NEUTROPHIL FUNCTION TESTING

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1. INTRODUCTION

1.1. Normal neutrophils become activated in the presence of serum factors, such as Complement C5a, kallikrein or bacterial peptides. Circulating neutrophils attach to the endothelium of the blood vessel, move along the gradient of a chemo attractant until coming in contact with organisms, adhering to the organisms, and ingesting and killing them. There are abnormalities intrinsic to neutrophils which can occur at each of these stages, either secondary to drugs or toxins or due to primary defects in the neutrophils.

1.2. Patients with neutrophil dysfunction present with chronic recurrent bacterial infections. Severe chemotactic defects in particular have been associated with chronic skin and mucosal infections. Primary neutrophil dysfunction significant enough to cause clinical disease accounts for less than 6% of all primary immune deficiency. Table 1 lists the currently recognized severe primary neutrophil dysfunction syndromes along with the screening tests and more detailed confirmatory laboratory tests. Tests that may detect the heterozygous state are indicated. Absent from this list are other primary immune syndromes and certain other metabolic diseases that have been associated with neutrophil dysfunction. Myeloperoxidase deficiency, although probably the most common inherited neutrophil defect, is not discussed since it is not generally considered to be clinically significant.

Table 1. Readily diagnosable neutrophil dysfunction syndromes

DIAGNOSIS	SCREEN	CONFIRMATORY/RESEARCH
Chronic Granulomatous Disease	NBT slide test[a], Flow cytometry for superoxide[ab]	Superoxide assay[b], cytochrome b assay, K_m for NADPH, cytosol factor level
Chediak-Higashi syndrome	Giant granules in neutrophils on PB film, Neutrophil chemotaxis	
Leucocyte adhesion deficiency	CD11a, CD11b, CD18 all [c], chemotaxis, bacterial killing	Western immunoblot, RFLP's
Specific granule deficiency	Chemotaxis, anti-lactoferrin staining of neutrophils	
Actin deficiency	Chemotaxis, bacterial killing phagocytosis by flow cytometry	F-actin content b

[a] Assays that can easily be set up in a routine laboratory
[b] May be useful in carrier detection.
[c] Measured with the use of commercially available fluorescent antibodies by flow cytometry.
(Table 1 adapted from Manual of Clinical Laboratory Immunology [3])

1.3. There are many clinical situations which lead to secondary neutrophil dysfunction (see Table 2). Certain neutrophil function assays are more sensitive to secondary dysfunction than others. In general, one should not hesitate to screen for neutrophil dysfunction even though the patient is currently sick. If the test is abnormal, however, interpretation of the results as indicative of a primary neutrophil dysfunction syndrome must be viewed with some caution. Usually the magnitude of the deficiency seen with the listed primary neutrophil dysfunction syndromes is much greater than that seen in any secondary dysfunction.

Table 2. Secondary neutrophil dysfunction

CAUSE OF DYSFUNCTION	FINDING[a]
Adriamycin	respiratory burst
Prednisolone	chemotaxis, adherence
Aspirin	chemotaxis, adherence
Penicillamine	respiratory burst
Alcohol	chemotaxis
Group A streptococcus	respiratory burst
Capnocytophagia	chemotaxis
Influenza	respiratory burst, chemotaxis

[a] decreased, markedly decreased
(Table 2 adapted from Manual of Clinical Laboratory Immunology [3])

1.4. Flow cytometric assays for neutrophil function currently exist for testing the respiratory burst, phagocytosis and bactericidal killing. As can be seen from the preceding paragraphs, these assays can be used to screen for a range of primary or secondary neutrophil dysfunction syndromes. This document will focus primarily on guidelines for neutrophil function testing for respiratory burst and phagocytosis.

2. SPECIMEN COLLECTION, TRANSPORT AND INTEGRITY

2.1. Specimen Collection

Universal precautions should be strictly observed when collecting blood samples (see 1.1 Safety Guidelines).

2.1.1. Heparin anticoagulated blood should be processed within 4 hours of collection. However, some laboratories may receive specimens collected in remote areas and overnight storage may be necessary. In this scenario, abnormal results are suspect. The use of other anticoagulants should be validated.

2.1.2. Any specimen over 24 hours old, or unlabelled or incorrectly labelled or of insufficient volume should be recollected.

2.1.3. Clinical history (including drug history) should be documented.

2.2. Specimen Transport

2.2.1. Packaging, labelling and transport of specimens should comply with all current local, state, national and international regulations for the regions through which the specimens will pass.

2.2.2. Specimens should be maintained at 18o - 22o C.

2.2.3. Temperatures below 10oC should be avoided, and above 30oC must be avoided.

2.3. Specimen Integrity

2.3.1. Visually inspect the specimen for clots, haemolysis or container defects. Where appropriate, recollect the sample if the specimen shows any visual signs of deterioration.

2.3.2. Specimens that are collected or transported outside of these guidelines should be treated with caution. The deficiencies in the sample should be noted and the report should reflect the effect that these deficiencies may have on the results.

3. SPECIMEN PROCESSING

3.1. Neutrophil Isolation

Operators may choose from a variety of methods. To separate neutrophils, a standard density gradient separation of mononuclear cells followed by dextran separation has commonly been used 3. A single step commercial density gradient medium is now available and will separate neutrophils and mononuclear cells into separate layers. Wherever possible whole blood lysis methodology should be employed. The advantage of this technique is that there is no activation of neutrophils (as a result of neutrophil separation from other blood cells). The latter method enables confident detection of low levels of neutrophil activation in the peripheral blood. It is important to maintain a constant temperature during preparation as radical temperature gradients activate neutrophils. Preparations containing clumps should be viewed with suspicion.

3.2. Neutrophil Superoxide Production

The principle of published assays relies on the action of superoxides to convert a non-fluorescent dye to a fluorescent one 4-13 (e.g. DHR –123, DCFH), which is then cell bound. Thus following the appropriate stimulus (e.g. S. aureus or phorbol ester) a normal neutrophil will produce superoxide species and oxidise the dye to its fluorescent and cell-bound form. It is important that the dye chosen can be demonstrated to be sensitive to the action of neutrophil superoxides and that the subsequent fluorescent particle remains within the cell for measurement by the flow cytometer.

3.3. Neutrophil Phagocytosis

The principle of these assays includes the staining of bacteria with a fluorochrome 10, 14, 15 (e.g. PI or FITC), incubation of these stained bacteria with patient neutrophils in the presence of complement and detecting the phagocytosis of stained bacteria. It is clinically useful to be able to demonstrate that the bacteria are in fact ingested as opposed to adherent to the neutrophil surface. This may be approached by either comparing fluorescence obtained with or without a cytoskeletal inhibitor 14 (e.g. Cytochalasin D), or using a dye that is sensitive to quenching agents (e.g. the fluorescence from adherent FITC-labelled S. aureus may be quenched by trypan blue).

3.4. Lysis

If using the whole blood lysis technique for neutrophil function, several lysing techniques are available. These include water, tris buffered ammonium chloride and hypotonic buffer 1, 2. Several proprietary lysing reagents are available from the instrument and monoclonal antibody manufacturers. When using commercial reagents, the manufacturer’s recommended protocol should always be followed unless data are available for confirming that any modifications do not adversely affect results.

4. CONTROLS

A method control must be prepared and run on a daily basis in parallel with patient samples.

5. SAMPLE ANALYSIS

5.1. Sample order. All control specimens should be run first and checked, before running the patient samples according to laboratory priority.
5.2. Test order within any panel. The first tube should be a gating control to maximise the cells of interest and minimise contamination. The appropriate isotype controls should be run next and then followed by the subsequent test panel to investigate the provisional diagnosis.
5.3. Set leucocyte gates as broadly as possible consistent with acceptable levels of contamination to minimise contaminating cells and maximise the inclusion of the cells of interest (the neutrophils). Live gating should be restricted to the setting of a forward light scatter threshold.
5.4. Each laboratory should establish limits of contaminating cells and debris, based on documentation that their inclusion does not significantly affect the measurement of interest. If levels of contamination exceed established laboratory limits, the corrective actions taken are to adjust the light scatter gates and reanalyse the immunofluorescent correlated two colour plot.
5.5. If levels of contamination by non lymphocytes cannot be minimised to within acceptable limits, then test results may be suspect.
If this contamination cannot be explained by reinterpretation of the data or by clinical diagnostic reasons then a second specimen should be requested.
5.6. Count at least 2000 gated events in each sample. This number assures with 95% confidence that the result is within 2% of the “true” value (binomial sampling). NB: This sample mode assumes that the variability of determining replicates is < 2%. The counting of 2000 gated events to ensure reasonable statistical confidence may not be achievable in severely leucocytopaenic specimens.
5.7. Instrument settings should be adjusted based on the expected fluorescence for normal neutrophils with maximal stimulation versus no stimulation at all.

6. DATA REPORTING

6.1. Report all unique patient identifiers including name/code, medical record number, laboratory ID/accession number and collection date/time as well as print date/time.
6.2. Each laboratory should establish reference limits for the antigens being tested (see 5.11 Appendix 2: Reference Range Determination).

7. QUALITY ASSURANCE

7.1. Analysis should include internal reliability checks of results, including:
  7.1.1. Light scatter patterns should be examined for each tube within the panel for variation from tube to tube. Similarly, the number of gated events and/or time to collect data should not vary greatly from tube to tube.
  7.1.2. Potential sources of error which are not necessarily covered by the above reliability checks may include inappropriate gating leading to exclusion of relevant cells, tubes in a panel run in the wrong order, inappropriate cut-offs between negative and positive cells and calculation or transcription errors. Individual laboratories may require procedures to cover such possibilities.
  7.1.3. Each laboratory should determine the level of test variability by preparing and analysing at least six replicates. This will provide a basis when changes to methodology are introduced.
7.2. Regulatory bodies currently require that a laboratory keeps all equipment maintenance and calibration records, staff training records, up-to-date method protocols, daily operator/reagent records, verification of transcription of results from machine printouts, procedures for amendment of results and checks by supervisors/pathologists.
7.3. The laboratory should belong to and participate in a recognised external quality assurance program with regular review of the results.

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