cytometric immunophenotyping of the BM granulocytic progenitor/precursor cells as a useful tool for establishing and confirming diagnosis of CIN between other neutropenias states especially MDS.

T CELL RECEPTOR VARIABLE BETA CHAIN ANALYSIS IN THE DIAGNOSIS OF CHRONIC NEUTROPENIAS

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Clonal expansions of cytotoxic T cells (CTL) in large granular lymphocyte leukemia (LGL) likely constitute an extreme pole in the spectrum of immune responses directed against mature or immature hematopoietic cells, while in aplastic anemia and idiopathic single lineage cytopenias immune responses are mostly oligo- or polyclonal. When hematologic manifestations in a cohort of patients (N=84) with LGL leukemia were investigated, neutropenia was present in 70% of patients. In a large proportion of LGL patients, clonal expansions can be detected by VB flow cytometry and the detection rate further increases when molecular clonotypic analysis is applied. When these sensitive technologies were applied as a useful diagnostic algorithm to a cohort of patients with idiopathic neutropenia (N=20), 15 expanded (immunodominant) CTL clones were detected in 12/20 (60%) patients. In comparison to LGL leukemia, these clones were less immunodominant but clearly discernible from sub-clinical lymphoproliferations in controls (N=90). As a surrogate of cytotoxic activity, we found markedly increased production of interferon-γ in most of the neutropenia patients, irrespective of the presence of immunodominant CTL clones. Of interest is that anti-neutrophil antibodies were found in 40% of these patients and their presence did not correlate with the oligoclonal expansion detected by flow cytometry. Similarly, there was no correlation with the myeloid colony formation inhibitory activity in serum and oligoclonal CTL expansions or anti-neutrophil antibodies. By analogy to autoimmune neutropenia, when patients with pure red cell aplasia (PRCA) without a known LGL process were clonotyped, LGL-like expansions were also found in 3/7 cases consistent with a discrete clonal CTL proliferation, similar to those seen in true LGL leukemia and PRCA/anemia (N=15). These results imply that immune-mediated inhibition in idiopathic cytopenias can involve both autoantibody production and clonal/oligoclonal CTL expansions; consequently, potential therapeutic manipulations may include immunosuppression targeting both humoral and cellular responses. It is possible that in LGL, a singular permissive target antigen is present determining the restriction of the immune-mediated pathology. The corresponding LGL clone may have evolved from the original polyclonal process. When NKG2D-MICA signaling was examined in cohort of 47 patients with LGL and neutropenia using flow cytometry, showed expression of NKG2D on CD8/CD57 cells and controls (p=0.576) but the absolute numbers of NKG2D were much higher due to the expansion of the aberrant CTL clone. When we measured MICA expression on neutrophils, likely corresponding to putative target cells, MICA levels appear highest in granulocytes derived from LGL patients; the signal intensity of MICA on CD15+ cells from patients with LGL was found to be significantly higher than controls (p=0.035) and the absolute neutrophil count was inversely correlated with MICA expression (R=0.50, p=0.035). When MICA levels in sera of LGL patients were studied, soluble MICA was found to be highly variable but overall significantly elevated in comparison to controls in whom MICA was virtually absent (p<0.001). Elevated soluble MICA levels above the mean levels found in patients were present in 80% of patients. It is possible that MICA is actively expressed and secreted as a result of an autoimmune attack on neutrophils and their precursors or MICA induction constitutes a marker of a pathologic process rendering myeloid cells as susceptible targets for clonal LGLs. For instance, viral infections have been associated with increased MICA expression. As MICA locus is highly polymorphic, we hypothesized that certain MICA alleles may be associated with CTL immune responsiveness. When we genotyped MICA in patients (N=66) with LGL, there was no increase in the frequency of any of the MICA alleles above to their prevalence in general population. Similarly, no association was found between specific KIR/HLA profiles and LGL. However, KIR/KIR-L match analysis revealed an increased frequency of mismatches between KIR3DL2 and KIR2DS1 and their ligands HLA-A3/11 and HLA-C group 2 (p=0.03 and 0.01, respectively). The association of LGL was also analyzed with a number of immunogenetic factors including CTLA-4 (+49 A/C), CD16 (-158V/F), CD45 polymorphisms and cytokine single nucleotide polymorphisms including TNF-α(-308G/A), TGF-beta1 (codons 10 C/T, 25 G/C), IL-10 (-1082 G/A), IL-6 (-

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174 C/G), and IFN-γ (+874 T/A). A statistically significant increase in the A/A genotype for TNF-α-308, IL10-1082 and CTLA-4 +49 was observed in T-LGL patients compared with controls, suggesting that the G allele serves a protective role in each case. These findings suggest that immunogenetic factors may play a role increased clonal reactivity in patients with LGL and possibly similar mechanisms are involved in cytopenias with less polarized CTL responses. The antigens triggering the aberrant immune response are not known but it is possible that serologically defined neutrophil antigens may also serve as targets for CTL responses.

**UPDATE ON THE CLINICAL ASPECTS AND MANAGEMENT OF IDIOSYNCRATIC DRUG-INDUCED AGRANULOCYTOSES**

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Idiosyncratic drug-induced agranulocytosis is characterized by a neutrophil granulocyte count below 5.109/L with no relevant decrease in haemoglobin and platelet counts. This is a very rare disease. The annual incidence is comprised between 1.6 and 9.2 cases per million population in Europe and is stable over the past 20 years despite the continuing development of new molecules. Almost all classes of drugs have been implicated in the development of this adverse event. Drugs with the highest estimated risk of agranulocytosis are mainly antithyroid drugs, dipyrene, trimethoprim-sulfamethoxazole, beta-lactams, carbamazepine, clozapine and ticlopidine. Pathogenesis remains unclear. Both immune and toxic mechanisms are probably involved and could be mediated by the drug itself or its reactive metabolites. The clinical aspects of drug induced agranulocytosis have changed since the historical reports of life threatening edema, necrosis and obstruction of the pharynx. Currently, patients usually present with fever, general malaise, chills, myalgia or arthralgia, aspecific sore throat and severe deep infection. Without intervention, most patients (>60%) develop septicemia while some have evidence of pneumonia, anorectal, skin or oropharyngeal infections and finally septic shock. It should be emphasized that agranulocytosis is sometimes discovered in an asymptomatic patients receiving a medication which justifies a close blood count monitoring such as antithyroid drugs. In our experience, clinical manifestations included isolated fever (41%), sepsicaemia and septic shock (34%), pneumonia (10%), sore throat and tonsillitis (7%), cutaneous infections (4%) and other deep infections as arthritis, urinary tract or digestive infection (4%). In the elderly patients, clinical manifestations are more severe with sepsicaemia and septic shock in 64% of the cases. The causative pathogen (typically Gram negative bacilli or Gram positive cocci) is isolated in a minority of cases (30%). The current mortality rate of drug induced agranulocytosis is less than 5%. Adverse prognostic factors are age ≥65 ans, number of neutrophil count at diagnostic <0.1×109/L, severe infection including sepsicaemia and septic shock and severe underlying disease or comorbidity such as renal failure.

There are no available guidelines for the management of drug induced agranulocytosis. The first therapeutic measure is the withdrawal of any potentially causative drug. The patient’s medication history (including over the counter drugs) must be carefully and chronologically reviewed in order to focus on the suspected agents. Patients with high risk of infection or with adverse prognosis factors should be managed in the hospital. Concomitant measures include aggressive treatment of any diagnosed or potential sepsis as well as the prevention of secondary infections. Preventive measures require good hygiene with special attention to high-risk areas including the mouth, skin and perineum. Patients isolation and empiric prophylactic antibiotics such as digestive decontamination have been proposed but their usefulness in limiting the infection risk is not validated. Patients with fever or afebrile patients who have signs or symptoms compatible with infection should receive promptly an intravenous broad-spectrum antibiotic therapy after blood, urine and any other relevant samples have been obtained for cultures. The choice of initial therapy should be based on potential infecting agent, site of infection, local antibiotic susceptibility, possible drug reactions, previous antibiotic therapy and duration of neutropenia. Empiric antibiotic therapy has to be active against Gram negative bacilli including Pseudomonas aeruginosa and against Gram positive cocci. A dual therapy including two of the three following drugs is usually effective: antipseudomonal beta-lactam or fluoroquinolone and aminoglycoside. The efficacy of hematopoietic growth factors (HGF) is not demonstrated according to evidence based criteria in the setting of drug induced agranulocytosis. Most studies, however, report a significant shorter duration of neutropenia in patients receiving G-CSF or GM-CSF. The use of these factors does not appear to decrease the global mortality rate but could reduce the rate of infectious complications or fatal issues in severe patients with an initial neutrophil count <100/mm³. Thus HGF use is usually recommended in the management of high risk patients with drug induced agranulocytosis such as those with one of the adverse prognostic factors listed above. Finally, the drug which is supposed or proved to be responsible of the episode is contraindicated all life long and the accident has to be declared in a database of adverse events for pharmacological surveillance.

**LATE ONSET NEUTROPENIA FOLLOWING ANTI-CD20 MONOCLONAL ANTIBODY TREATMENT IN LYMPHOMA PATIENTS. POSSIBLE PATHOPHYSIOLOGIC MECHANISMS WITH EMPHASIS ON T CELL LYMPHOCYTE IMBALANCES**

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Rituximab is a chimeric anti-CD20 monoclonal antibody containing human immunoglobulin (Ig)-G1 and kappa constant regions with murine variable regions. The reactivity of Rituximab is restricted to B cells and its mechanism of action is mainly mediated by complement binding and induction of antibody-dependent cellular cytotoxicity. The full impact of Rituximab on the hematopoietic and immune systems remains largely unknown. Since B cells are not simply passive recipients of T cell help but may also display effector functions beyond antibody production, e.g. modulation of T cell responses, depletion of B cells by Rituximab may have profound effects on immune regulation. In fact, a significant increase in activated CD4\(^+\) and CD8\(^-\) T-cells, as well as CD25\(^+\) FOXP3\(^+\) regulatory T cells, was recently demonstrated in patients with systemic lupus erythematosus after Rituximab-induced B cell depletion. Several groups have independently reported a syndrome of severe late-onset neutropenia (LON) in some patients with lymphoma treated with Rituximab±chemotherapy. The incidence of LON varies between series; this might be explained, at least in part, by the failure to detect some neutropenic episodes due to their short duration, the relatively long time to onset and the usually uncomplicated course. However, there is a gradual increase in the frequency of Rituximab-related LON probably due to the widespread use of Rituximab in the standard treatment of lymphomas but also to the ongoing awareness for this event. Previous studies from several groups, including ours, have provided indirect evidence to implicate an immune-mediated mechanism in the pathogenesis of Rituximab-associated LON. In 2002, we first reported two cases of neutropenia in Rituximab-treated lymphoma patients who showed evidence of T-large granular lymphocyte (TLGL) expansion characterized by a predominance of CD3\(^+\)CD8\(^+\)CD57\(^-\)CD28\(^-\) T-cells in blood and bone marrow (BM). Subsequent studies from our group on a series of 34 Rituximab-treated lymphoma patients confirmed T-lymphocyte subset imbalances with several hematological manifestations suggestive of immune-mediated myelosuppression. Given the well-established association between TLGL lymphoproliferative disorders and cytopneas, especially neutropenia, we recently investigated potential underlying mechanisms in 12 patients with various non-Hodgkin lymphoma (NHL) subtypes who developed LON without identifiable causes at a median of 95 (range 67–420) days after completion of the intended treatment with Rituximab ± chemotherapy (21). The study also included two control groups: (1) healthy donors (n=25) for comparative analysis of bone marrow (BM) functional parameters; (2) NHL patients treated with similar Rituximab ± chemotherapy regimens without LON for comparative evaluation of peripheral blood and BM lymphocyte subpopulations (n=38) and BM pathology findings (n=27). Ten of 12 patients with LON and 33/38 NHL controls developed profound B-cell depletion. Inverted CD4/CD8 cell ratios were observed in 10/12 LON cases vs. 13/38 NHL controls (p<0.01). Arise in CD8 cell count with systemic lupus erythematosus after Rituximab-induction. The 3rd Neutropenia Network Conference, Heraklion, Greece, September 26-27, 2008.

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year after 12 years of treatment. Patients receiving higher doses of G-CSF, i.e., the patients receiving G-CSF >8.0 mcg/kg/day, appeared to be at greater risk. Other than severity of disease as reflected by required G-CSF dose, the risk factors for leukemic evolution are not yet known. The risk of leukemia is equivalent for patients with or without mutation of the ELA 2 gene. Patients with cyclic and idiopathic neutropenia and several other categories of neutropenic patients are not at risk or have a much lower risk of developing leukemia.

Based on the SCNIR experience, treatment guidelines for SCN patient with history of recurrent fevers, infections or chronic inflammation are:

1. Start low and gradually increase dose. Find the minimal effective dose to give medial ANC of 1000 to 2000/mcL.
2. Initial daily dose for idiopathic 1.0 mcg/kg, cyclic 2.0 mcg/kg, congenital 3.0 mcg/kg.
3. Use daily therapy initially, the advantage of daily treatment is to avoid or minimize bone pain and other acute adverse effects.
4. Switch to every other day on MWF schedule for good responders (daily dose 3 mcg/kg/day or less).
5. If initial therapy is insufficient to reach target median count, increase dose by 1 mcg/kg/day at 1 to 2 week intervals; above 10 mcg/kg/day increase by 3 to 5 mcg/kg/day to max dose of 20-30 mcg/kg/day. Consult with a neutropenia specialist in all non-responding or poorly responding patients to consider alternate strategies, including hematopoietic transplantation.
6. Monitor CBC at least weekly in the initial 2 months on treatment, at least once per month for the first six months and quarterly thereafter for stable patients.
7. Evaluate all patients to be given G-CSF with bone marrow aspirate and cytogenetics pre-treatment. Repeat annually for severe congenital neutropenia, Shwachman-Diamond patients and any other category with known risk of leukemic transformation.

Other categories and issues beyond the evidence from randomized controlled trials:

1. Patients with autoimmune neutropenia, presumed or demonstrated by antibody testing, will respond to G-CSF, similar to idiopathic neutropenia.
2. Patients with autoimmune neutropenia as a part of SLE, RA, connective tissue disorders may respond to G-CSF, but the responses are more variable than with the above categories. These patients may also have increased adverse effects with worsening or recrudescence of prior autoimmune symptoms.
3. Patients with the LGL syndrome may not respond to G-CSF but may respond to the combination of methotrexate (5-10 mg once per week) plus G-CSF (e.g., 1.0 mcg/kg/day or MWF).
4. Patients with HIV infection usually respond to G-CSF.
5. Patients with MDS usually respond to G-CSF.
6. Patients with chronic neutropenia after chemotherapy or radiation therapy have not been studied systematically.
7. Pegylated G-CSF (peg G-CSF) has not been studied for treatment of SCN. Case studies and case reports indicated that excessive responses are frequent if a standard dose of 6 mg is used.

References

INFECTIONS IN PATIENTS WITH SEVERE CHRONIC NEUTROPIchia; PROPHYLAXIS AND TREATMENT

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Severe congenital neutropenia (Kostmann’s syndrome) and autosomal dominant sporadic cyclic neutropenia are disorders of neutrophil production predisposing patients to recurrent serious bacterial infections from early infancy. The primary goal of managing patients with severe chronic neutropenia, either cyclic or congenital, is to reduce the incidence of serious infections, while a secondary goal is the early and aggressive treatment of established infections. Nowadays the locus for autosomal dominant cyclic neutropenia has been mapped, and the disease is known to be associated with mutations coding the neutrophil elastase gene in approximately 60-80% of the cases. Many patients with autosomal recessive severe congenital neutropenia have also different homozygous mutations of the neutrophil elastase gene. Cautious prophylactic administration of trimethoprim-sulfamethoxazole and use of granulocyte colony-stimulating factor (G-CSF) support are the mainstays in preventing infections in these patients. At this point we should emphasize the difference compared to transient chemotherapy-induced neutropenia in patients with cancer, in whom G-CSF support allows chemotherapy dose intensification but in most cases does not reduce the infectious complications. Patients with severe, chronic, congenital neutropenia have to be monitored regularly for cytogenetic abnormalities and development of myelodysplasia. Risk factors for myelodysplasia/acute leukemia in these patients are younger age at diagnosis, and strong exposure to G-CSF. Prospective clinical trials with well-defined endpoints (e.g., infectious complications, antibiotic use, days of work loss, etc) are needed to define specific treatment groups who will mostly benefit from G-CSF support. In addition, randomized studies are required to evaluate the proper dosage and duration of G-CSF treatment. Hematopoietic stem cell transplantation remains the only curative option available for patients who are refractory to G-CSF. Regarding treatment of established bacterial infections in these patients, it is more difficult than in patients without neutropenia and associated with increased risk of failure and relapse.

NEUROLOGICAL MANIFESTATIONS AND NEURO-Psychological Abnormalities in Patients with Severe Chronic Neutropenia

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Background. The genetic cause of recessive severe congenital neutropenia (SCN, Kostmann disease) was recently revealed, and homozygous mutations of the HAX1 gene were demonstrated in patients of the original Kostmann family in northern Sweden. Our clinical observations suggested that these patients develop neurological and neuropsychological symptoms.

Methods. We performed comprehensive neurological and neuropsychological studies of the surviving Kostmann family patients, and in one Swedish SCN patient not related to this family. We also conducted mutational analyses of the HAX1 and ELA2 genes in these families, along with studies of the pattern of HAX1 alternative transcript expression in normal human tissues.

Findings. Five of six SCN patients in the Kostmann kindred harboured a homozygous HAX1 mutation (568C→T, Q190X), and one carried a heterozygous ELA2 mutation. The Swedish patient of Kurdish extraction carried an alternative homozygous HAX1 mutation (131G→A, W44X). The three SCN patients who were alive with the Q190X mutation all developed neurological disease (CNS-SCN) with decreased cognitive function. In addition, three of four evaluable patients that reached 10 years developed epilepsy. On the contrary, the patient with the ELA2 mutation and the patient with the W44X mutation showed no obvious neurological or neuropsychological abnormalities. Finally, quantitative RT-PCR demonstrated the presence of two alternative HAX1 splice variants in normal human tissues; both transcripts contained exon 5, harboring the Q190X mutation, whereas exon 2, containing the W44X mutation, was spliced out from the second transcript.

Interpretation. We describe neurological and neuropsychological abnormalities in patients with severe congenital neutropenia. This novel clinical presentation is associated with specific homozygous mutations in HAX1, a gene encoding a protein with anti-apoptotic properties.

Conclusion. We describe neurological and neuropsychological abnormalities for the first time in Kostmann disease patients. These central nervous system symptoms appear to be associated with specific HAX1 mutations.

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SEVERE CONGENITAL NEUTROPENIA

PRELIMINARY DATA ON USE OF PEGFILGRASTIM IN TWO CHILDREN WITH SEVERE CHRONIC NEUTROPENIA

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Objective. To evaluate efficacy and safety of pegfilgrastim in children affected with severe chronic neutropenia (SCN).

Methods. Pegfilgrastim was given to SCN children on daily G-CSF (filgrastim) with poor compliance/efficacy aiming to reach median ANC >500/mmc and/or maintain/reduce infection recurrence respect to daily filgrastim. Patient 1. A 6 year old SCN Ela-2 mutated girl on daily filgrastim (10 mcg/kg), had a median ANC of 883/cmm (0-4030) and 13 infections (otitis, bronchitis, mastoiditis, anal forunculosis) in 36 months (ratio 0.36). Due to recurrent bone pain and very poor compliance to daily injections on July 2006 started subcutaneous pegfilgrastim. A dose of 100 mcg/kg/9 days was found to maintain median ANC at 1248/cmm (0-33575) with 7 infections (otitis, mastoiditis) in 19 months (ratio 0.36). QOLimproved thanks to better tolerability of drug administration, as assessed by specific questionnaire. Pharmacokinetic showed that lowest pegfilgrastim concentrations, reached 72hs after administration were comparable to those observed 24hs after filgrastim. No side effects were observed. Patient 2 A 2 year old SCN Ela-2 mutated boy on daily subcutaneous filgrastim (10 mcg/Kg) with a median ANC of 132/cmm (0-1690) and 8 infections (otitis, mastoiditis, anal forunculosis) in 36 months (ratio 0.36) maintained a median ANC of 124/cmm (0-33575) with 5 infections (otitis) in 19 months (ratio 0.36). QOL improved thanks to better tolerability of drug administration, as assessed by specific questionnaire. Pharmacokinetic showed that lowest pegfilgrastim concentrations, reached 72hs after administration were comparable to those observed 24hs after filgrastim. No side effects were observed. Since March 2007 a dose of 100 mcg/Kg every 9/12 days maintained a median ANC of 468/cmm (0-5240) with 4 infections (otitis) in 12 months (ratio 0.3) and a remarkable reduction of hospitalisation days despite a similar qualitative infections pattern. QOL was improved and pharmacokinetic was similar to patient 1. On abdomen US spleen was mildly enlarged.

Conclusion. Pegfilgrastim was effective in rising ANC and in reducing infectious ratio/hospitalization. Pegfilgrastim kinetic showed that concentrations after 72 hs are comparable to lowest values obtained with filgrastim regimen. Longer follow-up and more patients are awaited to confirm these data.

MOLECULAR ANALYSIS OF HUNGARIAN PATIENTS WITH SEVERE CONGENITAL NEUTROPENIA

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Severe congenital neutropenia (SCN) is a heterogeneous group of genetic disorders characterized by impaired myelopoiesis and susceptibility to bacterial and fungal infections. We describe here molecular characteristics of 10 Hungarian patients diagnosed with SCN. Genomic DNA was isolated from blood leukocytes and bidirectional DNA sequencing of the HAX1, ELA2 genes and ELA2 promoter sequencing was performed by using standard assays. Ten SCN patients from nine unrelated families were studied. We found novel ELA2 promoter substitutions (-199 C to A) in 3 patients, of 2 families. We have identified one novel missense mutation in the ELA2 gene (serine 97 leucine substitution resulting from C to T transition). The heterozygous mutations in ELA2 of these four patients suggested autosomal dominant trait of SCN. Mutational analysis of CSF3R and GFI1 in patients with no ELA2 mutation is in process.
T-LGL RELATED NEUTROPENIA

THE SPECTRUM OF T (CD3+) LARGE GRANULAR LYMPHOCYTE (T-LGL) PROLIFERATIONS

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CD3+ T-LGL leukemia is a rare clonal lymphoproliferative disorder, usually of mild clinical behavior, often manifesting with cytopenias. It can precede, coexist or follow a wide spectrum of autoimmune and neoplastic diseases, including B-lymphoproliferations and de novo myelodysplastic syndromes (MDS). Benign, reactive, non clonal T-LGL (CD3+) lymphoproliferative disorders are even rarer and develop secondary to various entities, including Rituximab treatment in patients with lymphoma. Differentiating T-LGL leukemia from reactive hyperplasias is based exclusively upon detecting TCR monoclonality by PCR. We present clinicopathologic findings in a group of 42 patients with CD3+ T-LGL lymphoproliferations: (1) clonal, n=25, (2) non clonal, n=17. Group (1) included 13 patients with coexistence of T-LGL leukemia and various clonal B-lymphoproliferative disorders (multiple myeloma/MGUS: 4, Hodgkin lymphoma: 2, non-Hodgkin’s lymphoma: 7) and 7 patients with other coexisting diseases (lupus, Felty’s syndrome, autoimmune cytopenias, de novo MDS, renal transplantation). Group (2) included mainly lymphoma patients with increased CD3+CD8+CD57+ T-LGLs post-Rituximab (15/17 patients). The two groups did not differ with regard to the frequency of isolated cytopenias. In contrast, significant differences were noted between the two groups concerning the incidence of (1) pancytopenia (13/25 vs. 3/17, p=0.03), (2) lymphocytosis (7/25 vs 0/17, p=0.01), (3) splenomegaly (6/25 vs. 0/17, p=0.04), (4) pattern of bone marrow (BM) infiltration in the BM biopsy (BMB): specifically, a predominantly interstitial pattern of infiltration was observed in 12/25 patients of group-1 vs. 3/17 group-2 patients, while a predominantly dispersed pattern of infiltration was noted in 8/25 group-1 vs. 13/17 group-2 patients (p=0.004 for either comparison). Histological findings from the hematopoietic marrow are summarized as follows: (i) granulocytic series: increased, 17/42, decreased 22/42, dysplasia: 30/42 patients, frequent maturation arrest in both groups, (ii) erythroid series: increased, 34/42, decreased 6/42, dyserythropoiesis: 18/42 patients, (iii) megakaryocytic series: increased, 36/42, decreased 3/42, dysplasia: 33/42. In conclusion, establishing the diagnosis of T-LGL leukemia is a challenge both for clinicians and pathologists, since the disease usually manifests with cytopenias and BM dysplastic changes mimicking de novo MDS. Although the spectra of clinical manifestations and histopathological findings in T-LGL leukemia and reactive T-LGL proliferations show some overlap, important differences exist. Finally, the results presented here emphasize the decisive role of the pathologist in establishing the diagnosis of T-LGL leukemia in BMB samples and also in discriminating it from reactive and other, neoplastic T, T/NK or NK lymphoproliferative disorders.
AUTOIMMUNE NEUTROPENIAS

ANTI-NEUTROPIL ANTIBODIES IN YOUNG CHILDREN WITH PERSISTENT NEUTROPENIA

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Background. Chronic neutropenia in infants and toddlers may be due to autoimmune mechanisms, usually associated with the development of anti-neutrophil antibodies. The neutropenia observed in this condition, despite often being profound with a prolonged course, is of excellent prognosis and characterized by automatic remission. The treatment of this condition is mainly supportive. Objectives. In this study, we evaluated the rate and the significance of the detection of anti-neutrophil antibodies in children with persisting neutropenia. Methods. Blood samples from young patients with persisting neutropenia of unknown etiology were evaluated for the presence of anti-neutrophil antibodies, by the assays granulocyte immunofluorescence test (GIFT), granulocyte agglutination test (GAT) and monoclonal antibody immobilization of granulocyte antigens assay (MAIGA). All assays were performed in reference laboratories. Results. Eighty patients with persisting neutropenia (characterized by an absolute neutrophil count <1000/mm^3 for at least 3-6 months) were investigated. Antibodies were detected in 58 patients (72.5%), with at least one out of three methods (57/80 with GIFT, 19/80 with GAT, 10/10 with MAIGA). In 2 out of 4 initially antibodies-negative patients subsequent testing became positive. In 3 from 22 seronegative patients further evaluation established the diagnosis of congenital neutropenia (Barth syndrome, Kostmann syndrome, severe congenital neutropenia syndrome, respectively). The clinical course of seropositive patients was uncomplicated in the majority of them. Supportive treatment was required for patients with frequent or serious infections and included antibiotic chemoprophylaxis (15 patients), regular infusion of IVIg (2 patients) and short term G-CSF administration (2 patients). Conclusion. Anti-neutrophil antibodies are frequently observed in infants and toddlers with persisting neutropenia. The different assays used have limited and supplementary sensitivity. Their results need to be related with the clinical condition of the patients. Patients with neutropenia caused by anti-neutrophil antibodies have, in general, a benign course.

CHRONIC IDIOPATHIC NEUTROPENIA

TOLL-LIKE RECEPTOR 4 ACTIVATION IN THE BONE MARROW OF PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA MAY CONTRIBUTE TO THE INFLAMMATORY MARROW ENVIRONMENT

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Background. Chronic idiopathic neutropenia (CIN) is a bone marrow (BM) failure syndrome associated with local over-production of inflammatory mediators that induce the apoptotic death of the granulocytic progenitor cells. We have previously reported increased expression of TLR-4 in patient BM CD14^+ cells. Aim of the study. To probe the potential role of TLR-4 in the generation of the inflammatory BM milieu in patients with CIN. Methods. BM aspirates were obtained from 20 CIN patients and 11 healthy individuals after informed consent. To determine whether TLR-mediated signal transduction pathway is activated in CIN, we examined the expression of 84 genes involved in TLR-mediated signaling in immunomagnetically sorted BM CD14^+ by quantitative RT-PCR using the PCR array technology. To examine the involvement of TLR signaling in BM cytokine production, plastic adherent BM monocytes from CIN patients were treated for 24-hours with autologous BM plasma in the presence or absence of a specific TLR-4 inhibitor or a placebo and the levels of IL-6, IL-8, IL-1beta and tumor necrosis factor (TNF)α were evaluated. Finally, HMGB1 were evaluated in long-term BM culture (LTBMC) supernatants by means of ELISA. Results. Quantitative RT-PCR analysis of 84 genes involved in TLR-4 signaling, demonstrated increased expression of 43 genes in BM CD14^+ cells of CIN patients compared to healthy controls. The most prominent expression was obtained for genes considered as key-mediators of the TLR signaling such as TRAF6, MyD88, TICAM2, IRAK1, and TIRAP. These data suggest that the TLR downstream signaling pathway is activated in the BM CD14^+ cells of CIN patients. Furthermore, BM plasma from CIN patients induced the production of IL-6, IL-8, IL-1beta and TNFα by autologous BM monocytes in a TLR-4 dependent manner, since percentage of inhibition of cytokine production was significantly higher in the presence of TLR-4 inhibitor compared to placebo (p=0.022, p=0.009, p=0.0003, p=0.00003, respectively). Finally, CIN patients displayed significantly increased levels of HMGB1 in LTBMC supernatants compared to controls (p=0.045). Conclusion. TLR4 up-modulation in the BM CD14^+ cell compartment of CIN patients has a significant role in the pathophysiology of CIN contributing, at least in part, to the pro-inflammatory cytokine over-production in CIN BM. The increased levels of HMGB1, possibly derived from the late apoptotic/dead granulocytic progenitor cells, may represent the TLR-4 activating ligand.
CHRONIC IDIOPATHIC NEUTROPENIA

QUANTITATIVE CHANGES OF T-REGULATORY CELLS IN THE PERIPHERAL BLOOD AND BONE MARROW OF PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA

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Introduction. Functional and/or quantitative changes of T-regulatory (Treg) cells have been implicated in the pathophysiology of autoimmune diseases. Chronic idiopathic neutropenia (CIN) is a bone marrow (BM) failure syndrome characterized by the presence of activated T-cells with myelosuppressive properties in peripheral blood (PB) and BM. The role of Tregs in CIN has not been studied. Aim of the study. To investigate the frequency of Tregs in CIN patients and probe the underlying mechanisms implicated in their abnormalities, if any, by exploring the mutually exclusive relationship between Tregs and Th17 cells.

Patients-Methods. We have studied 50 CIN patients and 20 age and sex-matched healthy controls after informed consent. We evaluated the frequency of CD4+/CD25high/FOXP3+ Tregs in PB and BM using flow-cytometry and we also assessed IL-17 levels in serum and long-term BM culture (LTBMC) supernatants using ELISA. Results. CIN patients displayed lower number of total lymphocytes (1632±493) and CD4+ T-cells (765±281) compared to controls (2575±559 and 1096±308 respectively) (p<0.0001 and p<0.0001, respectively). The percentage of FOXP3+ cells within the CD4+CD25high cell fraction was significantly decreased in CIN patients (57.77±15.77%, respectively) compared to controls (72.95±12.23%, respectively) (p=0.0004). Interestingly, however, a parallel measurement of Treg cell proportion in PB and BM specimens of CIN patients (n=7) showed statistically significant increased percentage of FOXP3+ cells within the CD4+/CD25high T-cells of BM (68.51±11.88%) compared to PB (52.20±12.77%) (p=0.0005), suggesting a possible accumulation of Tregs in patients' BM. Because IL-17 levels actually reflect the Th17 numbers we evaluated cytokine levels in patient sera and LTBMC supernatants. Serum IL-17 levels did not differ significantly between CIN patients (5.1±7.98 ng/mL) and controls (5.99±9.40 ng/mL, p=0.8788). However, cytokine levels were statistically significant increased in patient LTBMC supernatants (4.09±6.20 ng/ml) compared to controls (0.69±1.82 ng/mL) (p=0.0268). Conclusions. CIN patients display decreased number of Tregs in the PB probably due to an accumulation of these cells in the BM in an attempt to suppress the local immune reactions mediated by activated T-cells and proinflammatory Th17 cells. The Treg cell function in CIN patients is also under investigation.

CYTOMEGALOVIRUS AS A POTENTIAL CAUSE IN THE PATHOGENESIS OF CHRONIC IDIOPATHIC NEUTROPENIA


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Background. Chronic idiopathic neutropenia (CIN) is a granulocytic disorder characterised by the prolonged, unexplained reduction in the number of circulating neutrophils. Increased apoptosis of bone marrow (BM) myeloid progenitor cells due to an inflammatory BM milieu has been implicated in the pathophysiology of the disease. The cause of these abnormalities, however, remains unknown. Aim. To evaluate the possible involvement of CMV in the pathophysiology of CIN.

Patients and Methods. BM cells, peripheral blood mononuclear cells and serum were examined from 16 CIN patients as well as from 13 healthy donors, serving as the control group. Viral detection in patient and control samples was performed by means of PCR and Fluorescent in situ hybridization (FISH) whereas long-term BM cultures (LTBMCs) were used for functional experiments. Results. CMV was detected by PCR in BM cells of 75% of CIN patients whereas it was not observed in the control group. Viremia in the serum was limited to 3 CIN cases. Further investigation for the identification of CMV genome in CD3+, CD14+ and CD34+ BM cells, representing T-lymphocytes, monocytes/macrophages and progenitor cells respectively and also in stromal cells, revealed the presence of viral DNA in the latter cell type, exclusively. FISH was also applied in LTBMCs from CIN patients and in stromal cells, confirming the presence of CMV genomes in 0.1% of the cell population. Infection of LTBMCs with a CMV strain expressing EGFP, resulted in viral latency at the stromal cells, whereas CMV could establish a lytic infection at the stromal cells in long-term cultures only when the non adherence bone marrow cells had been removed. The number and the clonogenic potential of non adherent cells in CMV-infected LTBMCs were lower compared to the mock-infected cultures. Conclusion. CMV is frequently detected in BM stromal cells of patients with CIN. Based on the functional assays, we postulate that the CMV latent infection of BM microenvironment cells may affect the growth of haemopoietic progenitor cells and may, therefore, have a role in the pathogenesis of neutropenia in the affected subjects.
STUDY OF BONE MARROW MESENCHYMAL STEM CELLS IN PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA

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Introduction. CIN is a BM failure syndrome characterised by increased apoptosis of granulocytic progenitor cells and abnormal functioning stroma. The possible primary defect of BM MSCs in CIN has not been investigated.

Aim. To evaluate the quantitative and functional characteristics and immunoregulatory properties of BM MSCs in patients with CIN. Methods. BM MSCs were expanded from CIN patients (n=14) and healthy controls (n=21). MSC identification was based on the morphologic and immunophenotypic characteristics and their potential to differentiate towards adipocytes (Oil red-O stain and aP2/PPAR-γ expression), osteoblasts (ALP/Von Kossa stain and ALP/CBFA1 expression) and chondrocytes (Alcian blue stain and Collagen II/Aggrecan expression). MSC quantification in the BM fraction at day-0 was evaluated by limiting dilution assay. The functional characteristics of MSCs were studied by evaluating (a) their clonogenic potential using a CFU-F assay and enumerating the CFU-Fs/100MSCs through passages(P), (b) their proliferative potential time-course using MTT and the cell-doubling time, (c) their immunosuppressive properties by a 3H-thymidine-based mixed lymphocyte reaction (MLR) using normal purified CD3+ cells stimulated with PHA (2 mg/mL) or IL-2 (500IU/mL) in the presence or absence of allogeneic normal or patient MSCs from P2.

Results. Patient MSCs displayed the anticipated morpholo- gy and immunophenotype and normal chondrogenic/osteogenic/adipogenic potential. Patient MSC numbers at day-0 (14.64±14.53/10.000 BMMCs) did not differ significantly from the controls (23.78±16.49 MSCs/10.000 BMMCs). Compared to controls, however, patient MSCs displayed impaired CFU-F potential time-course (p<0.001; P1-P6) and defective proliferative capacity. This was demonstrated by the cell doubling-time (p<0.001; P1-P7) and the MTT assay (p<0.01 at P1). MLR assay showed that the percentage of inhibition of PHA- or IL-2-activated T-lymphocytes by MSCs did not differ significantly between CIN patients (75.77±7.48% and 80.41±15.97%, respectively) and controls (84.08±12.43% and 80.73±10.76%, respectively) suggesting normal immunosuppressive properties of BM MSCs in CIN. Patient MSC culture supernatants at P2 displayed normal levels of VEGF, IL-6, IL-1β, TNFα, SDF-1, LIF, but increased levels of TGF-beta1, compared to controls (p<0.05).

Conclusions. CIN patients display normal number, differentiation potential and immunosuppressive properties of BM MSCs but impaired clonogenic and proliferative potential probably due to the increased TGF-β1 production by the MSCs.

T-CELL RECEPTOR Vβ REPERTOIRE ANALYSIS AND CDR3 SPECTRATYPING IN PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA

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Objective. Cytotoxic T-cells (CTL) display a pathogenetic role in bone marrow (BM) failure syndromes. CIN is a BM failure syndrome characterized by presence of activated T-lymphocytes in BM and peripheral blood (PB). Aim of this study is to determine if clonal CTL expansions with possible pathogenetic significance occur in CIN patients. Methods. PB and BM samples from the 85 CIN patients were studied by a) flow cytometric analysis of TCRV, repertoire in CD3+, CD4+ and CD8+ cells and b) PCR analysis of CDR3 in CD4+ and CD8+ cells using a mixture of V, and J, primers. Family expansions were defined as above of 2SD from the mean values of 85 healthy controls.

Results. 69.41% of the patients displayed one or more prominent clones in PB CD3+ cells. The V,16 and V,12 were the most frequently expanded subsets in PB, identified in 52.94% and 14.12%, respectively. Furthermore, CIN patients as a group displayed statistically increased proportion of V,16 and V,12 subsets within the CD3+ PB cells (2.00±1.39% and 2.45±2.52%, respectively) compared to controls (0.90±0.29 and 1.66±0.54, respectively) (p<0.001 and p<0.01, respectively). In BM a proportion of 82.61% presented one or more prominent clones in CD3+ cells and V,16 and V,12 were also the most frequently expanded subsets (65.22% and 17.39%, respectively). Further analysis of CD4+ and CD8+ cells demonstrated that 58.62% and 74.19%, respectively, in PB and 93.75% and 75.0%, respectively, in BM of CIN patients displayed statistically increased proportion of V,16 and V,12 subsets within the CD3+ PB cells (2.00±1.39% and 2.45±2.52%, respectively) compared to controls (0.90±0.29 and 1.66±0.54, respectively) (p<0.001 and p<0.01, respectively). CDR3 spectratyping by PCR showed that oligo/monoclonality was present in BMCD8+ cells in PB and/or BM almost in 100% of the CIN patients. PCR analysis of CD4+ revealed that only three patients displayed a different pattern than a gaussian-like peak distribution of CDR3. Conclusion. These data support the hypothesis that CIN belongs to the immunemediated BM failure syndromes and elucidate the role of CTLs in the pathogenesis of the disease.
DISTURBANCES OF IMMUNOGLOBULINS IN PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA CORRELATE WITH INCREASED SERUM LEVELS OF TGF-β1 AND DECREASED B CELL EXPRESSION OF CD40

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Introduction. The immunoglobulin production by B-cells is under the surveillance of the T-cell system either through interaction of CD40/CD40L or through the production of a variety of cytokines that may impact the immunoglobulin class/subclass switching. CIN is characterized by the presence of activated T-cells in both peripheral blood and bone marrow. Immunoglobulin levels in CIN patients have not been extensively studied. Aim of the study. To evaluate the immunoglobulin classes/subclasses levels in CIN patients and probe the mechanisms associated with the underlying abnormalities, if any.

Patients and Methods. We studied 295 CIN patients and 82 healthy subjects. We evaluated: (a) serum IgM, IgA, IgG1, IgG2, IgG3, IgG4 levels, (b) CD40L induction on T-cells following incubation with PMA (10 ng/mL) and Ionomycin (500 ng/mL), (c) CD40 expression on CD19+ cells, and (d) serum TGF-β1 levels by ELISA. Results. CIN patients had higher IgM (160.4±90.7 mg/dL) but lower IgG3 and IgG4 (40±20 mg/dL, 55±21 mg/dL, 75.1±31.2 mg/dL, respectively) levels compared to controls (103.4±30.9 mg/dL, 55±21 mg/dL, 75.1±31.2 mg/dL, respectively) (p<0.0001, p<0.0001 and p<0.0001 respectively). Patient IgG1, IgG2, and IgA levels were normal. Interestingly, an inverse correlation was observed between the levels of IgM and the number of neutrophils (r=-0.2544, p<0.0001) and the levels of IgG3 (r=-0.3968, p<0.0001) and IgG4 (r=-0.3546 p<0.0001). CD40L induction on patient T-cells (42.46±41.07%, n=17) did not differ significantly from the controls (46.09±38.81%, n=17) (p=0.558) but the proportion of CD40+ B-cells was significantly lower in CIN patients (18.87±9.85%, n=12) compared to controls (33.15±13.53%, n=19) (p=0.0037). Serum TGF-β1 levels were significantly higher in CIN patients (56.68±31.18 ng/ml) compared to controls (19.21±10.69 ng/ml; respectively) (p<0.0001; respectively) and correlated positively with serum IgM (r=0.3610, p<0.0001) and inversely with serum IgG3 (r=-0.2544, p=0.008) and IgG4 (r=-0.3664, p<0.0001) levels. Conclusions. The increased IgM and decreased IgG3 and IgG4 levels in CIN patients, correlate with the severity of neutropenia and may be partly affected by the increased levels of serum TGF-β1, as well as, the decreased expression of CD40 on B-cells.

ANALYSIS OF FAS-LIGAND, TUMOR NECROSIS FACTOR-α AND TRANSFORMING GROWTH FACTOR-β1 GENE POLYMORPHISMS IN PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA

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Background-Objective. CIN patients display increased levels of FasL, TNFα, TGF-β1 in the bone marrow (BM). Single-nucleotide genetic polymorphisms at codon 10 (+869 T/C), codon 25 (+915 G/C) and promoter (-509 C/T) of TGF-β1 gene have been associated with increased cytokine concentrations in a variety of disease states. Polymorphisms of TNFα -308 G/A influences the gene expression and in the promoter of FasL, (-844 T/C) can modify gene transcriptional activity. The aim of this study was to investigate the presence of TNFα, TGF-β1 and FasL genetic polymorphisms in CIN.

Materials-Methods. We studied 57 CIN patients and 60 normal subjects. Genotype analysis for polymorphisms was performed using a PCR-based restriction fragment length polymorphism (RFLP) assay. TGF-β1 levels in long-term BM culture (LTBMC) supernatants were measured using ELISA. Polymorphism analysis was performed using χ² and Fisher’s exact tests. Levels of TGF-β1 were analysed by Mann-Whitney. Results. No statistically significant difference was found between patients and controls in genotype distribution of FasL, -844, TGF-β1 codon 10 and codon 25 polymorphisms. A significant difference was found in the number of patients carrying a C/T transition at the promoter (-509) of TGF-β1 compared to controls (p=0.0372). An increased risk was associated with the TT genotype (odds ratio [OR]=12.95% confidence interval =1.24-115.4; p=0.02) compared with wild-type genotype. TGF-β1 levels in LTBMCs were significantly increased in patients (135.5±461 pg/mL) compared to controls (60±200 pg/mL; p<0.0001). Patients with the C/T -509 polymorphism, displayed higher cytokine levels in LTBMC supernatants compared to patients with non-polymorphic genotype (p<0.0001). There was a different frequency of mutated A/A genotype of TNFα -308G/A between patients and controls but the association remained non significant (p=0.07). Conclusion. The presence of the C/T genetic polymorphism at the promoter of TGF-β1 (-509) is associated with CIN and with increased levels of this cytokine in the BM. Since TGF-β1 has been implicated in the pathophysiology of the disease by altering the homeostasis of the BM microenvironment, we postulate that genetic factors may have a role in the pathogenesis of CIN.
SEVERE CONGENITAL NEUTROPENIA

COMPOUND HETEROZYGOUS HAX1 MUTATIONS IN AN ITALIAN PATIENT WITH SEVERE CONGENITAL NEUTROPENIA ASSOCIATED TO NEURODEVELOPMENT ABNORMALITIES

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Objective. Homozygous mutations in the antiapoptotic gene HAX1 have recently been identified in some individuals, from Middle East, Japan and in the original Swedish Kostmann family, with autosomal recessive form of severe congenital neutropenia (SCN). In this study we describe an Italian patient with SCN associated to neurodevelopment abnormalities carrying compound heterozygous mutations in HAX1 gene. Methods. The HAX1 gene was amplified from genomic DNA extracted from peripheral blood of the patient and the other members of the family and directly sequenced on ABI PRISM 3100. The ELA2 gene was also analyzed. Results. Sequencing analysis of HAX1 in genomic DNA of patient identified two different heterozygous mutations within exon 3. A frame-shift mutation c.430_431insG leading to a premature stop codon Val144GlyfsX5 inherited from his father and a missense mutation c.389T>G generating a non conservative amino acid substitution Leu130Arg inherited from his mother. The latter is a novel mutation not detected in 80 healthy controls, excluding the possibility of a polymorphic change. Both parents and one brother, heterozygous carrier of the c.430_431insG, had no detectable clinical phenotype. Sequence analysis of the patient ELA2 gene showed a wild type genotype. Conclusion. This is the first described Italian patient with SCN associated to neurodevelopment abnormalities carrying compound heterozygous mutations in HAX1 gene. Since both mutations were found in exon 3, so affecting both published transcript variants of HAX1, we confirm the hypothesis that mutations affecting both isoforms are associated to SCN with an additional neurologic phenotype. Moreover our result indicates that HAX1 mutations are not limited to patients with specific ethnic origin. Because HAX1 is a ubiquitously expressed gene, but HAX1 mutations are relatively uncommon, further accumulation of the patients should be needed to better characterize the clinical features of SCN-HAX1 mutated.

T-LGL RELATED NEUTROPENIA

SEVERE NEUTROPENIA IN PATIENTS WITH T-CELL LARGE LYMPHOCYTIC LEUKEMIA

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Introduction. T-cell large lymphocytic leukemia (T-LGL) is a clonal lymphoproliferative disorder. Presenting features are usually neutropenia, anemia and/or thrombocytopenia. Three patients with neutropenia are presented. Methods. Three patients were admitted to our department for evaluation of neutropenia. Laboratory evaluation consisted of 1) morphological examination of peripheral blood smear, 2) bone marrow aspiration and biopsy, 3) radiological imaging, 4) immunophenotypic analysis of peripheral blood lymphocytes, 5) TCR-Vb repertoire analysis using flow cytometry, 6) PCR examination for the detection of clonal rearrangement of gamma-chain. T-LGL was confirmed as the final diagnosis in all 3 patients examined. Results. Patient No=1: presented with absolute neutropenia. Treatment with granulocyte growth factor (G-CSF), Cyclosporine (CyA), methotrexate (MTX) was ineffective. During a follow-up period of 8 month she developed 2 episodes of neutropenic fever, treated successfully with broad spectrum antibiotics. Finally she responded to treatment with 2-deoxycoformycine and she remains in remission for the last 4 years. Patient No=2: presented with an absolute neutrophil count (ANC) of ANC=600/µL. Treatment with G-CSF was effective although of transient duration. He responded to treatment with MTX and currently 2 years later he remains in remission. Patient No=3: presented with absolute neutropenia. Administration of G-CSF, and CyA had no effect. Treatment with 2-deoxycoformycine was associated with a transient response. During a follow-up period of 18 months she developed 3 episodes of neutropenic fever treated successfully with broad spectrum antibiotics. Currently she is on treatment with MTX, but without any response so far. Conclusion. More effective therapeutic modalities need to be developed for patients with T-LGL and severe neutropenia.
FAMILIAL CD3+ T LARGE GRANULAR LYMPHOCYTE PROLIFERATION PRESENTING WITH CYTOPENIAS: HISTOPATHOLOGICAL, FUNCTIONAL AND MOLECULAR STUDIES

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CD3+ T large granular lymphocyte (T-LGL) proliferations often present with cytopenias (especially neutropenia) and splenomegaly. We report results from detailed analysis of two relatives (father and son) with CD3+ T-LGL proliferation. Case 1 (father): thrombocytopenia, splenomegaly, positive test for anti-nuclear antibodies at the age of 55 (1999). Partial response to corticosteroids. 2002: splenectomy (initial pathology assessment non-specific findings), mild persistent neutropenia ever since. Case 2 (son): pancytopenia since the age of 17 (1993). Routine laboratory investigations (including bone marrow aspiration and biopsy); non-diagnostic. 2002: pancytopenia, splenomegaly, splenectomy. Partial recovery of hematologic values, persistent thrombocytopenia since 2003. Strikingly similar results were obtained for both cases on detailed laboratory investigations.

Flow cytometry (repeated tests 2003-2006): inverted CD4/CD8 ratio, increased CD3+CD8+CD57+ cells (father>30%, son: 18-22%). Antineutrophil antibodies (GIFT, GAT assays): negative. (3) Molecular testing for elastase mutations: negative. (4) Bone marrow biopsy: 10-15% interstitial T (CD3+CD20-) lymphocytic infiltration (CD8>CD4), increased cellularity, hyperplasia of all series with concomitant dysplastic changes. Spleen biopsy: (i) white pulp: hyperplasia of both the B zone (mantle/marginal zone, CD20+CD3-) and the T zone (CD20-CD3+), CD20>CD8 (ii) red pulp: moderate CD3+CD20- lymphocytic infiltration (CD8>CD4) of the splenic cords and sinuses. The most important difference between father and son concerned the patterns of T cell receptor beta (TRBV) rearrangements. (1) Father: monoclonal TRBV rearrangement amidst a polyclonal background. TRBV gene repertoire: 10 identical TRBV12-3/TRBD2/TRBJ2-6 rearrangements (major subclone) and 5 identical TRBV7-2/TRBD1/TRBJ1-2 rearrangements (minor subclone) from two timepoints over a 3-year period among a total of 51 colonies. (2) Son: polyclonal TRBV rearrangement. TRBV gene repertoire: heterogeneous. In conclusion, this is the first report of familial CD3+ T-LGL proliferation. The results from TRBV repertoire analysis support a role for antigen in lymphomagenesis, in the sense that persistent antigenic drive may initially lead to polyclonal proliferation and, under certain circumstances (genetic predisposition?), evolve to clonal disease (T-LGL leukemia). Finally, the findings reported here indicate that TCR antigen specificity may determine the clinical presentation by specifically recognizing and destroying distinct hematopoietic lineages.

NEUTROPENIA INVESTIGATION

LONG TERM FOLLOW-UP OF PATIENTS WITH ABNORMAL DIFFERENTIAL WHITE CELL COUNT-RELATIVE NEUTROPENIA AND/OR LYMPHOCYTOSIS

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Objective. The significance of the abnormalities of the differential white blood cell count is often obscured by their triviality. In this paper we report the long-term outcome of 60 patients with persistent and apparently inexplicable relative neutropenia and/or lymphocytosis. Patients with absolute lymphocytosis or with neutropenia attributed to definite medical conditions were excluded. Patients and Methods. 60 patients, 19 male/41 female, aged 18-77 years with abnormal differential count lasting more than 6 months, underwent complete physical examination and laboratory work-up including serial complete blood counts, morphology examination, biochemical and serological tests, ultrasonogram of the abdomen, immunophenotyping of the peripheral blood and/or bone marrow, bone marrow aspiration, karyotyping and bone marrow biopsy (47/60). The patients were followed-up for 1-18 years (median time 4 years). Results. 14/60 patients had absolute neutropenia; 4/14 subsequently presented myeloid disorders, while 10/14 remain asymptomatic. In 9/60 (5 absolutely neutropenic) there was evidence of drug-related neutropenia. 18/60 eventually developed lymphocytosis; after a median follow-up of 1-11 (median 2.5) years, 14/18 manifested a lymphoproliferative disorder, one had monoclonal B lymphocytosis and 3/18 reactive polyclonal lymphocytosis. 8/60 had high titers of anti-TPO and anti TG antibodies and 4/60 presented other collagen and autoimmune disorders. In 21/60 patients (30%) the reason of the relative neutropenia/lymphocytosis remains obscure. Of note, these patients have persistently abnormal counts of CD4/CD8 and NK lymphocytes. Conclusions. The persistence of unexplained relative neutropenia and/or lymphocytosis may be the early sign of an emergent disease. An extended laboratory diagnostic work-up is absolutely warranted.
SYSTEMIC EVALUATION OF NEUTROPENIA IN 183 PATIENTS FROM A SINGLE HEMATOLOGIC UNIT


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Objective. Neutropenia is a common medical problem. The aim of this study was to define the causes of neutropenia in a series of patients using a systemic approach.

Methods. 183 patients seen the last three years in our Department were included in this study. Patients’ evaluation included medical history, physical examination, complete blood counts (CBC), serial CBC, assessment of folate, vitamin B12, iron status, serum liver enzymes, serum proteins, immunoglobulin levels, tests for hepatitis B and C virus, cytomegalovirus, mononucleosis, human immunodeficiency virus and toxoplasmosis, serologic tests for thyroid and autoimmune diseases, abdominal ultrasonography, blood lymphocyte immunophenotyping, molecular detection of TCR monoclonality, bone marrow aspiration plus biopsy and karyotypic analysis.

Results. Patients median age was 55 (range, 18-83) with a male to female ratio 1:3. Chronic idiopathic neutropenia (CIN) was identified in 50%, autoimmune diseases in 14%, T-gamma lymphocytosis in 10%, infectious diseases in 5.5%, haematological diseases in 4%, nutritional deficiencies in 11%, drug-related neutropenia in 4%, neutropenia due to exposure to chemicals in 1% and ethnic neutropenia in 0.5%. Among patients with CIN, 46% had coexisting thyroid disorders. The presence of thyroid disorders in a significant proportion of patients with CIN is an interesting finding possibly indicating that thyroid disorders may cause immune or T-cell mediated neutropenia. The detection of antineutrophil antibodies was not performed in this study so it is possible that some cases of CIN may in fact represent primary autoimmune neutropenias.

Conclusions. Systemic evaluation of incidental neutropenia is highly needed in order to identify and possible treat the underlying cause, and provide clinicians a systemic approach for evaluating patients with neutropenia.

BONE MARROW FAILURE SYNDROMES

CLOPIDOGREL-ASSOCIATED APLASTIC ANAEMIA: SUCCESSFUL MANAGEMENT WITH CYCLOSPORIN-A

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Clopidogrel is an anti-platelet agent with chemical structure and function similar to ticlopidine, it has a proven efficacy in cardiovascular diseases and is considered to be safer to ticlopidine, hence its increasing usage. Although bleeding is the commonest adverse event there have been reported some rare non bleeding side effects potentially fatal which are rather under-estimated. Herein, we present a case of Clopidogrel-associated aplastic anaemia with a favourable outcome probably due to the prompt initiation of treatment. A 78 year old lady was started on clopidogrel 75 mg per day following a diagnosed transient ischemic attack. Three months after clopidogrel was commenced a routine FBC showed pancytopenia, Hb 9.2 g/dL, WCC 1.87×109/L, neuts 0.24×109/L, PLT 67×109/L, and reticulocytes 6×109/L. A bone marrow aspirate was performed which showed marked hypocellularity and absence of myelodysplastic features. A tentative diagnosis of aplastic anaemia was confirmed with a trephine bone-marrow biopsy. There was no clinical, biochemical, serological or molecular evidence of hepatitis, autoimmune disorders or recent infection by EBV, CMV, Parvovirus or HIV. Given its previously reported link with aplastic anaemia Clopidogrel was discontinued. In the following 3 weeks the patient’s pancytopenia became worse and the patient eventually became transfusion-dependent. Two weeks after presentation, treatment with cyclosporine (CSA) 5 mg/kg per day was started. Ten and 13 weeks after CSA commencement, neuts recovered to higher than 1.5×109/L and PLTs exceeded 100×109/L respectively and the patient became transfusion independent. Five months after CSA commencement the FBC recovered completely with Hb11 g/dL, neuts 4.5×109/L, PLT 230×109/L. The patient discontinued CSA after seven months and retains normal counts for over 18 months.

BLACKFAN-DIAMOND ANAEMIA: DEMONSTRATION OF A CASE

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Objective. Blackfan-Diamond anemia is a rare congenital hypoplastic anemia with an incidence rate about 1 in 200,000. Patients and methods. We report a sporadic case of 32 year-old woman with Blackfan-Diamond anemia, diagnosed in the first few months of her life. Results. Anemia is severe, nonresponsible to erythropoietin therapy and the patient is transfusion-dependent. Neutropenia (absolute neutrophil count between 0.5-2.5×109/L)
was intermittently registered. Despite from iron chelation therapy anemia is complicated by secondary hemosiderosis. Growth retardation but with normal somatotropic hormone secretion and endocrine abnormalities (hypothyroidism and hypogonadism) due to hemosiderosis are found. Cytogenetic analysis does not reveal any abnormalities. Conclusions. We report a rare case of bone marrow failure with severe complications.

KINETICS, FUNCTION AND BONE MARROW TRAFFICKING OF CD4+CD25HIGH FOXP3+ REGULATORY T CELLS (Tregs) IN MYELODYSPLASTIC SYNDROMES

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Objectives: Tregs prevent autoimmunity, but may convey excessive suppression of antitumor immunity. Treg compartmentalization influences their suppressor potential and, in bone marrow (BM), is mediated via CXCL12/CXCR4 signals. Malignancy and autoimmunity coexists in MDS, therefore we speculated that these processes might be subject to aberrant regulation by Tregs. Patients and methods. Fifty-four MDS patients and 14 normal age-matched individuals were recruited. Based on IPSS, patients were subdivided in early-stage MDS (E-MDS, low/intermediate-1 risk, n=37) and late-stage MDS (L-MDS, intermediate-2/high risk, n=17). Treg frequency and CXCR4 expression were measured by flow cytometry. Treg suppressor activity was assessed by CFSE-based proliferation assays and inhibition of IFN-γ and TNF-α secretion of MACS-isolated CD4+CD25+ OR - cells. PB CD4+ cells from patients and controls were induced to migrate towards CXCL12 or BM fluid (BMF). CXCL12 was detected by ELISA. Results. Peripheral blood (PB) (17.8±3.3 cells×10⁶/L) and BM (2.28±0.4%) Tregs were expanded in L-MDS patients compared to controls (PB: 7.5±0.9 cells×10⁶/L, p=0.01, BM: 1.1=0.33%, p=0.01) and E-MDS patients (PB: 6.5±0.6 cells×10⁶/L, p=0.01. BM: 0.79±0.14%, p=0.01). Tregs were profoundly increased in two L-MDS patients who progressed to overt leukemia, while they remained unchanged in either E-MDS or L-MDS patients (n=13) with stable disease over a period of 9 months. In E-MDS patients, Tregs were dysfunctional compared to controls and L-MDS patients (p<0.001 in both PB and BM) and failed to suppress IFN-γ and TNF-α production compared to controls (IFN-γ: p<0.001 and p=0.088, TNF-α: p=0.015 and p=0.1 for PB and BM, respectively) and L-MDS patients (IFN-γ: p=0.046 and p=0.036, TNF-α: p=0.13, and p=0.4, for PB and BM, respectively). In E-MDS, CXCR4 was downregulated on PB Tregs (15.8±1% vs 24.9±1.3%, p=0.002 in controls vs 25.9±3.3%, p=0.001 in L-MDS), which also exhibited decreased chemotaxis towards CXCL12 compared to controls (p=0.036) and L-MDS patients (p=0.086). Additionally, E-MDS patients displayed significantly decreased Tregs in BM (1.4±0.22%) compared to PB (0.79±0.14%, p<0.001). Finally, normal Tregs migrated equally towards BMF from all groups and BM CXCL12 levels were comparable among all groups. Conclusions. Defective suppressor function and BM trafficking of Tregs may foster the autoimmune process and BM failure of E-MDS, but increased Treg activity could favour leukemic clone progression in L-MDS. Our findings provide novel evidence for a link between Treg-mediated cancer immunomodulation and malignant hematopoiesis.
MISCELLANEOUS

DYNAMICS OF HOST-PARASITE IMMUNE RESPONSES INCORPORATING PIGMENT CONTAINING NEUTROPHILS AND MONOCYTES, AND COMPLIMENTARITY TO CONVENTIONAL PERIPHERAL PARASITE COUNT IN MALARIA DIAGNOSIS

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Seven malaria patients (5 mild and 2 severe), clinically diagnosed presumptively for malaria and referenced for laboratory test were diagnosed by microscopy. 2 of the 5 mild malaria (MM) patients recorded positive outcome for presence of malaria parasites, using peripheral parasite count (PPC) at 2.5% and 2.0% levels of parasitemia. 3 of the 5 MM patients recorded no presence of parasites (0% parasitemia) while one of the 2 severe malaria (SM) patients recorded 0% parasitemia, despite manifesting symptoms of mild (uncomplicated) and severe malaria respectively, indicating uncertainty and need to compliment this diagnostic parameter of the PPC. Malaria pigments were not observed in any of the MM patients but in the SM patients with PCM: PCN ratios of 1:2 and 1:4 respectively. The period encompassing haemozoin release and accompanying chemical metabolites, and its consequent phagocytosis by neutrophils (N) and monocytes (M) are periods of more pronounced damages and manifested pathology in the SM patients in which PCM and PCN counts were observed. Factors that could have influenced PCM and PCN counts which are aftermaths of phagocytosis through two independent events in time and space, having haemopoietic embryonic stem cell origin with an accompanied immune coordination include: physiological pH of the body, circulating antibodies and complements, chemo-attractants, rate of active metabolism and globin digestion by parasites, and the influence of antimalarials taken prior to blood smear examination. The results from this study indicates that the PCN and PCM could compliment uncertain diagnosis by PPC and in monitoring progress made on treatment; with impacts on epidemiological.
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