Disorders of Neutrophil Number and Function

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This review of disorders of neutrophil number and function will discuss important research advances in the field and then provide a clinical diagnostic approach. The focus will be on two recent clinical developments in the field of phagocyte disorders. First, an important natural history study from the Severe Chronic Neutropenia International Registry has recently quantitated the incidence and risk factors for death from sepsis and for progression to myelodysplastic syndrome and acute myeloid leukemia in a large cohort of severe chronic neutropenia patients, many of whom were followed 10 or more years on treatment with granulocyte colony-stimulating factor. Second, in the past year, a multinational group has announced successful gene therapy of two adults with chronic granulomatous disease, the most common disorder of neutrophil function. However, monitoring of retroviral insertion sites revealed expansion of the multiclonal population of gene-modified cells, raising concerns about eventual leukemogenesis. The review also provides a pragmatic approach to the evaluation of a patient with a suspected disorder of neutrophil number or function.

Severe Congenital Neutropenia and Cyclic Hematopoiesis

The seemingly distinct disorders of severe chronic neutropenia (SCN; also known as Kostmann syndrome) and cyclic hematopoiesis (CH; also known as cyclic neutropenia) have recently been shown to derive largely from heterozygous germline mutations in a common gene, ELA2, encoding neutrophil elastase. Although the exact molecular pathophysiology remains unknown, the mutations show an autosomal dominant inheritance pattern and induce accelerated apoptosis in differentiating myeloid cells. The autosomal dominant pattern of inheritance has been demonstrated most dramatically by the discovery of SCN in 5 children from 4 kindreds, all of whom shared the same ELA2 mutation—and the same sperm donor.

Additional, very rare cases of SCN derive from mutations in the Gfi1 gene (encoding a transcriptional repressor that regulates ELA2 expression), constitutional mutations of the granulocyte colony-stimulating factor (G-CSF) receptor gene, or allelic variants of Wiskott-Aldrich syndrome. However, a sizeable minority of SCN patients have no identified causative mutation and may indeed represent a separate, autosomal recessive disease as originally proposed by Kostmann. The genetics and cellular pathology of SCN and CH were reviewed at a previous ASH Education Session and will not be further discussed here.

Most SCN patients and virtually all CH patients respond to therapy with G-CSF (filgrastim), with SCN patients generally requiring higher doses. Prior to the G-CSF era, approximately half of SCN patients died of bacterial sepsis in the first year of life and the remainder in early childhood. Those who survived longer appeared to be at increased risk for the development of myelodysplastic syndrome and acute myeloid leukemia (MDS/AML). However, now that G-CSF therapy has made SCN a chronic disease, the relative incidence of these two major complications has reversed: the development of MDS/AML is now more common than death from bacterial sepsis. During long-term therapy with G-CSF, some SCN patients acquire mutations in the G-CSF receptor gene, then myelodysplasia characterized by monosomy 7, and finally myeloid leukemia. A key question has been whether long-term treatment with G-CSF contributes to the development of this complication or only permits its evolution by prolonging survival. A recent report from the Severe Chronic Neutropenia International Registry (SCNIR; http://depts.washington.edu/registry/) has quantitated the incidence and some of the risk factors for sepsis and MDS/AML through a registry-based study of 374 SCN patients, many of whom were followed for 10 years or more. The cumulative incidence of death from sepsis was 8% after 10 years of G-CSF therapy, and the cause-specific hazard of death from sepsis was stable over time at 0.9%/year. In contrast, the cumulative incidence of MDS/AML was 21% after 10 years on G-CSF and 36% after 12 years, with the cause-specific hazard of MDS/AML increasing over time from 2.9% per year at 6 years up to 8% per year after 11.7 years of therapy. Thus, the SCNIR analysis showed for the first time an increasing risk of MDS/AML as patients receive G-CSF for longer periods of time. Importantly, the SCNIR analysis also identified a subgroup of SCN patients at greatest risk for MDS/AML. Table 1 shows the relative hazard of MDS/AML (white boxes) and death from sepsis (shaded boxes) in four subgroups of the 238 SCN patients with adequate data for the analysis. The subgroups are classified by G-CSF dose above or below the median dose and by on-treatment absolute neutrophil count (ANC) above or below the median. Using the "best respond-
ers” (G-CSF dose < 8 µg/kg/day and ANC at 6-18 months on therapy of ≥ 2188) as a referent group, the relative hazard of the two major complications was increased only in the “worst responder” group with G-CSF dose ≥ 8 µg/kg/day and ANC < 2188. These patients had a 4.5-fold increased risk of MDS/AML (significant at p = 0.008) and a 3.8-fold increased risk of death from sepsis (trend not significant at p = 0.094). However, comparison of the worst responder group to the pool of the other three quadrants of the table resulted in relative hazards of 3.1-fold for MDS/AML and 5.6-fold for sepsis (both comparisons significant, at p = 0.002 and p = 0.004, respectively). In the worst responder group, the cumulative incidence at 10 years was 40% for MDS/AML and 14% for death from sepsis, compared to 11% and 4%, respectively, for the best responder group. However, it is notable that even the best responders were not free of the risk of MDS/AML or even death from sepsis. Because data are not available on the patients’ blood counts at the onset of sepsis, it is not known whether these patients are at risk for sepsis at any time or only when below some ANC threshold.

The SCNIR data confirm the previously-published findings of the French Severe Chronic Neutropenia Study Group,11 which reported that the risk of MDS/AML rose over time and correlated with higher doses of G-CSF. However, in this somewhat smaller study the cumulative incidence of MDS/AML was lower — overall 2.7% at 10 years and 8.1% at 20 years — and the French group reported no deaths from sepsis in patients receiving G-CSF therapy. Notably, both the current and previous studies showed a higher risk of MDS/AML in patients with Shwachman-Diamond syndrome but no progression to myelodysplasia or leukemia in cyclic hematopoiesis.9,10 The latter finding is particularly interesting because, as discussed above, both cyclic hematopoiesis and most cases of SCN derive from mutations in the same gene, ELA2.

Despite the risk of MDS/AML, the use of G-CSF for SCN patients still represents a major therapeutic advance over the previous practice of antibiotic therapy alone. The SCNIR and French studies also reinforce the importance of yearly monitoring of bone marrow morphology and cytogenetics in patients with SCN or Shwachman-Diamond syndrome. The appearance of cytogenetic abnormalities, particularly monosomy 7, usually precedes the development of MDS or AML.9 As hematopoietic stem cell transplantation is more likely to be successful when undertaken prior to progression to MDS/AML,12,13 elective transplantation should be considered at that point. For patients in the “worst responder” category of high G-CSF dose requirements and low ANC on therapy, early transplantation should also be considered, depending upon the availability of a matched (preferably sibling) donor.

### Chronic Granulomatous Disease

Chronic granulomatous disease (CGD), the most common disorder of phagocytic function, generally presents early in life with severe recurrent infections, primarily affecting natural barriers such as the respiratory tract and lymph nodes, and eventually internal organs, such as the liver, spleen, bones and brain.14-16 Infections are generally caused by catalase positive bacteria, such as *Staphylococcus aureus* and gram-negative bacilli, as well as fungi, including *Aspergillus* and *Candida*.17 The molecular defects causing CGD result in the absence, low expression, or malfunction of one of the phagocyte NADPH oxidase components responsible for the generation of microbicidal reactive oxygen species. X-linked CGD results from mutations in the *CYBB* gene encoding the cytochrome b heavy chain protein, gp91-phox. The molecular genetics and pathophysiology of CGD were thoroughly reviewed by Dr. Mary Dinauer at the 2005 ASH education session.18

CGD has long been considered a prime target for gene therapy, because clinical improvement should occur with replacement of a low level of oxidase activity (as occurs in relatively asymptomatic patients with “variant” CGD19) or with high level correction of only 5-10% of phagocytes (as occurs in asymptomatic carriers of X-linked CGD). Most approaches have relied upon retroviral transduction of bone marrow or peripheral blood hematopoietic stem and progenitor cells. Although considerable success has been achieved in murine models of CGD,20,21 scale-up to humans has proven difficult. For example, a phase I trial of *ex vivo* gene therapy with p47-phox for autosomal recessive CGD resulted in the production for 2-6 months of oxidase-normal neutrophils, but only in a low proportion (1:5000) of peripheral blood leukocytes.22

However, in the past year, a multinational group has announced successful gene therapy in 2 adult X-linked CGD patients.23 After conditioning with liposomal busulfan, the patients were infused with autologous CD34+ peripheral blood stem cells transduced with a monocistronic gp91-phox–expressing retroviral vector, termed...
SF71gp91\textsuperscript{phox}. This vector drives gp91-\textit{phox} expression from the spleen focus-forming virus (SFFV) long terminal repeat, which is transcriptionally active in hematopoietic stem cells and myeloid progenitors. Correction of the CGD phenotype was highly successful in patient 1, whose peripheral blood leukocytes showed phagocyte oxidase activity in 10-20\% of cells at day 100 post infusion, with a peak of 57\% at day 300, and then maintenance at levels of 40-50\% beyond day 500. The expressed gp91-\textit{phox} formed functional heterodimers that supported superoxide production at rates 25-30\% of normal in the corrected peripheral blood granulocytes from this patient and improved bacterial killing of \textit{Escherichia coli} to a level intermediate between CGD and normal cells. As the phagocyte oxidase has also been proposed to be critical to the degradation of microbes,\textsuperscript{26} it is notable that ingested \textit{E coli} and \textit{Aspergillus fumigatus} organisms showed signs of necrosis and irregular morphology in electron micrographs of corrected phagocytes.

In patient 2, although initial assays showed 35-40\% oxidase-positive leukocytes, the proportion fell to 10-15\% by day 100 post infusion and remained at that level to day 500. His cells produced superoxide, but at a lower rate than those from patient 1, and did not show significant improvement in bacterial killing activity. Nonetheless, both patients appeared to benefit clinically, with resolution of \textit{S aureus} liver abscesses in patient 1 (who was able to discontinue antibiotics at day 65) and of invasive pulmonary aspergillosis in patient 2. However, patient 2 had reactivation of chronic bacterial hidradenitis after the gene therapy procedure.

The striking and unusual increase in gp91-\textit{phox}-expressing cells in patient 1 more than 100 days after infusion of the transduced stem cells appeared to derive from an expansion of multiple transduced clones, which was observed in both subjects.\textsuperscript{23} Unlike gene therapy for severe combined immunodeficiency or Fanconi anemia, replacement of the defective gp91-\textit{phox} gene should not confer any growth or survival advantage on the stem cell or its progeny. Nonetheless, the investigators’ careful monitoring of retroviral insertion sites in the subjects’ genomic DNA revealed expansion of the population of gene-modified cells with emergence of multiple predominant clones, starting 5 months after the infusion of transduced cells. These clones contained insertions at only three genetic loci: \textit{MDS1/EVI1} and \textit{PRDM16}, which encode zinc finger transcription factors, and \textit{SETBP1}, a candidate tumor suppressor gene.\textsuperscript{23} These sites are homologous to, or associated with, genes previously associated with immortalization by insertional mutagenesis or with naturally occurring leukemias.\textsuperscript{25-27} The common integration sites were first detectable in the 2 subjects’ peripheral blood at 149 and 157 days after infusion, and reached predominance, representing > 80\% of insertions in circulating transduced cells, over the next 100-150 days. However, the insertional clones showed stable proportionate contributions, remained polyclonal, and were not associated with any elevation of peripheral blood leukocyte numbers.

The clonal expansion of cells transduced with a retroviral vector integrated at leukemia-associated genetic loci raises concerns about leukemogenesis, as observed in the recent French gene therapy trials for severe combined immunodeficiency.\textsuperscript{28,29} Despite the stability of clonal expansion and the regulated proliferation and differentiation of transduced progenitors, the possibility of additional somatic mutation presents a risk of malignant evolution. This process may be latent for a long period, as demonstrated by the 3-year latent period for the appearance of leukemia in the immunodeficiency trial\textsuperscript{20} and a 5-year delay recently reported in a rhesus macaque model.\textsuperscript{30} Thus, the long-term outcome of this otherwise promising trial of gene therapy will need careful evaluation before the treatment can be recommended to other CGD patients.

### Diagnostic Approach to Neutrophil Disorders

As there is no proven, evidence-based method for the evaluation of a child with a suspected disorder of neutrophil number or function, this review will present the author’s personalized pragmatic approach.

**Table 2** presents a summary of primary and secondary
forms of neutropenia. The differential diagnosis in any individual case depends upon the severity and duration of neutropenia, leukocyte and bone marrow morphology, and associated hematological or congenital abnormalities. Acquired neutropenia, which is far more common than congenital forms, often accompanies viral infection and requires only monitoring of blood counts until recovery. Although most often due to suppression of myelopoiesis during or after infection, destructive depletion of bone marrow reserves can also markedly reduce the ANC in overwhelming sepsis, particularly in the newborn. Drug-induced neutropenia, either due to myelosuppression or antibody-mediated destruction, can be caused by almost any drug, including antibiotics, anticonvulsants, anti-inflammatory agents, antithyroid agents, diuretics, and phenothiazines. If the offending drug can be discontinued, time provides both the diagnosis and the cure. Sequestration and destruction of neutrophils by autoantibodies is relatively common, both the diagnosis and the cure. Sequestration and destruction of neutrophils by autoantibodies is relatively common, both the diagnosis and the cure.

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Although practice parameters have been published recently for the diagnosis of a wide range of primary immunodeficiencies, including phagocyte defects, few tests are widely available for specific evaluation of phagocyte function, and the consensus guideline’s algorithm (http://www.guideline.gov/algorithm/4445/NGC-4445.4.html) can be refined, as follows, to guide testing in accordance with the clinical presentation.

The most important step in the diagnosis of a functional disorder of neutrophils is to have a clinical suspicion of a phagocyte disorder. Many patients experience a delay in diagnosis; for example, in the National CGD Registry, the mean ages at diagnosis for X-linked and autosomal recessive CGD were 3.0 and 7.8 years, respectively. As noted by Richard B. Johnston, Jr., “These ages are surprisingly high. At least, they are much higher than they should be for the sake of the patient.”14 Internists should note that several CGD patients, generally with milder “variant” phenotypes, have been diagnosed in adulthood, including one at age 69 years.35

Specific clinical features characteristic of phagocyte defects are listed in Table 3. This list includes only the more typical histories and physical or laboratory findings, as functional disorders of neutrophils can present with highly variable clinical features. Many clinicians in the field have also had the unfortunate experience of making the post-mortem diagnosis of a phagocyte disorder. With the exception of hyper-IgE syndrome (also known as “Job’s syndrome” because of the associated recurrent boils), invasive, rather than superficial, infections are the hallmark of an underlying phagocytic disorder.

Physical examination may discern diagnostic features such as partial oculocutaneous albinism in Chédiak-Higashi...
syndrome, the characteristic facies of hyper-IgE syndrome, or the complex phenotype of congenital disorder of glycosylation, type IIc (also termed leukocyte adhesion disorder, type 2). Although delayed separation of the umbilical cord is associated with leukocyte adhesion disorder type 1, most such babies represent one end of the normal distribution rather than this rare disease and thus do not require evaluation of leukocyte CD18 (see below) unless they also demonstrate marked leukocytosis.

Laboratory evaluation should proceed by a focused set of tests (listed in Table 4), directed toward specific diseases on the basis of clinical features of a case and the prevalence of each disorder. Assays required for the diagnosis of some of the rare or newly defined disorders can be performed only in a research laboratory and should be discussed first with an expert in the field.

In general, my approach is to test for CGD in virtually all cases of suspected phagocyte disorder, unless there is already a compelling reason to focus on another diagnosis, because CGD is the most common of these rare disorders and most protean in clinical manifestations. Phagocyte oxidase assays for the diagnosis of CGD are provided by many hospital and reference laboratories. The classic histochemical assay of nitroblue tetrazolium (NBT) dye reduction has been largely replaced by flow cytometry, using a fluorescent dye such as dihydrorhodamine (DHR). These assays can diagnose classic CGD, variant CGD with reduced but measurable respiratory burst activity, and the carrier state for X-linked CGD. Further classification of affected patients by oxidase component or gene mutation—provided by a research or commercial genetic testing laboratory—may be useful for prognosis, genetic counseling, or eventual gene therapy.

The morphology of giant granules evident in Chédiak-Higashi leukocytes (including granulocytes and large granular lymphocytes) may be difficult to detect in the peripheral blood but are displayed unmistakably in bone marrow aspirate smears. Examples may be found in most blood cell atlases or the ASH image bank.

Diagnostic tests for chemotactic disorders are generally available only in research laboratories, because the in vitro assays are difficult to standardize and prone to artifact, and the in vivo Rebuck skin window procedure is time consuming and operator-dependent. As the treatment for chemotactic disorders consists primarily of continuous antibiotic prophylaxis (which would probably be indicated

Table 3. Clinical features of disorders of neutrophil function.

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Associated Neutrophil Disorders*</th>
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<tbody>
<tr>
<td>Recurrent bacterial and/or fungal infections</td>
<td>All</td>
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<tr>
<td>Single opportunistic infection</td>
<td>All</td>
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<tr>
<td>Organisms associated with specific disorders:</td>
<td></td>
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<tr>
<td>Aspergillus</td>
<td>CGD</td>
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<tr>
<td>Atypical mycobacteria</td>
<td>Atypical mycobacteriosis† (OMIM 209950)</td>
</tr>
<tr>
<td>Bacillus Calmette-Guerin (BCG) – disseminated</td>
<td>CGD, atypical mycobacteriosis†</td>
</tr>
<tr>
<td>Burkholderia cepacia</td>
<td>CGD</td>
</tr>
<tr>
<td>Candida – invasive</td>
<td>CGD</td>
</tr>
<tr>
<td>Candida – mucocutaneous</td>
<td>Hyper-IgE syndrome (OMIM 147060)</td>
</tr>
<tr>
<td>Serratia marcescans</td>
<td>CGD</td>
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<tr>
<td>Marked leukocytosis with neutrophilia</td>
<td>Leukocyte adhesion disorder, type 1 (OMIM 116920);</td>
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<tr>
<td>(absolute neutrophil count 30,000-100,000/mm³)</td>
<td>congenital disorder of glycosylation, type IIc (OMIM 266265)</td>
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<tr>
<td>Delayed separation of the umbilical cord (&gt; 30 d)</td>
<td>Leukocyte adhesion disorder, type 1</td>
</tr>
<tr>
<td>Partial oculocutaneous albinism</td>
<td>Chédiak-Higashi syndrome (OMIM 214500)</td>
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<tr>
<td>Impaired resorption of primary teeth; coarse facies</td>
<td>Hyper-IgE syndrome</td>
</tr>
</tbody>
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†Hereditary atypical mycobacteriosis represents a defect in monocyte and macrophage, rather than neutrophil, function. It is included because of the overlap in presentation with congenital disorders of neutrophil function, most of which also affect monocyte/macrophage function.

In vitro

Table 4. Laboratory tests for disorders of neutrophil function.

<table>
<thead>
<tr>
<th>Disorders</th>
<th>Assays</th>
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<tbody>
<tr>
<td>Atypical mycobacteriosis (hereditary)†</td>
<td>Interferon-gamma and interleukin-12 pathways*</td>
</tr>
<tr>
<td>Chronic granulomatous disease (CGD)</td>
<td>Phagocyte oxidase activity</td>
</tr>
<tr>
<td>Chédiak-Higashi syndrome</td>
<td>Leukocyte morphology</td>
</tr>
<tr>
<td>Chemotactic disorders</td>
<td>In vitro chemotaxis,* Rebuck skin window</td>
</tr>
<tr>
<td>Congenital disorder of glycosylation, type IIc</td>
<td>CD15 expression</td>
</tr>
<tr>
<td>Hyper-IgE syndrome</td>
<td>Serum IgE, chemotaxis*</td>
</tr>
<tr>
<td>Leukocyte adhesion deficiency (LAD), type 1</td>
<td>CD18 expression (or function*)</td>
</tr>
</tbody>
</table>

*Usually available only in a research laboratory
†See footnote to Table 3
for recurrent cutaneous infections in any case), clinical use of these assays remains very limited.

Widely available flow cytometric assays for CD18 are usually sufficient for the diagnosis of leukocyte adhesion disorder type 1. However, some patients have mutations that inactivate the beta-2 integrin without interfering with its surface expression; so functional testing in a research laboratory may be warranted if CD18 antigenic testing by flow cytometry is normal in a clinical setting consistent with the disease. Deficiency of CD15 (Lewis*)40,41, among the many glycoproteins abnormally fucosylated in congenital disorder of glycosylation, type IIC, can also be detected readily in most clinical flow cytometry laboratories.

All of the tests discussed above are focused on single diseases, generally caused by one or more gene mutations transmitted by classical Mendelian inheritance. However, future evaluations for defects in innate immunity are likely to involve simultaneous testing of many single nucleotide polymorphisms (SNPs), both within and outside coding regions of the human genome. Several specific SNPs have already been associated with infection susceptibility that is less severe, but perhaps more common, than the recognized phagocyte disorders.40,42 Although the influence of single SNPs may be detected most readily in populations with a background of immune deficiency, such as cancer therapy or CGD, combinations of less extreme defects (most likely analyzed by microarray) may provide clues to the genetic basis of the susceptibility of patients with recurrent infections but no major disorder of phagocyte function.

References