

24. Clinical and laboratory assessment of immunity

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Clinicians order specific laboratory tests and interpret laboratory data that will be useful in establishing a diagnosis of immunodeficiency, either inherited (primary) immunodeficiencies or acquired (secondary) immunodeficiencies. The clinical immunologic evaluation of patients for immunodeficiency proceeds in an orderly fashion, from screening tests to sophisticated tests. The medical history and physical examination of the patient frequently provide clues guiding the level of entry into this testing program. Specific tests have been designed to screen for the four basic mechanisms of host defense: antibody, T cell, phagocyte, and complement. The clinical immunology laboratory is a powerful adjunct to the clinician in the initial evaluation of immunodeficiency disorders and in the design of more sophisticated testing for selected patients. (*J Allergy Clin Immunol* 2003;111:S702-11.)

Key words: Cellular or T-lymphocyte immunodeficiency, chronic granulomatous disease, complement deficiencies, humoral or B-lymphocyte immunodeficiency, immunophenotyping; innate immunity, in vitro lymphocyte proliferation, mitogens, natural killer cells, polymorphonuclear leukocytes

In the past 10 years, there has been a rapid increase in scientific knowledge about the nature and pathogenesis of a number of inherited primary immunodeficiencies. Research in basic and clinical immunology and genetics has provided important insights in the understanding of immunodeficiency disorders. Evaluations or assessments of immunity have increased in complexity and sensitivity. Many of the new diagnostic methods and testing require sophisticated methods and reagents and remain in the realm of the research laboratory. In this section, we will review an approach that uses the most basic tests and clues to begin an assessment of immunity, and then move on to a review of the more specific and sophisticated methods. Figures 1 and 2 give schematic approaches for the assessment of B- and T-lymphocyte numbers, products, and functions. A brief review of an approach to evaluate the components of innate immunity, natural killer (NK) cells, polymorphonuclear neutrophil (PMN), and complement is also included. The approach outlined in this section should permit the initial assessment of immunity and will provide a guide for further evaluation.

ASSESSMENT OF HUMORAL IMMUNITY

There are a number of relatively well-defined defects in

Abbreviations used:

B cell:	B lymphocyte
CBC:	Complete blood count
CGD:	Chronic granulomatous disease
CH50:	Total hemolytic complement
cpm:	Counts per minute
C1, C2, C3, etc:	Complement component 1-9
DTH:	Delayed-type hypersensitivity
ELISPOT:	Enzyme-linked immunospot
FCM:	Flow cytometry
Ig:	Immunoglobulin
LAD:	Leukocyte adhesion deficiency
LPA:	Lymphocyte proliferation assay
NADPH:	Nicotinamide adenine dinucleotide (phosphate reduced)
NBT:	Nitroblue tetrazolium
NK:	Natural killer
PBCM:	Peripheral blood mononuclear cells
PMN:	Polymorphonuclear neutrophil
PPD:	Purified protein derivative
PWM:	Pokeweed mitogen
T cell:	T lymphocyte

the humoral limb of the immune response.¹ In general, certain clues, such as the development of infections in early life and the failure to clear an infection even after appropriate antimicrobial therapy, lead to the suspicion of a humoral or B-lymphocyte deficiency. The B-lymphocyte deficiency may be relatively mild and create only rare problems, or it may result in a severe deficiency with a complete inability to synthesize and secrete antibodies.

The nature of the infecting agent and the age of the patient are almost always the first indications of the need to evaluate for B-lymphocyte immunodeficiencies. A pattern of infections starting in infancy is important to note. Repeated infections caused by common pyogenic organisms, such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* type b, and *Neisseria meningitidis* may serve as significant indicators for evaluation for most of the inherited immunodeficiencies (Table I). Recurrent or incompletely cleared conditions, such as otitis, sinusitis, bronchitis, pharyngitis, or conjunctivitis are important factors to be considered. Some patients with severe agammaglobulinemia may not respond to toxoid vaccines and may develop serious infections when given attenuated viral vaccines. (See Chapter 12 for a more complete description of humoral immunodeficiencies.)

Figure 1 is an outline of the laboratory tests for assessing B-lymphocyte immunocompetence. The initial testing can be performed in basic hospital laboratories. Depending on those results, more specific testing can continue for a more detailed investigation.

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Reasonable Suspicion of B-Lymphocyte Defect (based on clinical history)

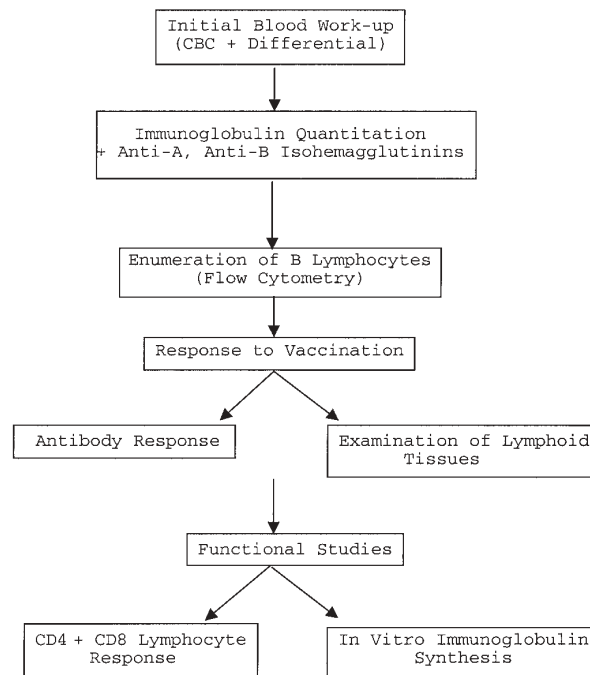


FIG 1. Flow chart for the evaluation of humoral immunity.

Reasonable Suspicion of Cell-Mediated Immune Defect

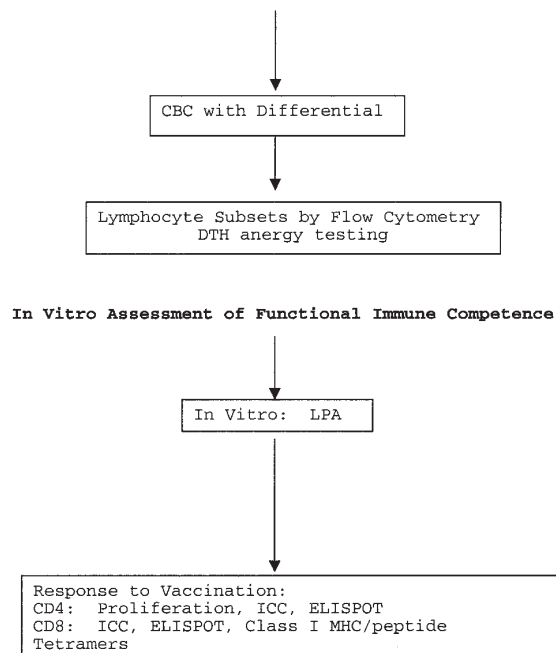


FIG 2. Flow chart for evaluation of cellular immunity.

Evaluation of the humoral immune response usually begins with a complete blood count (CBC) and a differential to assess the lymphocyte and other blood cell numbers. In most B-lymphocyte immunodeficiencies, the

lymphocyte count may be normal to slightly decreased. However, this blood cell count can be a useful first step in documenting the presence of lymphocytes.

The next step in an evaluation of humoral immuno-

TABLE I. Infectious agents frequently associated with immunodeficiency disorders

Humoral or B-lymphocyte deficiencies	
<i>Streptococcus pneumoniae</i>	
<i>Streptococcus pyogenes</i>	
<i>Haemophilus influenzae</i>	
<i>Ureaplasma urealyticum</i>	
<i>Giardia lamblia</i>	
Cellular or T-lymphocyte deficiencies	
Mycobacteria species	
Salmonella species	
<i>Listeria monocytogenes</i>	
<i>Toxoplasma gondii</i>	
<i>Pneumocystis carinii</i>	
Fungi	
Viruses	
Combined humoral and cellular deficiencies	
<i>Candida albicans</i>	
<i>Pneumocystis carinii</i>	
Aspergillus species	
Viruses (especially herpes viruses)	
Phagocyte or phagocyte cell deficiencies	
<i>Staphylococcus aureus</i>	
Klebsiella species	
<i>Escherichia coli</i>	
Enterobacter	
<i>Serratia marcescens</i>	
Salmonella species	
Pseudomonas species	
Complement deficiencies	
<i>Neisseria meningitidis</i>	
<i>S pneumoniae</i>	
<i>H influenzae</i>	
<i>Neisseria gonorrhoeae</i>	

TABLE II. CD Designations for commonly used monoclonal antibodies (single or combinations) in FCM

Antigen	Target cell recognized
CD3	Pan T cell
CD3 + CD4	T helper cells
CD3 + CD8	T cytotoxic cells
CD16 + CD56	NK cells
CD19 or CD20	B cells
CD25	Activated cells
CD38	Activated cells
CD45	Pan leukocyte
CD45RO + CD4 or CD8	Memory T cells
CD45RA + CD62L + CD4 or CD8	Naïve T cells

competence involves quantitating the level of serum immunoglobulins. Studies are performed to measure the immunoglobulin levels and to establish the immunoglobulin classes. Normal immunoglobulin levels depend on a number of factors, including the age and sex of the individual, as well as ethnic and genetic background. The primary immunoglobulins routinely measured in serum are IgG, IgA, and IgM. Determination of IgE and IgD levels requires more specialized testing. Under certain circumstances, it may be important to measure the serum levels of subclasses of IgG. Normal values or reference ranges for all the immunoglobulin classes and subclasses

of IgG are published and are obtainable for age of the patient and for different methods of measurement.² Immunoglobulin levels should be determined in any patient suspected of being immunodeficient.

A number of standardized and well-defined methods are available for immunoglobulin level determination.³ The classic method, single radial immunodiffusion, was the standard for many years, but most clinical laboratories now use some form of nephelometry for measuring total immunoglobulin levels, classes, and IgG subclasses.

Nephelometry is a modification of the precipitin reaction. It depends on measuring the rate of soluble immune complex formation as detected by light scattering. Results are given in optical density units that are related to a standard curve and converted to milligrams per deciliter. These precipitin reactions are formed in antibody excess, so when the antigen concentration increases, the amount of light scattering increases. Automated nephelometric methods provide highly reproducible assays for quantitating IgG, IgA, IgM, and IgG subclasses in serum or other fluids.³

Depending on the age of the patient and blood group (not in type AB), it may be possible to measure anti-A and anti-B isohemagglutinins in the serum.¹ These are predominantly IgM-class antibodies against blood groups. A patient suspected of being hypo- or agammaglobulinemic is capable of making antibodies against a blood group antigen if isoagglutinins are detected. Low or absent isohemagglutinin titers in a patient without type AB blood would be indicative of poor IgM synthesis and would suggest that further studies are required.

If serum immunoglobulin levels are abnormally high in a patient with repeated infection, particularly an adult, it may be important to perform serum protein electrophoresis to determine if a paraprotein is present in the form of a monoclonal spike as evidence of a lymphocyte malignancy. High serum immunoglobulin levels can be a sign of HIV infection.

Under certain circumstances, it may be helpful to measure the number of circulating B lymphocytes to help clarify the particular defect causing the immunodeficiency. B lymphocytes have characteristic surface antigens that can be detected by a number of methods, most frequently by using a combination of monoclonal antibodies and flow cytometry (FCM).⁴ Monoclonal antibodies have been developed for cell surface markers on blood cells, and these antibodies have been carefully defined and standardized through the use of FCM.⁵ A number of specific B-lymphocyte markers are used to identify B lymphocytes (Table II). The most commonly used specific B-cell markers are CD19 and CD20. Normal peripheral blood B lymphocytes (defined with the use of anti-CD19, anti-CD20, or both) make up between 4% and 10% of the lymphocytes. A markedly depressed number of B cells in peripheral blood may suggest a possible immunodeficiency relating to a lack of B-cell differentiation.⁶

To further characterize B-lymphocyte and other blood cell populations and B- and T-lymphocyte function, monoclonal antibodies and flow cytometry can be used to

quantitate the number of helper and cytotoxic T lymphocytes in peripheral blood. Helper T cells are defined by reactivity with anti-CD4 antibody, and cytotoxic T cells are identified by reactivity with anti-CD8 antibodies. Decreased numbers of CD4⁺ T lymphocytes are associated with HIV infection and may contribute to an inability to mount a primary humoral immune response.⁷

Depending on the age and vaccination history of a patient suspected of having a B-cell immunodeficiency, one can measure antibody responses to the agents used for vaccination. Most infants will have received a battery of vaccinations by the time they are 1 year old. These vaccinations usually include tetanus and diphtheria toxoids, common viral vaccines, and *H influenzae* type b polysaccharide vaccine. The presence of antibodies elicited by one or more of these vaccinations should be indicative of an intact humoral immune system. However, abnormally low levels of one or more of the antibodies against the vaccines might support a diagnosis of B-cell deficiency. If it can be documented that the patient has recurrent or multiple infections with the same microorganism, it may be possible to isolate that particular organism to determine if the patient is unable to respond to that specific organism. In specific IgG subclass deficiencies, individuals do not respond to certain microorganisms with pronounced polysaccharide capsules.⁸ These are not usually life-threatening infections but may be related to a specific IgG2 subclass deficiency. These types of immunodeficiencies are usually detected by detailed clinical history and a study of the infecting microorganisms. However, with the IgG subclass deficiency, the total immunoglobulin levels in serum may be normal or slightly depressed. A determination of IgG subclass levels may reveal a decrease in one or more IgG subclasses. Deficiencies of IgG2 and combined deficiencies of IgG2 and IgA have been reported.⁹

In some cases of suspected B-lymphocyte immunodeficiency, an examination of peripheral lymphoid tissues may be useful. Histologic examination of draining peripheral lymph nodes after vaccination may show a lack of germinal centers and secondary follicles of germinal centers. In most cases of B-lymphocyte deficiency, all the lymphoid tissues, except the thymus, are hypoplastic, lack a defined germinal center, and are deficient in plasma cells.¹⁰

Functional studies of B lymphocytes may be useful in defining the nature of a humoral immunodeficiency. These studies are not run routinely in a clinical laboratory but can be especially useful for investigating specific defects in subpopulations of regulatory T cells and other defects concerning immunoglobulin synthesis and secretion.

Lymphocyte function may be evaluated in vitro by isolating the peripheral blood lymphocytes from the patient and culturing them in vitro with mitogenic agents; that is, substances that cause proliferation or differentiation of lymphocytes. The effects of mitogenic stimulation can be measured as DNA turnover by a number of established methods.¹¹ Pokeweed mitogen (PWM) is a plant derivative that induces both T and B cells to undergo proliferation and differentiation. As with most mitogens, both T

and B cells need to be present, and PWM requires the presence of T cells to induce a mitogenic effect on B lymphocytes. Other mitogens, such as killed *S aureus*, (protein A, Cowan 1 strain), may be used to stimulate B cells to proliferate and differentiate. These types of studies may be useful in determining the nature of the B-cell defect in individuals with suspected B-lymphocyte deficiencies.

The ability of B lymphocytes to synthesize and secrete immunoglobulin in vitro can be used to evaluate B-lymphocyte function.¹² B lymphocytes (from patients and controls) can be stimulated in vitro with either antigens or mitogens and, with the use of sensitive methods, immunoglobulin secretion can be detected. Obviously, very small amounts of immunoglobulin would be secreted in the medium in vitro, and radioimmunoassay or enzyme-linked immunosorbent assay would be used for detection. A number of methods are used for either antibody or immunoglobulin secretion, and these highly specialized studies would be used to further delineate the defect.

Humoral immunity can be evaluated through a number of laboratory methods, ranging from very routine, simple methods such as CBC and differential or serum immunoglobulin quantitation, to complex research laboratory techniques that measure potential immunoregulatory defects. It is possible to accurately detect and characterize the known B-lymphocyte deficiencies through available methods.

ASSESSMENT OF CELL-MEDIATED IMMUNITY

Cell-mediated immune responses result from complex interactions between antigen-presenting cells that take up, process, and present foreign antigens in an MHC-restricted fashion, to T lymphocytes possessing antigen-specific receptors. This recognition phase is followed by cell activation, elaboration of soluble mediators, proliferation, and cytotoxic activity. Defects in any of these broad categories of activities may lead to increased susceptibility to infections or cancers (Table I). Isolated T-cell deficiencies are relatively rare because of the important role that T cells play in the generation of an antibody response.

As with defects in humoral immunity, cell-mediated defects are associated with frequent or serious infections, usually of the respiratory system, skin, or gut, that may be difficult to treat with standard therapies. In contrast to defects in the humoral immune system, defects in the cell-mediated immune system are associated with infections caused by intracellular organisms, particularly viral and fungal organisms that, in the immunocompetent host, are not virulent. Congenital defects in cell-mediated immunity may present in the first few months after birth and may manifest as a failure to thrive in addition to infection.¹ Maternal antibody does not delay the manifestation of cell-mediated defects.

Assessment of the cell-mediated immune system is obviously important for the diagnosis and monitoring of cell-mediated immune deficiencies.^{11,13} In addition, it has applications for the diagnosis of infection,¹⁴ moni-

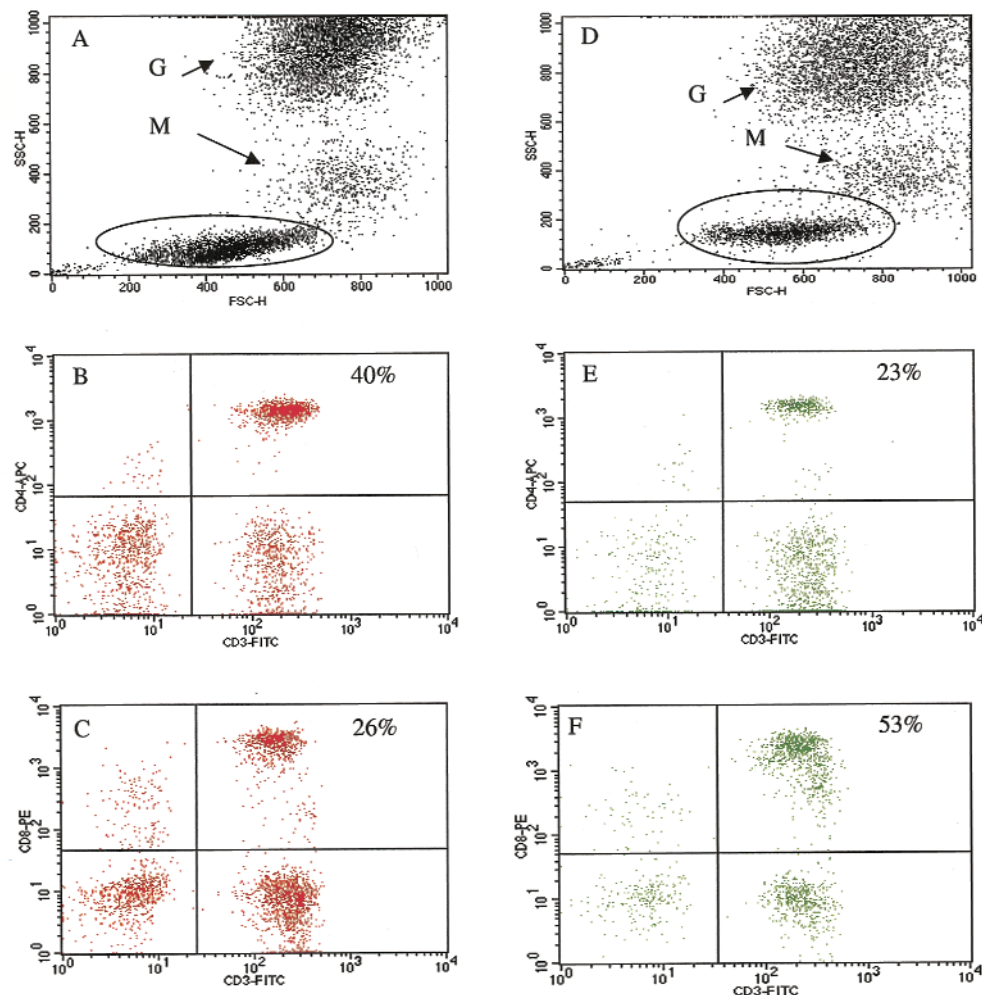


FIG 3. Flow cytometric analysis of CD4 and CD8 T lymphocytes. Panels A through C are representative dual parameter dot plots from a normal (HIV seronegative donor). Panels D through F are plots from an HIV seropositive donor. A and D are forward versus side scatter plots demonstrating the presence of lymphocyte (oval), monocytes (M), and granulocytes (G) populations. B and E demonstrate the proportion of CD3/CD4 dual positive T-helper lymphocytes in the lymphocyte gate (oval). C and F demonstrate the proportion of CD3/CD8 dual positive cytotoxic T lymphocytes. Note the inversion of the CD4:CD8 ratio in the HIV seropositive donor.

toring the effects of immunomodulatory therapy,¹⁵ and assessing the response to vaccination. The list of applications will likely grow as therapies for immune-mediated disorders are discovered and newer technologies come into more common use.

An initial assessment of the integrity of the cell-mediated immune system is obtained with a CBC and differential. From this evaluation, the proportion and absolute number of the major cell lineages are determined. Severe congenital cell-mediated deficiencies may demonstrate lymphopenia. However, this is not the case in all deficiencies nor consistently observed within specific types of deficiencies. As such, a more detailed analysis, including enumeration of specific cell subsets and assessment of their phenotypic and functional characteristics, is warranted.¹

ENUMERATION OF CELLS BY FCM IMMUNOPHENOTYPING

Flow cytometry (FCM) is used to determine the proportion and absolute number of lymphocyte subsets and other cell types.⁴ The advantage of this technology for cell enumeration is its ability to analyze precisely a large number of cells rapidly and objectively.

The power of FCM lies, in part, with the use of fluorochrome-labeled monoclonal antibodies. With newer instruments that can measure fluorescence from four or more fluorochromes simultaneously, multiple monoclonal antibodies, each labeled with a distinct fluorochrome, can be used to identify very specific subsets of cells in complex mixtures, such as whole blood (Fig 3). Mono-

clonal antibodies that specifically bind to any of a large number of cell surfaces or intracellular antigens can be used to define the state of maturation, activation, and functional capacity of specific cell types (Table II). A basic immunophenotyping panel to assess the proportion of the major cell subsets in peripheral blood includes antibodies to CD3, CD4, CD8 to identify the T-helper and cytotoxic subsets; CD19 to enumerate B cells and CD16 and CD56 to enumerate NK cells (Table II).⁵ Standard flow cytometers provide the proportion of these cells in the lymphocyte population. The absolute number of these various cell types can also be calculated as the product of the subset proportion and the absolute lymphocyte count determined on a hematology analyzer. More recently, reliable absolute counts have been obtained directly from the flow cytometer using counting beads¹⁶ or a volumetric analyzer that finds the frequency of cells in a defined sample volume.¹⁷ Cell subset enumeration is only one application of immunophenotypic analysis by FCM. This technology can also be used to identify the presence or absence of cell surface or intracellular antigens critical to the functioning of the cellular immune system. For example, one can assess the presence of molecules critical to the activity of cells, such as CD154 (CD40 ligand) on T cells, that is deficient in patients with hyper-IgM syndrome.¹ Flow cytometry is also the method of choice to assess depletion of CD3-positive T cells in transplant patients receiving OKT3 or thymoglobulin therapy.¹⁸

Enumeration of immune system cells is a primary means of assessing the integrity of the immune system. However, the functional capacity of these cells with possible defects may not be reflected in cell number. Both in vivo and in vitro techniques are available to assess the integrity of cell-mediated immunity. More recently, a variety of newer in vitro methods have been developed that enable enumeration and assessment of the functional capacity of the cellular immune system. Included on this list are the enumeration of antigen-specific CD8 T lymphocytes with tetrameric major histocompatibility complex (MHC)/peptide complexes and flow cytometric detection of antigen-induced intracellular cytokine production. These newer methods will very likely play important roles for assessing immunity in the future.

DELAYED-TYPE HYPERSENSITIVITY SKIN TESTING

The delayed-type hypersensitivity (DTH) reaction is considered an in vivo correlate of cell-mediated immunity.¹⁹ DTH skin testing can be used as a method to screen for defects in cell-mediated immunity.¹³

DTH skin testing is performed by intracutaneous inoculation of a defined amount of a standardized test antigen with a tuberculin syringe (Mantoux method).¹⁴ For assessment of cell-mediated immunity, three antigens are applied: purified protein derivative (PPD), *Candida albicans*, and mumps. Tetanus toxoid and trichophyton antigen may also be used. The use of multiple antigens

decreases the chance of a false negative result from a lack of prior exposure to an antigen. The injection site is monitored for the presence of induration 48 to 72 hours after administration of antigen. A positive result is characterized by induration of at least 2 mm.

The majority of immunocompetent individuals will demonstrate a DTH response to at least one of the three antigens. A lack of response to all three is indicative of anergy. Congenital T-cell deficiencies, cancer, some viral infections (eg, HIV), and immunosuppressive therapy may result in a lack of DTH response.¹⁹

IN VITRO LYMPHOCYTE PROLIFERATION

The standard in vitro test of lymphocyte function is the lymphocyte proliferation assay (LPA). It has been used to diagnose and monitor patients with primary and secondary immunodeficiencies; monitor immunomodulatory therapy; assess reconstitution of immune function in bone marrow transplants and in HIV patients receiving highly active antiretroviral therapy; assess histocompatibility and donor-specific hyporeactivity in transplantation; assess exposure to pathogens or allergens; and assess the response to vaccination.¹¹

The LPA assesses the ability of lymphocytes to proliferate in response to a variety of different stimuli. Peripheral blood mononuclear cells (PBMC) from anticoagulated peripheral blood are cultured for 3 to 7 days in the presence of a variety of stimulants. The length of culture depends on the specific stimulant used. PBMC are also incubated with no stimulus to assess the background proliferation rate of the preparation. At the end of the incubation period, the wells of the culture plate are pulsed with tritiated thymidine (³H-thymidine) that is incorporated into replicating DNA. After 6 to 18 hours to allow incorporation of the ³H-thymidine, the cells are harvested and the DNA captured onto filter paper discs. The amount of incorporated ³H-thymidine is quantitated with a liquid scintillation counter and expressed as counts per minute (cpm). Quantitation of incorporated ³H-thymidine allows one to assess the proliferative capacity of the cells, as the amount incorporated is proportional to the degree of proliferation. The results of LPA are presented as the stimulation index, that is, the cpm of the stimulated wells divided by cpm of the control wells. Results can also be presented as net cpm that equals the cpm of stimulant wells minus the cpm of the control wells. Ideally, both types of results should be provided.

Several types of stimulants may be used in LPA and provide distinct information on the functional capacity of the cellular immune system. Mitogens, including plant lectins such as phytohemagglutinin or anti-CD3 antibodies, are potent stimulants for proliferation. They are polyclonal activators and stimulate most T-lymphocytes in the culture to proliferate. An absent or poor mitogen response suggests a severe defect in cell-mediated immunity. Recall antigens, including tetanus toxoid, *C albicans* antigen, and streptokinase, induce proliferative responses only in subjects who have been previously

exposed. The responder cell frequency to recall antigens is much less than for the potent polyclonal stimulators. Evaluation of responsiveness to recall antigens is particularly useful in studies of immune reconstitution.²⁰

LPAs are complex tests not available in most clinical laboratories. These assays provide clinically relevant information on the status of cellular immunity. However, longitudinal monitoring of individuals or interindividual comparison is complicated by the high degree of variability of this assay. As such, LPA is best suited as a qualitative rather than quantitative indicator of lymphocyte function.

ANALYSIS OF CD8 T CELLS

Cytolytic activity is a critical effector mechanism for viral infections and is mediated by CD8 T cells. Cytotoxicity assays for CD8 T-cell function are very complex, available in research laboratories, and will not be considered here. More recently, the development of class I MHC/peptide tetrameric complexes has simplified the enumeration of antigen-specific CD8 T cells.²¹

MHC/peptide tetrameric complexes are reagents consisting of four class I MHC molecules linked together with a biotin/avidin bridge. The class I molecules are loaded with a specific peptide from the antigen of interest and also labeled with a fluorochrome such as phycoerythrin (detectable with standard flow cytometers). This complex mimics class I restricted antigen presentation to CD8-bearing T cells. The tetrameric configuration increases the affinity of binding to CD8 T cells, compared with single MHC class I/peptide configuration. The increased affinity results in more stable cell surface binding, enhancing utility for flow cytometric analysis. Class I MHC/peptide tetramers can be added to whole blood or PBMC preparations along with antibodies to CD8 T cells to enumerate the frequency of antigen-specific CD8 T cells in these biological samples.

The simplicity of this approach has greatly facilitated the analysis of CD8-mediated T-cell responses to viral pathogens, such as HIV, and assessment of response to vaccination. Their application is limited by the requirement that one must know the class I HLA phenotype of the patient and the relevant peptide from the infectious agent, malignancy, or vaccine for production of the tetramer. Class II tetramers are more problematic to produce and are not routinely available.

MITOGEN/ANTIGEN-INDUCED CYTOKINE PRODUCTION

In addition to proliferation and cytotoxicity, the other major function of T cells is to produce soluble mediators, such as cytokines, that are involved in various aspects of the immune response. There are several scenarios in which the detection or enumeration of these effectors is applied. The plasma levels of various mediators can be assessed to study their levels in normal and disease states.²² Analysis of multiple cytokines can be used to assess the qualitative nature of the immune response such

as TH1/TH2 analysis. Mitogen or antigen-induced cytokine secretion in whole blood or PBMC cultures can be used to assess whether the cytokine of interest is produced in expected amounts in a disease state or to assess the response to vaccination. For these applications, cytokine levels are determined by enzyme-linked immunosorbent assay.

A third and increasingly applied purpose is the determination of antigen-induced cytokine secretion at the single cell level as a marker for enumeration of antigen-specific cells.²³ Detection of cytokine production at the single cell level allows one to identify and enumerate antigen-specific CD4 or CD8 T cells. Single cell cytokine determinations offer increased simplicity and reproducibility compared with previously used methods, especially for antigen-specific CD8 T-cell enumeration. The two approaches most frequently used for assessment of cytokine production on a single cell basis are the enzyme-linked immunospot (ELISPOT) and FCM-based detection of intracellular cytokine production.

ELISPOT

The ELISPOT²⁴ assay quantitates antigen-specific cells by their production of cytokines, usually interferon gamma (IFN γ). PBMC, or purified CD4 or CD8 T cells, are incubated in wells containing a membrane that is coated with an anti-IFN γ capture antibody. A stimulant, mitogen or specific antigen (peptide or recombinant vaccinia virus), is added and the plates cultured for several hours. If antigen-specific T cells are present in the culture, they will produce IFN γ that is captured on the membrane at the site of its production. After removal of the cells, bound IFN γ is detected by addition of a second monoclonal antibody specific for IFN γ . This second antibody is labeled with an enzyme that will cause a precipitate to form at the site of IFN γ production when substrate is added. The number of spots counted per well is divided by the input cell number to yield the number of spot-forming cells per input cell number.

This assay has provided a relatively simple means of quantitating antigen-specific cell frequency, which has been difficult to do with proliferation and cytotoxicity-based assays.

INTRACELLULAR CYTOKINE DETECTION

Also known as cytokine FCM, this method involves incubation of PBMC or whole, anticoagulated peripheral blood with a specific antigen and costimulatory antibody, followed by treatment with an agent to prevent secretion of cytokines from the cell. After as little as 6 hours, cells can be stained for surface antigens to identify cell type, such as CD4 or CD8 T cells, and for intracellular cytokine production. The proportion of cytokine-secreting cells in the sample is rapidly determined by flow cytometric analysis. This method, like ELISPOT, is a simpler alternative for the enumeration of antigen-specific CD4 and CD8 T cells. Cytokine FCM has an advantage over

Assessment of Polymorphonuclear Neutrophils

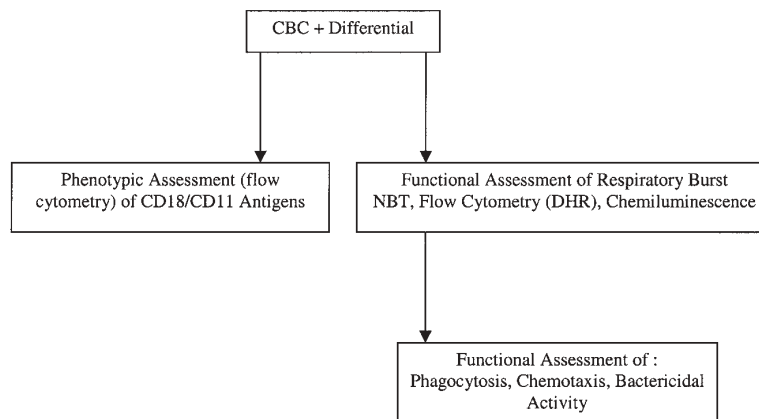


FIG 4. Flow chart for evaluation of neutrophil function.

ELISPOT in that it does not require depletion of cell subsets to determine the source of cytokine secretion. This assay has been used in the study of response to a variety of antigens²⁵ and has been particularly useful in the assessment of CD4 and CD8 T-cell responses to viral pathogens.

ASSESSMENT OF NK CELLS

NK cells are an important component of the innate immune defense system. They are capable of lysing virally infected or tumor cells and are also the source of a variety of cytokines. NK cell defects increase the susceptibility to viral infections but are rare entities. The quantitative assessment of NK cell number is achieved by FCM. NK cells typically fall within the lymphocyte region of a forward versus right-angle light scatter dot plot. In addition, they coexpress the CD16 and CD56 molecules, so multiparameter flow analysis combining light scatter and surface marker expression provides a reliable estimate of the NK cell number.

NK cell function is assessed by the ability of PBMC preparations to lyse NK-sensitive tumor target cells in a chromium release assay.²⁶ The K562 cell line is an erythroleukemic line that is susceptible to NK cell-mediated lysis. PBMCs are prepared from peripheral blood and incubated with K562 cells that have been prelabeled with radioactive chromium 51. Functional NK cells will lyse the labeled target cells, resulting in release of radioactive chromium into the culture supernatant. The amount of radioactive chromium released from the target cells is proportional to the NK lytic activity. This assay is not available in most clinical laboratories because of the need for maintenance of radioisotopes and cell culture. In addition, cytotoxicity assays are variable and difficult to standardize. Other methods using FCM²⁷ are available. However, they still rely upon the use of an NK-susceptible cell line.

ASSESSMENT OF PMN

PMN are major cellular components of blood and are important in acute inflammatory responses. Their ability to respond and clear infections is the result of a series of phenomena including migration to sites of infection (chemotaxis), adherence at the site of infection, recognition and uptake of invading organisms, metabolic upregulation, and killing. A deficiency resulting in neutropenia or a marked decrease in functional activity of any of the pathways may result in significant morbidity and mortality.¹

As with other major cell lineages in the peripheral blood, deficiencies in cell number are readily determined with routine CBC and differential. PMN numbers below 1500/ μ L are associated with increased susceptibility to infection. Functional assessment of PMN activity may include an assessment of any of the steps in PMN function as outlined above. However, most of these tests are complex and not routinely available. Cell surface adhesion molecule expression and the assessment of the respiratory burst activity are the defects identified with these tests and occur more frequently than defects in chemotaxis or phagocytosis (Fig 4).

Of the various adhesion molecules on the surface of PMN, defects in the upregulation of the beta-2 integrins (CD18, CD11a-c) are the most notable. These surface adhesion molecules promote adhesion to ligands on vascular endothelial cells, and defects in their expression result in leukocyte adhesion deficiency (LAD). Flow cytometric phenotyping can be used to determine their basal level of expression, and with appropriate stimulation, their ability to upregulate on the cell surface.²⁸

Upon recognition and ingestion of foreign material, PMN undergo metabolic changes that result in the generation of reactive oxygen species and hydrogen peroxide (respiratory burst). These molecules, in addition to other nonoxidative mechanisms, result in the killing of ingested microbes. The importance of the respiratory burst is evi-

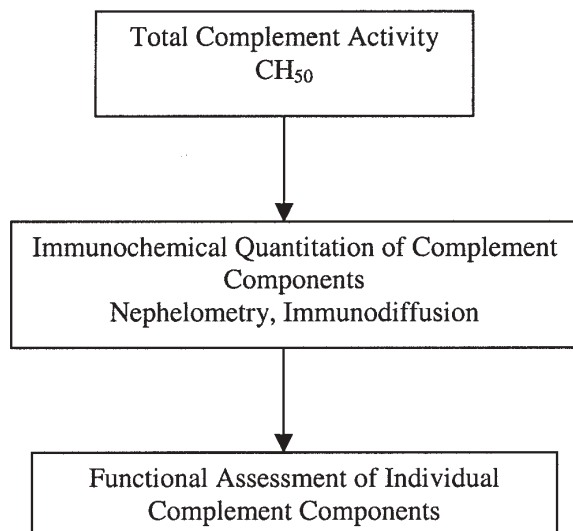


FIG 5. Flow chart for evaluation of the complement system.

denced by the morbidity and mortality associated with defects in this system. Several mutations have been described that result in chronic granulomatous disease (CGD), including X-linked (the most common) and autosomal mutations in genes of the nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase system. Respiratory burst activity can be assessed by several methods, including the nitroblue tetrazolium (NBT) dye reduction test, chemiluminescence, and FCM.²⁸

The NBT test involves the uptake of NBT by cells, provision of an activation stimulus, such as latex particles, and monitoring (photometrically or microscopically) for reduction of the dye, causing it to change to a blue color. A second method relying upon reduction of a dye taken up by PMN is FCM using dihydrorhodamine-123 or DHR. This dye is taken up by PMN and is nonfluorescent in resting cells. When the respiratory burst is induced, the dye is oxidized and emits fluorescence that can be detected with the flow cytometer. Both methods, NBT and FCM, detect CGD patients and can identify carriers.

Chemiluminescence is the generation of light that results from the interaction of reactive oxygen species generated during the respiratory burst with ingested organisms.¹³ With the addition of enhancing agents, this light can be detected in a scintillation counter, with the amount released reflective of the respiratory burst activity of the cells. Chemiluminescence is reduced in patients with CGD and carriers. This method offers increased sensitivity relative to the NBT test.

ASSESSMENT OF THE COMPLEMENT SYSTEM

The complement system is another major component of the innate immune system. It works in concert with other components of innate immunity and the adaptive immune system to provide pathways and mechanisms for

removing foreign microorganisms or damaged cells. The complement system consists of more than 40 proteins that are primarily glycoproteins that become activated to help remove any unwanted microorganism or other affected material through various pathways. The complement system is under considerable regulation to prevent uncontrolled activation that could cause host tissue damage.

Complement proteins work in a cascade fashion and they exist as precursors in plasma. After activation, the proteins interact in a sequential fashion to become active enzymes that interact with the next protein in the system. There are several pathways whereby the complement cascade can become activated and produce profound results as a result of its activation.²⁹ The complement system, its various pathways, and active products provide a formidable first line of defense for the host. At least three pathways of complement activation have been defined (see Chapter 12 for review), and they all contribute to an intact overall immune system.

There are distinct inherited deficiencies of the complement system.²⁹ Because there are multiple redundancies in the system, mild or partial complement defects do not usually result in disease. The majority of the complement proteins are coded for by autosomal genes. Therefore, individuals who have a heterozygous defect in one of the complement components usually are not at risk for infection because of this defect. Individuals who are homozygous for a single component defect are usually symptomatic and can be detected by specific complement functional testing. For example, an individual with a defect in C5 would have a break in the cascade of reactions involving complement activation, and the process would stop at that point. Depending on where the defect is in the cascade, some of the redundant features may bypass the defect and some residual activity would remain.

It has been shown that inherited defects in the early complement proteins, C1, C4, C2, C3, usually result in an increase in rheumatic disorders, an increase in pyogenic infections, or both.²⁹ The infecting organisms are either unusual infecting agents or of low pathogenic capacity. Inherited deficiencies of the later components of the complement cascade (C5, C6, C7, C8, or C9) are usually associated with a greater susceptibility to infections with *Neisseria* species, usually *N meningitidis*. The infecting organisms may be uncommon species that normally do not cause infections in healthy individuals. There is also a slight increase in the incidence of autoimmune diseases with defects in C5, C6, C7, or C8.

The conditions that would lead to suspicion of a complement system defect would be autoimmune disorders, especially unusual or noncharacteristic presentations, recurrent infections with unusual or mildly pathogenic organisms, or disseminated neisserial infections in young adults.

Two basic screening tests are used for assessing complement activity (Fig 5). The total hemolytic complement (CH₅₀) test measures the function of the classic complement cascade, whereas the AH₅₀ measures the function of the alternate pathway.³⁰ It measures the complement activity in dilutions of the patient's plasma on sheep ery-

throcytes that have been coated with anti-sheep erythrocyte antibody. The antibody-coated sheep-erythrocyte immune complex activates the complement cascade. If all components are present and functioning, the sheep erythrocytes are lysed and the hemolysis can be measured. Normal reference ranges are established for the assay system, and a patient with a total defect in one of the components of the classic pathway would show no hemolysis. Patients with mild (heterozygous) defects of C1, C4, C2, C3 may not show a decrease in hemolysis because of the redundancy in the system.

To confirm the specific complement component defect, an immunochemical method would be used to quantitate the individual components. Nephelometric assays similar to those used for detection of immunoglobulin levels are available for all the major complement components and reference ranges for each are available.³⁰ Immunochemical assays measure only the presence or quantity of each component and will not detect an altered or dysfunctional complement component.

If one suspects that a deficiency in complement activity may be the result of activation of the complement system, split components such as C4a, C4d, or Bb can be quantitated.³⁰ Their presence suggests activation of the complement system.

There are reference laboratories offering functional assays for specific component deficiencies. These assay systems assess the ability of the patient's serum to lyse antibody-coated sheep erythrocytes in the presence of reagents lacking specific complement components. If the patient's serum contains the component missing in the above system, lysis occurs. If not, no lysis occurs. By using a process of elimination, it may be possible to identify the missing or dysfunctional components in the patient's serum.

Overall, detection of inherited deficiencies of complement are available and can detect most of the possible defects. Complement deficiencies are rare, about 0.03% of the population, and the patient's history and clinical presentation are important in making the correct diagnosis.

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