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TITLE: Neutrophil Defects

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Slide 1: Introduction

Hello, my name is Vijaya Knight. I am the Director of the Diagnostic Immunology Laboratory and Assistant Professor in the Department of Medicine at National Jewish Health in Denver, Colorado. Welcome to this Pearl of Laboratory Medicine on "Neutrophil Defects."

Slide 2: Background

Neutrophils or polymorphonuclear leukocytes (PMN) are bone marrow-derived, terminally differentiated innate immune cells. They are the most abundant white blood cell in peripheral circulation. They are maintained at 3000-6000 cells/mm^3, comprise 30-50% of the white blood cell population, and are produced at a rate of 10^9 per hour in a healthy adult. Neutrophils are short-lived cells and are constantly replenished from the bone marrow. In fact, the bone marrow represents 90% of the total available PMN pool, with just 3% in circulation. PMNs are characterized by their segmented nuclei, divided into 3 to 5 lobules, and are thus readily identified by light microscopy. Cytoplasmic granules are also a characteristic feature of PMNs. These granules mediate a variety of antimicrobial activities.

Granules are classified into:

- primary or azurophilic (examples include proteinase 3, elastase, and alpha-defensins)
- secondary or specific granules that contain lactoferrin and cathelicidin peptides
- tertiary granules that contain gelatinase and are markers of terminal neutrophil differentiation
- secretory granules that contain various receptors that insert into the cell membrane following activation and enable the neutrophil to respond to a variety of stimuli

Neutrophils are key components of the innate immune system and form the first line of defense against bacterial and fungal infections. Given the central role that neutrophils play in the innate immune response to infection, defects in neutrophil numbers or function can lead to infections with serious consequences. In the following slides, I will discuss neutrophil defects, clinical presentation, and laboratory evaluation.

Slide 3: Neutrophil Defects

Lack of neutrophils or compromised neutrophil function can lead to overwhelming infections with bacteria and fungi. Primary immune deficiencies occur due to inherited genetic defects and often affect a component or components of the immune system. Inherited neutrophil defects account for about 5% of primary immune deficiencies. Such defects characteristically present with an increased frequency of infections at epithelial surfaces. The causative agents are most often bacteria or fungi; these infections tend to be aggressive and can be life-threatening. Management of neutrophil defects includes antibiotic treatment and in some cases, lifelong prophylaxis. Genetic defects are treated with bone marrow transplant or gene therapy.

Slide 4: Neutrophil Defects

Neutrophil defects can be broadly divided into those associated with neutrophil development and those associated with neutrophil function. Developmental defects, resulting in neutropenia (defined as < 1500 cells/mcL), are intrinsic and are due to inherited genetic defects that involve genes along the neutrophil developmental pathway, for example *ELANE*, the gene encoding neutrophil elastase. Neutrophil development may also be compromised by extrinsic or acquired causes, examples of which include neutropenia due to chemotherapy or autoimmune processes.

Defects of neutrophil function manifest as defects of the various biological processes that are involved in the bactericidal activity of neutrophils and include defects of arrest on the endothelium or adherence, defects of motility or chemotaxis, defects of oxidative burst, and defects of extracellular bactericidal mechanisms such as the release of neutrophil extracellular traps or NETS.

Similar to neutrophil number, both intrinsic and extrinsic factors may affect neutrophil function. Examples of intrinsic or genetic defects include mutations of the *CYBB* gene that encodes one of the membrane bound units of the NADPH complex. Such mutations have deleterious effects on oxidative burst and result in overwhelming bacterial infections. Extrinsic factors that affect neutrophil function are less well-defined. Infectious disease (e.g., influenza), burns, or alcoholism are examples of extrinsic factors that can affect neutrophil chemotaxis, oxidative burst, or adherence to varying degrees and increase susceptibility to bacterial and fungal infections.

In this Pearl, I will focus on intrinsic and extrinsic causes of neutropenia, as well as genetic defects of adherence, oxidative burst, and chemotaxis. The next two slides list some examples of intrinsic and extrinsic factors leading to compromised neutrophil development and therefore, to neutropenia.

Slide 5: Examples of Intrinsic Defects

This table lists a few examples of intrinsic genetic defects that lead to compromised development of neutrophils and thereby to neutropenia. Severe congenital neutropenia (SCN) is a heterogeneous disorder resulting from mutations in various genes. Although SCN is considered a monogenic disorder, mutations in multiple genes have been noted and while one of the mutations may be the dominant cause of SCN, a second deleterious mutation may

worsen disease. Genetic defects leading to SCN may be inherited in an autosomal dominant or recessive manner, or may arise sporadically.

Schwachman-Diamond syndrome, an autosomal recessive condition, is characterized by moderate to severe neutropenia with growth retardation. Other autosomal recessive conditions leading to neutropenia as well as defective neutrophil granule formation include specific granule deficiency, Griscelli syndrome, and Chédiak–Higashi syndrome. These are just a few of the genetic defects that affect neutrophil number and function. A more comprehensive list of defects can be found in the 2010 British Journal of Hematology paper by Gerben Bouma et al, listed at the end of this Pearl.

Slide 6: Acquired Neutropenia

Acquired neutropenia is due to extrinsic causes that affect neutrophil generation or survival, or prevent access to peripheral circulation. Acquired neutropenia may be due to decreased output, as noted in the case of bone marrow suppression due to chemotherapy with therapeutics such as cyclophosphamide, methotrexate, azathioprine, and colchicine.

Increased peripheral destruction leading to neutropenia may be an alloimmune process whereby transplacental passage of anti-neutrophil antibodies that recognize paternal neutrophil-specific antigens target fetal neutrophils.

Autoimmune disorders such as ALPS (Autoimmune Lymphoproliferative Syndrome) or ITP (Idiopathic Thrombocytopenic Purpura) may be associated with anti-neutrophil antibodies that lead to peripheral destruction of neutrophils.

Finally, neutropenia may be a result of sequestration of the cells in the spleen due to secondary disease processes that lead to splenic enlargement. Some examples of sequestration of neutrophils due to splenomegaly are malaria, portal hypertension, and Felty’s syndrome.

Slide 7: Mediators of Leukocyte Adhesion

We now move on to defects of neutrophil function. Leukocyte Adhesion Defects (LAD) arise from defects in adhesion of neutrophils to the vascular endothelium, a process that is necessary to arrest neutrophils at the site of infection, so that they are able to migrate through the endothelium into the tissues.

Bidirectional communication between neutrophils and the endothelium is necessary for migration of circulating neutrophils into the tissues and the site of infection or injury. Signals from inflamed tissues or invading organisms lead to activation of neutrophils. During this process, interactions between CD15s or sialyl-Lewis X, a fucosylated surface receptor, with endothelium-associated P-selectin, mediates the initial low affinity tethering of neutrophils to the endothelium. Additionally, cytoplasmic granules containing adhesion molecules CD18 and CD11b translocate to the surface, and constitutively-expressed adhesion molecules, CD11a and CD11c, undergo qualitative changes and associate with CD18. These groups of molecules, collectively called ‘integrins,’ interact with ICAMs (endothelial intracellular adhesion molecules) and selectins, resulting in arrest of neutrophils on the endothelium. Chemokine-mediated

signaling from within the cell mediated by the protein Kindlin3, further activates the integrins to strengthen adhesion. Adhesion to the endothelium is followed by transmigration into the tissues.

In the following slides, I will discuss genetic defects that affect these key players that mediate neutrophil adhesion.

Slide 8: Comparison of LAD I, II, and III

There are 3 variants of the Leukocyte Adhesion Defect (LAD).

1. LAD I is a result of mutations in ITGB2, encoding CD18, thereby leading to loss of surface expression of all three integrins, CD11a, b, and c, that require association with CD18 for surface expression. LAD I is the most common of the three defects. Its classical presentation is delayed separation of the umbilical cord at birth together with omphalitis and life-threatening infections. Expression of surface integrins is low and treatment is early recognition followed by a bone marrow transplant.
2. LAD II is a result of mutations of FUCT1, leading to a defect in fucose metabolism, thereby leading to an absence of sialyl-Lewis X or CD15s, a ligand for P-selectin. LAD II has only been described in a few patients. This defect has a milder course than LAD I and is characterized by recurrent infections, moderate-severe mental retardation, dysmorphism short stature, and a Bombay blood phenotype. Treatment with oral fucose effectively restored surface expression of CD15s in one patient to date.
3. LAD III, a variant of LAD I, is a result of mutations in the *FERMT3* gene, encoding Kindlin-3 that normally activates integrins for optimal expression and function. LAD III presents similar to LAD I with severe recurrent bacterial and fungal infections. Additionally, these patients present with severe bleeding tendency because the Kindlin-3 mutation affects beta integrins expression on multiple cell types including platelets, leading to defective clotting. This defect is very rare and treatment is through early intervention with a bone marrow transplant.

Slide 9: Diagnosis of LAD I

Flow cytometry for surface expression of integrins is used for the diagnosis of LAD I. Surface expression of CD18, the beta chain, as well as CD11a, b, and c are all decreased. LAD II is diagnosed by analysis of CD15s or sialyl-Lewis X, while a diagnosis of LAD III requires genetic analysis of *FERMT3*, since surface expression of integrins is normal.

Slide 10: Expression of CD18 and CD11a Post Bone Marrow Transplant

This slide shows an example of flow cytometry performed for LAD I. A healthy donor sample is shown above, with normal levels of CD11a and CD18 on the gated neutrophil population, shown in red. In comparison, the majority of the patient's neutrophils, M1, in the lower row of plots, show no expression of CD18 and CD11a. This patient received a bone marrow transplant to correct the defect and the engrafted cells, indicated by M2, show good expression of CD11a and CD18. This simple flow cytometry assay, performed on whole blood, is very useful not only for the identification of LAD 1, but also for following engraftment of donor cells post-transplant.

Slide 11: The NADPH Complex

We now move on to neutrophil oxidative burst, a critical mechanism employed for clearance of infection. Neutrophil oxidative burst is mediated by the NADPH oxidase complex, shown in this figure, that is assembled in the phagosome or at the plasma membrane upon neutrophil activation. The main membrane bound subunits are $p22^{phox}$ and $gp91^{phox}$, while the other 4 subunits, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac2, are recruited from the cytosol upon activation. Assembly of the complex then mediates the transport of electrons from NADPH to FAD along the electron transport pathway eventually leading to generation of reactive oxygen species.

Slide 12: The NADPH Complex

Mutations in the various subunits that form the NADPH complex lead to defective neutrophil oxidative burst of varying degrees of severity.

The most common of these defects is due to mutations in the *CYBB* gene, encoding $gp91^{phox}$. Defects in $gp91^{phox}$ lead to chronic granulomatous disease (CGD), characterized by severe, life-threatening infections including osteomyelitis, lymphadenitis, pulmonary infections, abscesses, and septicemia. *CYBB* is present on the X chromosome, and therefore, X-linked CGD due to $gp91^{phox}$ mutations is seen in boys.

Mutations in *CYBA* encoding p22, NCF-1 encoding p47, NCF-4 encoding p-40, and NCF-2 encoding p67 are rarer and are inherited in an autosomal recessive manner. The disorder, termed autosomal recessive CGD, can therefore manifest in both girls and boys, generally later in life, and the disease course is milder. To date, only one case of an NCF-4 mutation leading to a mutated p40 protein has been described. The clinical presentation of this patient was one of granulomatous colitis rather than infection.

Finally, mutations of Rac2, a protein that is essential for both motility and oxidative burst, can manifest chiefly as a defect in chemotaxis with reduced but not absent oxidative burst.

On the next slide, we will examine laboratory testing for CGD.

Slide 13: Dihydrorhodamine 123 Assay for Neutrophil Oxidative Burst

Laboratory testing for chronic granulomatous disease (CGD) is carried out with a simple flow cytometry test using the dye dihydrorhodamine 123 (DHR-123). The dye readily permeates most membranes and in the presence of reactive oxygen intermediates generated during the respiratory burst, is rapidly oxidized to produce a brightly fluorescent compound – rhodamine 123, which can be detected by flow cytometry.

In brief, 100 microliters of whole blood is incubated with DHR allowing the neutrophils to take up the dye. The blood sample is then stimulated with PMA or phorbol 12 myristate 13-acetate, which stimulates oxidative burst. Following a 10-minute stimulation, RBCs are lysed and the fluorescence of the sample is read on a flow cytometer.

Shown here is a typical flow cytometry analysis of normal neutrophil oxidative burst. The first histogram, on the upper right hand side, indicates neutrophils that have not been loaded with the dye; the second one below shows neutrophils loaded with dye, thereby increasing

fluorescence, and the third histogram shows a normal oxidative burst response, with fluorescence increasing and shifting the peak all the way to the right.

The next slide demonstrates the application of this simple flow cytometry assay in diagnosing X-linked-CGD and AR-CGD.

Slide 14: Flow Cytometry in CGD

The schematic shown here indicates the various flow cytometry patterns seen in oxidative burst defects. The two plots, A and B, indicate normal oxidative burst from healthy neutrophil populations. The cells fluoresce brightly following stimulation with PMA. In contrast, neutrophils from the CGD patient, shown in C, have no detectable oxidative burst. The patient's mother, shown in D, carries the *CYBB* mutation on one of her X chromosomes and has a typical carrier profile with one population of neutrophils exhibiting normal oxidative burst and the other, defective oxidative burst. Plot E shows an autosomal recessive variant, as seen in mutations of NCF-1, 2, and 4, or *CYBA*. The patient's neutrophils have some residual oxidative burst activity accounting for the milder clinical phenotype in autosomal recessive variants of CGD.

Integrity of the neutrophil population is critical for accurate assessment of oxidative burst. Since neutrophils are fragile cells, samples must be analyzed as soon as possible following collection to avoid artifacts and false positive or uninterpretable results due to compromised stability of the neutrophils. We recommend analysis of neutrophil oxidative burst within 24 hours of sample collection for optimal results.

In addition to the diagnosis of X-linked and AR-CGD, the DHR assay can also be used to monitor patients for engraftment following bone marrow transplant. Although CGD patients, both X-linked and autosomal recessive variants, have defective neutrophil oxidative burst, there is considerable heterogeneity in their ability to produce reactive oxygen intermediates (ROI). It has been demonstrated that a combination of mean fluorescence intensity of the DHR assay and superoxide production in neutrophils was predictive of long-term survival in CGD patients. Patients with modest production of reactive oxygen intermediates and a higher DHR MFI tended to have less severe infections and illness, and survived longer than those who did not have residual ROI production. The DHR assay, in combination with evaluation of ROI, therefore, has prognostic value in CGD patients.

Slide 15: Neutrophil Chemotaxis Defects

The last of the neutrophil functions to be discussed is chemotaxis, or the ability of neutrophils to move towards a chemical stimulus. This stimulus may include microbial products or acute phase reactants generated by complement activation such as C3a and C5a. Chemotactic defects, generally a result of failure to regulate the actin cytoskeleton or defective vesicular trafficking, rarely occur in isolation and are generally associated with other neutrophil defects, such as oxidative burst as in the case of Rac2 mutations, or neutropenia as in severe congenital neutropenia due to defects of Glucose-6-phosphatase.

Other syndromes that are associated with defective neutrophil chemotaxis are the Papillon Lefevre syndrome, characterized by planoplantar warts and periodontitis; the Chédiak-Higashi syndrome, characterized by recurrent pyogenic infections, neutropenia, and neurological

symptoms; Down's syndrome, characterized by increased susceptibility to infection and developmental defects; and the Schwachman Diamond syndrome, characterized by pancreatic insufficiency, predisposition to leukemia, and pancytopenia.

Thus, unlike the characteristic clinical features of CGD or LAD, chemotactic defects of neutrophils may be seen in a diverse range of clinical conditions.

Slide 16: Assessing Neutrophil Chemotaxis

Chemotaxis is assessed in the laboratory using the modified Boyden chamber. Here, neutrophils are placed in the upper chamber of the apparatus and a chemotactic stimulus, generally activated serum containing split complement products, placed in the lower chamber. The two chambers are separated by two filter paper discs; the upper one allows the neutrophils to pass through while the lower one does not.

Following incubation of the neutrophils in the Boyden chamber for about 30 minutes in a 37°C degree incubator, the filter paper discs are removed, stained and observed under a light microscope. Neutrophil migration is assessed by focusing on the cells first on the upper filter and then on the lower filter and calculating the distance travelled in microns.

Slide 17: Summary

In summary, neutrophil defects may be both inherited through genetic mutations or acquired, either due to autoimmune, iatrogenic, or other causes. The common feature of neutrophil defects, whether acquired or inherited, is increased susceptibility to infections. While, in many cases, lifelong antibiotic prophylaxis may be required to protect against infection, severe neutrophil defects, such as CGD or LAD, can be treated with bone marrow transplants or gene therapy. Finally, there are several laboratory tests that can be used to assess neutrophil function, follow success of engraftment following bone marrow transplant, or assess carrier status.

Slide 18: References

Listed here are some useful references dealing with neutrophil defects, intrinsic and extrinsic, as well as with the diagnosis of these defects and brief descriptions of clinical intervention.

Slide 19: Disclosures

Slide 20: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on "Neutrophil Defects."