Hot Topic

Laboratory Testing for Chronic Granulomatous Disease: Challenges and Recommendations
Laboratory Testing for Chronic Granulomatous Disease: Challenges and Recommendations

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Disclosures

• None

I have no disclosures.
As you view this presentation, consider the following important points regarding testing:

- How is the testing going to be used in your practice?
- When should the tests be used?
- How will results impact patient management?
Disclaimer

• This presentation does not provide extensive detail about clinical phenotype of CGD (only brief contextual information), no information on treatment or management of CGD, and very limited information on genetic testing in CGD

• The primary focus of this presentation is on DHR / Dihydrorhodamine (DHR) Flow Cytometric Test, Blood, used for diagnosis of CGD

I have provided a disclaimer related to the content of this presentation and what it will cover and not cover.
This slide provides the most recent classification of primary immunodeficiencies, which includes CGD, or chronic granulomatous disease, based on the International Union of Immunological Societies (IUIS) classification schema. CGD is grouped under the phagocytic defects due to its association with neutrophil dysfunction.
Neutrophils are part of the innate immune response and can be considered an essential first responder in the immune system. There are 3 primary mechanisms of neutrophil killing, and the control of pathogens is part of the immune response. The first is NADPH-dependent oxidative damage, activation of various neutrophil-specific proteases, and production of neutrophil extracellular traps or NETs. While most people may be familiar with the first 2, the latter refers to extracellular extrusion of chromatin fibers, which facilitate neutrophil killing of pathogens while minimizing damage to host cells. The formation of NETs is an active process, and it contains neutrophil granule proteins and chromatin. NETs have been reported to be cleaned by macrophages.
Some of the key neutrophil granule proteins involved in the neutrophil immune response are listed here. They include the azurophilic granules, which are myeloperoxidase and cathepsin G, neutrophil elastase, defensins, and lysozyme. There are specific proteases in the granules that contain lactoferrin and lysozyme, and there are tertiary granules that contain gelatinase.
This slide provides some background information on the specific primary immunodeficiency (PID) called chronic granulomatous disease or CGD. CGD is caused by germline defects in the 5 individual components that comprise the NADPH oxidase complex. This NADPH oxidase enzyme is integral to the generation of the neutrophil oxidative burst, which is mentioned a few slides hence, is a component of the neutrophil immune response. Four of the 5 components of the NADPH oxidase complex are required for superoxide generation. Defects in this enzyme complex affect all aspects of neutrophil killing.
The incidence of CGD is considered to be approximately 1 in 250,000 births in the United States, and the X-linked form is the most common, accounting for approximately 70% of cases. One of the noteworthy features of CGD is the observation of skewed lyonization, that is skewed X-chromosome inactivation in carrier females of X-linked CGD, and this is a phenomenon that has been shown to increase with the age of the individual.
The X-linked form of CGD is caused by mutations in the *CYBB* gene, including the gp91phox protein. The autosomal recessive forms of CGD are due to defects in the *NCF1*, *NCF2*, *NCF4*, and *CYBA* genes that encode the p47phox, p67phox, p40phox, and p22phox proteins, respectively. With the exception of gp91phox and p22phox, which are membrane-bound, the other 3 proteins are cytosolic components of the phagolysozyme. CGD involving p40phox is quite rare and has a milder phenotype, while X-linked CGD usually has a more severe phenotype. Carriers for *NCF1* gene mutations are quite common in the general population. Mutations observed in these genes include missense, nonsense, frameshift, deletions, splice-site, intronic, and regulatory.
In carrier females of X-linked CGD, as previously mentioned, due to skewed lyonization, there is the presence of 2 populations for neutrophil oxidative burst, and this does not remain static over time but can change with age-related skewing of lyonization observed with increasing age. In general, female carriers become symptomatic when they have less than 10% cells with normal neutrophil oxidative burst. However, in practice, we have seen clinically symptomatic females with higher proportions of normal neutrophils. The age of presentation is variable, depending on the genetic defect and residual NADPH oxidase activity. The primary assays used for diagnosis of CGD measure oxidative burst, and these will be described later.
The infectious phenotype in CGD is typically associated with 5 organisms, including *Staphylococcus aureus*, *Burkholderia cepacia*, *Serratia marcescens*, *Nocardia* and *Aspergillus* spp. Other rarer organisms have been reported, particularly in different geographical areas.

- Frequent sites of bacterial infection are lung, skin, LN, and liver. Osteomyelitis, gingivitis and perianal abscesses are also common.
- Infectious pathology in CGD is quite specific with the majority of infections due to 5 organisms (bacterial infections are due to catalase-positive organisms) - *Staphylococcus aureus*, *Burkholderia cepacia*, *Serratia marcescens*, *Nocardia* and *Aspergillus* spp. Other rarer organisms have been reported, particularly in different geographical areas.
- Cutaneous inflammatory responses are often dysregulated in CGD and can form exuberant granulomas. There is also a hyperinflammatory response seen in CGD patients in response to infections that requires steroids for management, in addition to antimicrobials.

The infectious phenotype in CGD is typically associated with 5 organisms, including *Staphylococcus*, *Serratia*, *Burkholderia*, *Nocardia*, and *Aspergillus*. *Candida* infections can occasionally be seen, though it is not systemic. Other rare bacterial infections with chromobacterium and other species have also been reported. Granulomas in skin and other organs are frequent, and there is often a hyperinflammatory response seen associated with infection in CGD that does not respond to antimicrobials but requires steroids.
This slide shows a schematic of the organization of the NADPH complex subunits within the phagolysozyme of the cell. The components are not constitutively present in this form, but are brought together on activation of the neutrophil on activation with a nonspecific stimulus such as phorbol myristate acetate or PMA in the in vitro diagnostic test. There is electron transfer and generation of hydrogen peroxide in other compounds, ultimately resulting in the production of superoxide, which exerts a powerful antimicrobial effect.
The major pathogens in CGD are catalase-positive because they can neutralize hydrogen peroxide. Defective function of the NADPH oxidase complex affects neutrophil degranulation and granule enzyme function.
The laboratory diagnosis of CGD has traditionally involved the use of the nitroblue tetrazolium test, which is useful for measuring NADPH oxidase function in neutrophils. The NBT is considered an outdated test at the present, but it measures NADPH oxidase activity neutrophils after activation with PMA, which is a plant-derived mitogen. NBT is an oxidant and BCIP is a dye that gives a dark blue color with alkaline phosphatase substrate if there is normal neutrophil oxidative burst. The disadvantage of this test is that it is highly subjective and dependent on user expertise.
The more recent and standard testing for CGD involves the dihydorhodamine, or DHR test, which is now the most commonly used test for diagnosis of CGD, and measures reactive oxygen intermediates (ROIs) produced during neutrophil respiratory (oxidative) burst. It is a highly sensitive and specific test and is performed by flow cytometry. The test is based on the principle that DHR123, a nonfluorescent, uncharged compound, which can passively diffuse across membranes can be oxidized by H₂O₂ (produced during neutrophil respiratory burst by oxidase activity) and myeloperoxidase (MPO) to cationic rhodamine 123, a green fluorescent compound, which localizes in mitochondria and can be detected by flow cytometry. In the flow test, neutrophils are activated by stimulation with PMA. Comparison is made between stimulated and unstimulated samples.
This slide shows the DHR test, which was performed by flow cytometry in a healthy control. The green histogram is the unstimulated sample, and the red histogram is the neutrophil oxidative burst observed on stimulation with PMA. As you can see, there is a strong shift on the X-axis, indicating normal and robust respiratory burst or neutrophil NADPH oxidase activity.
This slide is an example of the DHR test in a male patient with X-linked CGD. As you can see, the green and the red histograms for unstimulated and stimulated samples completely overlap, indicating that there is an absence of neutrophil oxidative burst due to defective NADPH oxidase function.
In the autosomal recessive forms of CGD, the DHR test usually shows a different pattern where there is diminished neutrophil oxidative burst and decreased DHR fluorescence as observed on the X-axis with the red histogram. The green histogram is the unstimulated sample, which is used as the control for comparison. This patient had a mutation in the *NCF1* gene, encoding the p47phox protein.
This is another patient with autosomal recessive CGD with a mutation in the NCF2 gene, encoding the p67phox protein, and as seen in the case of the previous slide with the NCF1 gene mutation, the pattern is similar with diminished neutrophil respiratory burst and reduced DHR fluorescence on the X-axis for the stimulated sample marked by the red histogram.
The DHR results can give false-positive if there is complete myeloperoxidase deficiency. In complete myeloperoxidase deficiency, there is a shift of the PMA-stimulated histogram, marked by the red line. Patients with complete myeloperoxidase deficiency are usually asymptomatic, but approximately 5% of patients with complete myeloperoxidase deficiency can have significant infections, often in the context of diabetes.
This is an example of a male patient with X-linked CGD who presented with an atypical phenotype with 1 episode of *Burkholderia pneumonia*. As you can compare and contrast to the previous example of X-linked CGD, this patient does show reduced but present neutrophil oxidative burst in contrast of what is typically observed, which is completely absent oxidative burst. This result can be confused with complete myeloperoxidase deficiency or an autosomal recessive form of CGD and, therefore, genetic testing is essential to determine the inheritance pattern and the specific genetic defect.
This is an example of skewed lyonization in a female carrier of X-linked CGD. This is an elderly woman in her seventh decade of life who presented with an episode of *Burkholderia pneumonia*. She had a known history as carrier of X-linked CGD with a male offspring who was clinically symptomatic. This patient shows age-related skewing of lyonization with 100% skewing of lyonization in completely absent neutrophil respiratory burst as shown by the superimposition of the green and red histograms.
This test is very dependent on the quality of the sample; and in samples that are shipped, it is important to ensure that there is optimal handling of the specimen. This is an example of a sample shipped under optimal conditions. The Y-axis shows side-scatter. The X-axis shows forward-scatter. The neutrophils are represented in the gate marked unstimulated neutrophils.
The DHR test performed in samples that are shipped correctly show a complete separation of positive and negative peaks, the positive peak being represented by the red histogram and the negative, the unstimulated sample.
In this test, we assess neutrophil oxidative burst on viable neutrophils; and as demonstrated in this slide, the left-most histogram or slide shows the forward- and side-scatter plots for neutrophils. The middle plot shows the viability assessment, and the right plot in the top panel shows the background in the unstimulated control. This shows that there was in vitro activation of neutrophils prior to stimulation. The lower panel shows the PMA-stimulated samples and, again, the left-most panel and the middle panel show the forward- and side-scatter and the viability assessment. The right-most lower panel shows the neutrophil oxidative burst or DHR fluorescence after PMA stimulation. This sample had a viability of 90%. Viability of 80% or higher is considered optimal for this assay.
This is an example of a sample transported under suboptimal conditions. As you will notice on the X-axis and the Y-axis, which is the forward- and side-scatter, respectively, there is a lot of dead neutrophils marked by cells with high side-scatter.
When the DHR fluorescence assay is performed on such a suboptimal sample, as you can see with the red histogram, there are 2 peaks for DHR fluorescence, 1 with bright intensity, which is the right-most peak, and 1 with a dimmer fluorescence intensity, which is the left red peak. The green histogram represents the unstimulated sample and is shown by its wide spread vitro activation of neutrophils due to suboptimal transportation.
Thank you.