CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY 24, 122–138 (1982)

Use and Abuse of Laboratory Tests in Clinical Immunology: Critical Considerations of Eight Widely Used Diagnostic Procedures[1]

Report of a Joint IUIS/WHO Meeting on Assessment of Tests Used in Clinical Immunology

IUIS/WHO Working Group

Geneva, Switzerland, May 18–20, 1981

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[1] This article has appeared in the *WHO Bulletin*, 59(5), 717–728, 1981.

0090-1229/82/070122-17$01.00/0

Methodological Considerations

Many methods for the quantitative assessment of immunoglobulins have been described. Two of them are currently of the most value and of comparable accuracy: (1) radial immunodiffusion (RID) and (2) nephelometry.

When patient load is relatively low, RID will probably remain the method of choice. However, with a high patient load and if nephelometer is already available, nephelometry is useful.

Radial immunodiffusion has a constant coefficient of variation which, under optimal conditions, may be less than 10% except at extremely low concentrations. The limit of accurate protein measurements, using low concentrations of antisera, is about 10 mg/liter (10 μg/ml). Techniques using limited diffusion are more accurate than those with timed diffusion. With normal sera, results can be obtained after 24 hr of diffusion but more time may be required for the assessment of very high or very low levels.

Pitfalls. RID is sensitive to differences in diffusion constants; special precautions should be taken to ensure that immunoglobulins in the standard and test sera are not split or aggregated and are in the same form. For instance, reliable measurements of such proteins as low-molecular-weight IgM and secretory IgA cannot be made unless a standard preparation of these kinds of immunoglobulin is used.

Nephelometric techniques are increasingly used for quantitating serum immunoglobulin levels. Both turbidimetric procedures and the detection of antigen–antibody complexes by light scattering can be applied. The advantages are that results can be obtained within a very short time, they can be fully automated, and there are no problems with polymeric immunoglobulin.

Pitfalls. Expensive instrumentation is required and turbid serum samples may need to be clarified.

Standards and antisera. Discrepancies in results have arisen from the use of different standards by different laboratories. WHO makes available reference preparations for the five classes of human serum immunoglobulins and it is recommended that working standards should be related to these preparations.

All antisera, including those from commercial sources, must be shown to be specific in the test for which they are being used. Hybridoma-derived monoclonal antibodies may be useful in the future; however, many monoclonal antibodies do not precipitate antigen when used alone so that mixtures of such antibodies may be required. With these antibodies it may become also easier to quantitate subtypes and subclasses of the immunoglobulins.

Normal values. Concentrations of immunoglobulins in sera vary with age, geographical environment, and sex. Each laboratory should measure serum immunoglobulin concentrations on a matched control group.

Clinical Indications

Serum

Quantitation of serum immunoglobulins is ESSENTIAL in suspected primary or secondary immunodeficiency (ID) even when no abnormality is seen in electro-

phoresis. Concentrations of immunoglobulins cannot be used, however, as the sole criterion for diagnosis of primary ID. Reports have been made of persons with selective IgA deficiency without any evidence of associated disease, and IgA is undetectable in approximately 0.03–0.2% of the normal population.On the other hand, failure to respond to one or more antigens can sometimes be observed in patients with normal or high levels of all immunoglobulins. Thus, normal immunoglobulin concentrations do not exclude antibody deficiency. Monitoring of serum immunoglobulin levels is *ESSENTIAL* in patients with severe forms of hypogammaglobulinemia who receive gammaglobulin substitution therapy.

Quantitation of serum Ig is *HELPFUL* in distinguishing "benign" idiopathic monoclonal gammapathies from paraproteinaemias caused by myeloma. In the latter case, the levels of *normal* immunoglobulins are usually decreased while they usually are unaltered in the "benign" form. In this context it should be stressed that monoclonal immunoglobulins tend to give falsely high values in immunodiffusion assays. When large enough amounts of the monoclonal protein are present, it is more accurate to measure the protein by the area under the spike on serum protein electrophoresis.

The value of quantifying serum immunoglobulins for other clinical purposes has been established in only a few additional instances, such as the determination of IgM levels in the cord blood of infants suspected of congenital infections, and as an aid in the diagnosis of trypanosomiasis and of tropical splenomegaly.

Optionally, *FOR RESEARCH PURPOSES*, immunoglobulins may be quantitated in diffuse hypergammaglobulinemia in conditions such as some lymphoproliferative diseases, liver cirrhosis, and systemic lupus erythematosus.

More promising might be immunoglobulin studies in the families of patients with immunodeficiency or homogeneous immunoglobulins which may clarify the role of genetic factors.

Other Body Fluids

Urine. Quantitation of immunoglobulins in the urine is possible but fraught with problems. For instance, Ig molecules may be split, urinary light chains exist as monomers, making standardization difficult. For the demonstration of Bence Jones proteins, the combination of protein electrophoresis and immunoelectrophoresis is more useful.

Cerebrospinal fluid (CSF). Quantitation of immunoglobulin should be performed on unconcentrated CSF since concentration procedures will lead to the aggregation of immunoglobulins, especially IgG, and a falsely low value by RID.

Quantitation of immunoglobulins in CSF is of interest in diseases such as multiple sclerosis and subacute sclerosing panencephalitis where the concentration of IgG relative to the total protein or albumin is often, but not always, increased. In African trypanosomiasis the increase of CSF Ig is an indication of invasion of the central nervous system by the parasites.

IMMUNOELECTROPHORETIC ANALYSIS OF IMMUNOGLOBULINS IN BIOLOGICAL FLUIDS

Immunoelectrophoresis (IEL) permits ready identification of the major immunoglobulin classes. It is the method of choice for the identification of monoclonal

immunoglobulins since it detects simultaneously their electrophoretic and antigenic homogeneity. It is not a good quantitative technique. It should not be used for the systematic screening of serum proteins.

Methodological Considerations

IEL is a useful method to study immunoglobulins in other fluids in addition to serum, e.g., urine, cerebrospinal fluid, saliva, and intestinal juice. In the latter instances, it is usually necessary to concentrate the proteins before performing IEL and to simultaneously run a serum sample from the same patient.

The medium of choice for IEL is either agar or agarose, using where possible, the same type of gel for the serum protein electrophoresis.

IEL requires the use of potent and specific antisera. It is recommended to use, in the first step, polyvalent antisera containing precipitating antibodies to the various Ig classes and light chain types. In order to identify monoclonal immunoglobulins, monospecific antisera to the various Ig heavy and light chains are often required. These antisera are commercially available and should always be checked for their content of precipitating antibodies and for their specificity.

The determination of the heavy chain class of a monoclonal immunoglobulin requires sometimes, but not always, the use of class-specific antisera. Such antisera are necessary for the diagnosis of IgD or IgE myeloma. The identification of the heavy chain subclass of monoclonal IgG or IgA components is mainly of value in research. The identification of the κ or λ light chain type is necessary for the diagnosis of Bence Jones proteins and optional for myeloma proteins. The light chain type may, however, have prognostic significance in myeloma. IEL with anti-κ and anti-λ antisera allows the detection of small monoclonal components in the presence of diffuse hyperimmunoglobulinemia, and sometimes the detection of multiple monoclonal components.

Pitfalls. Because of poor availability of antigenic determinants for crosslinking, many anti-light chain antisera are unable to precipitate some whole monoclonal immunoglobulin molecules, especially IgA-λ and/or some free light chains (Bence Jones proteins). Thus for the diagnosis of heavy chain diseases (in particular α-chain disease), the use of additional procedures is necessary; e.g., IEL with antisera containing precipitating antibodies to conformational determinants of the Fab region or immunoselection combined with IEL, using potent antisera to light chains or to Fab which are incorporated into the gel. As in all immunoprecipitation procedures, antigen excess may preclude the visualization of a precipitin line especially when horse antisera are used. This is particularly the case when analyzing Bence Jones proteins.

When a cryoglobulin is present in the serum, the immunoelectrophoretic analysis of the whole serum should be performed after heating at 37°C and resolution.

In order to permit ready identification of some IgM proteins and to ascertain their monoclonal nature by light chain typing, additional procedures may be necessary, such as the addition of a reducing agent to the fluid under study (in order to convert 19 S IgM into 8 S subunits) or preliminary separation of IgM from IgG by physicochemical techniques. Immunofixation, a technique more recently devel-

oped for the identification of monoclonal immunoglobulins, may be particularly useful in such instances.

In interpreting immunoelectrophoretic patterns one should be aware of possible associations of monoclonal immunoglobulins with other proteins, such as serum albumin, α-1 anti-trypsin, and lipoproteins.

Clinical Indications

Serum

IEL is ESSENTIAL in certain cases:

(a) When the clinical hematological, and/or pathological findings lead to the diagnosis or suspicion of the following diseases: myeloma, Waldenström's macroglobulinemia, heavy chain diseases, amyloidosis, and immunoglobulin deposition disease.

(b) In the presence of the following biological abnormalities:

(i) an abnormal narrow band on serum protein electrophoresis; however, it should be stressed that IEL allows the detection of monoclonal components in situations without a distinctive electrophoretic pattern.

(ii) presence of a cryoglobulin; IEL is necessary to identify the proteins of the cryoprecipitate and to distinguish single class homogeneous cryoprecipitating immunoglobulins from mixed cryoglobulins with or without a monoclonal component. IEL should also be done on whole serum.

(iii) presence of a Bence Jones type of proteinura.

(iv) Pyroglobulin; serum hyperviscosity; discrepancy between immunoglobulin level as appreciated by electrophoretic and immunochemical procedures.

IEL may be USEFUL in some immunoproliferative disorders such as chronic lymphocytic leukemias (detection of μ-chain disease and of monoclonal immunoglobulins) and cold agglutinin disease and in diseases such as Gaucher's disease or mucinar papulosis (monoclonal components) and trypanosomiasis (elevated polyclonal IgM).

IEL may be useful for RESEARCH PURPOSES in several instances such as primary immunodeficiencies (in addition to measurement of Ig levels which is necessary), bone marrow grafts in patients with leukemias, marrow aplasia, or severe combined immunodeficiency; some autoimmune diseases; some hematological conditions, such as myelomonocytic leukemias; various infections, such as cytomegalovirus or congenital toxoplasmosis; systematic survey of family members of patients with monoclonal gammapathies.

Urine

IEL is ESSENTIAL in myeloma (with or without whole homogeneous serum Ig); amyloidosis, immunoglobulin deposition disease; in all cases in which a monoclonal Ig has been found in serum, whatever the clinical conditions; in cases in which an abnormal narrow band has been found on the urinary protein electrophoresis pattern.

IEL is OPTIONAL in malignant lymphoproliferative diseases other than myeloma (macroglobulinemia, chronic lymphatic leukemia, lymphoma, heavy chain diseases) and in primary immunodeficiencies.

Other Fluids

IEL of *cerebrospinal fluid* proteins is also USEFUL in the search for oligoclonal components in patients with subacute sclerosing panencephalitis, or in myeloma or macroglobulinemia with neurological involvement. In multiple sclerosis, the technique of isoelectrofocussing is more productive.

IEL of the *intestinal juice* is ESSENTIAL in "immunoproliferative small-intestinal disease" with suspicion of α-chain disease, when the abnormal protein is not detected in the serum of the patient.

MEASUREMENT OF TOTAL IgE

IgE is the most important mediator in atopic disease. Moreover it is highly increased in some parasitic diseases. The clinical usefulness of IgE level determination, however, is of limited value.

Methodological Considerations

The recommended methods to measure serum IgE (usually present in μg/liter quantities) are: ELISA techniques and solid phase radioimmunoassay.

The common principle of the two methods is to use insolubilized anti-IgE antibody. This reagent can be used either in a competitive binding assay using radiolabeled IgE and IgE standard, or in a noncompetitive assay using radiolabeled anti-IgE. Like other competitive assays, the first one is subject to nonspecific inhibition by other serum factors and of limited sensitivity and is not recommended. The advantages of noncompetitive assays are: increased sensitivity and precision and the fact that they are usually free from interference by nonspecific serum factors.

Although radioimmunoassays were used initially, the advantages and potential of ELISA enzymoimmunologic assays, especially in developing countries, should foster their increasing use.

The main advantages of ELISA techniques are the avoidance of isotope markers; the long shelf life of the reagents; the evaluation by means of a photometer instead of the gamma counter. The only limitation is that the ELISA techniques developed to date are not sufficiently sensitive to measure very low IgE levels.

Radioimmunoassay is therefore the method of choice in pediatric patients, in immunodeficiencies, and for analysis of cord blood, supernatants of cell cultures, etc. For the higher sensitivity and reproducibility required for research purposes, suitable double antibody assays have been described.

The values obtained must be compared with those of a control group matched according to age and geographical location. A WHO international reference preparation is available.

Indications

Determination of total IgE is NOT ESSENTIAL except in the diagnosis of the rare hyper-IgE syndrome associated with eosinophilia and recurrent infections described by Buckley.

Determination of total IgE may be USEFUL in differentiating IgE-mediated from non-IgE-mediated disorders when this cannot be done by clinical means.

Properly used, measurement of specific IgE can reduce the frequency of provocation test.

Measurements of specific IgE are used for RESEARCH purposes in various IgE-mediated diseases and in some parasitic infestations.

Measurement of specific IgE should be not regarded as a "screen" for allergic diseases or requested for the evaluation of allergic conditions in which IgE-mediated mechanisms are not involved (e.g., contact dermatitis).

COMPLEMENT MEASUREMENTS

Complement consists of a series of proteins that undergo sequential activation as a consequence of interaction with a variety of agents.

Measurement of complement can be achieved either by functional measurement of the whole system, by functional measurement of individual components, or by immunochemical measurement of individual components, using specific antisera. These measurements are static, representing the balance between synthesis and consumption. Elevated complement levels occur due to increased synthesis, especially following acute inflammation and trauma, and low levels are found due to increased consumption and/or decreased synthesis. The latter may be genetically determined.

Methodological Considerations

The total complement hemolytic assay (CH50), assesses the ability of serum to lyse a standard suspension of sheep erythrocytes optimally sensitized with anti-sheep red cell rabbit antibody. The test, as usually performed, principally assesses the functional activity of the components which generate the classical pathway C3 convertase, and of C3 itself. It is also a test of the presence of functionally active terminal components C5–C9, although it is not sensitive to variations in the level of these components.

There are many ways of performing this test, but the technique which is most reproducible and clinically applicable is that described by Mayer. Variations in this procedure use different concentrations of cells, and/or different volumes of reactants and incubation times. It is also possible to measure CH50 by automated methods.

Pitfalls. The value is dependent on the conditions of the test, and variations in level may occur if the red cells are aged or not standardized properly, or are low in potassium, or not adequately sensitized. For this reason, a standard serum with a known value should be included in all batches of estimations. Also, inadequate collection and storage of test specimens may give falsely low values. Sera should be separated within one hour after collection of blood, and stored at −70°C before testing. Where this is not possible, the use of EDTA plasma has been recommended. In sera containing cryoglobulin, falsely low functional and immunochemical complement levels may be obtained. A reference standard preparation for CH50 is available through WHO.

Functional measurement of *individual components* is seldom necessary in clinical practice unless a genetic complement defect is suspected. Antisera are available to most complement proteins, in particular C3, C4, C1q, C1 esterase inhib-

itor, and Factor B. Estimation of individual components by immunochemical techniques is adequate for the vast majority of clinical purposes, and is particularly useful in poorly stored specimens. Although rarely, genetic defects occur which result in the synthesis of abnormal molecules without functional activity; in general, immunochemically estimated component levels reflect *in vivo* functional levels.

The immunochemical estimation of C3 and C4 and other complement proteins can be carried out either by the single radial diffusion test, or by some form of nephelometry. Rocket electroimmunodiffusion is not recommended because of the changes in electrophoretic mobility of the molecules on storage. International reference standard preparations for C3, C4, C1q, and Factor B are available through WHO. The specificity of the antiserum used in the analysis is important and, for C3, antisera specific for C3c only should be used.

Estimation of C3 and C4 together form the most useful routine measurements of complement components. In some conditions the C4 may be abnormally low although the CH50 may be normal. Sometimes a low CH50 is due principally to a low C2, but antisera to C2 are not widely available, and functional tests of this protein are difficult to carry out in routine laboratories. C1 esterase inhibitor levels are principally of value in the differential diagnosis of angioedema.

Clinical Indications

CH50 complement estimations are ESSENTIAL only in those conditions in which a genetic defect in complement is suspected, e.g., in patients presenting with recurrent infections, especially recurrent meningitis, with hereditary angioedema or with established immune complex diseases occurring in families. For the confirmation of angioedema, estimation of the C1 esterase inhibitor level is essential, and if a normal immunochemical level is obtained then a functional assay should be performed since 10–15% of kindreds are associated with the production of nonfunctional molecules. If the CH50 is normal, functional assays of individual components are unnecessary except to detect heterozygous states.

Complement estimations (CH50, C3, and C4) are HELPFUL in the assessment and monitoring of patients with glomerulonephritis, in established immune complex diseases such as systemic lupus erythematosus, and certain forms of vasculitis, and in conditions such as dengue haemorrhagic fever. In conditions in which low levels are found, these frequently return to normal in remission, and complement levels can be used to monitor treatment.

Routine complement tests are of little value in most other acute and chronic inflammatory or infectious diseases.

DETECTION OF IMMUNE COMPLEXES IN HUMAN BIOLOGICAL FLUIDS

There is a good evidence that immune complexes (IC) are involved in the pathogenesis of tissue lesions in a variety of human diseases.

Since 1972, more than 30 methods for the detection of circulating IC have been devised and used extensively. It was expected that this type of technology would provide ideal tools for the diagnosis of diseases due to immune complexes. However, these expectations have not been fully realized.

Methodological Considerations

Most methods have been designed for the detection of immunologically aggregated immunoglobulins without considering the nature of the antigen(s) involved in the IC. These methods are the most widely used for clinical purposes.

Some methods are based on *physicochemical* differences between monomeric Ig and aggregated Ig. Precipitation in polyethylene glycol (PEG) has been widely used as a routine method. Although it may be useful to concentrate complexes, it is not specific for immune complexes since, even at low concentrations, a variety of large serum proteins are also precipitated. The quantitation of total protein or even individual proteins in PEG precipitate is not recommended as a measurement of IC levels.

Biological methods are based on the recognition of IC in humoral or cell receptor systems. Although all of these methods detect IC they do not allow for a direct quantitation of IC proteins. Tests using Fc receptors on macrophages, K cells, or platelets have been largely abandoned for two reasons: (a) high sensitivity to interfering factors, (b) difficulty in achieving reproducibility.

Although interfering factors can lead to false-positive results, it appears now that, in most cases, a positive result is likely to indicate the presence of IC when the following methods are used: C1q solid-phase or fluid-phase binding tests; conglutinin assays; monoclonal RF inhibition; RAJI cell assay. These tests have been found the most acceptable in recently WHO/IUIS collaborative studies. Some of them (e.g., solid-phase C1q or conglutinin) can be used to detect the class of antibody present in the complex by the use of appropriate specific antisera at the final stage.

Pitfalls. The main pitfalls of these four methods are the following:

(a) These methods will detect nonspecifically aggregated Ig as well as immunologically aggregated Ig. Some of the methods require a pretreatment of the sample (heating at 56°C) which may induce Ig aggregation.

(b) The collection and the storage of samples for IC detection should be done with care, avoiding bacterial contamination and repeated freezing–thawing. Blood should be allowed to clot for 2 hr at 37°C before separation of serum. The temperature of storage should be −70°C.

(c) Tests using C1q may be influenced by the presence of heparin, endotoxins, or free DNA in the test sample.

(d) Methods using rheumatoid factors (RF) are unsuitable for IgM containing IC and cannot be used with sera containing RF or in presence of elevated IgG levels. It has also proved difficult to standardize RF preparations.

(e) In the case of the RAJI cell assay, false positive results may be obtained in the presence of antilymphocyte antibodies. The cells require particular care in culture conditions to avoid variations in sensitivity.

(f) For the above reasons, the results of the different tests for IC may not *always* be directly comparable.

(g) The quantitation of IC has been done until now without comparable reference preparations. Therefore published results expressed in micrograms of complexes or as equivalent of micrograms of heat-aggregated IgG are not comparable from one laboratory to the next one. Reference preparations of aggregated IgG

and of preformed IC (tetanus toxoid antigen–antibody complexes) are now available on request.[1]

Although antigen-specific detection of immune complexes should be the main goal in this type of investigation, information regarding the nature of the antigen(s) involved in the *in vivo*-formed IC has only been obtained in restricted clinical conditions, using methods developed for that particular purpose (e.g., microbial antigens, DNA, etc.). Information obtained through the analysis of IC purified from serum indicates that IC may often result from specific interactions between immunoglobulin molecules (RF, anti-idiotypes). Thus the presence of IC in serum samples does not imply the presence of a particular antigen of exogenous, microbial, or autologous origin.

Clinical Indications

The detection of IC is NOT ESSENTIAL in any clinical condition. The presence of IC in serum is not specific for an immune complex disease. IC-induced lesions (e.g., glomerulonephritis) can exist without detectable circulating IC while IC are often present in serum without evidence of typical immune complex-associated lesions.

The detection of IC may be HELPFUL for assessment and monitoring of disease activity in conditions such as rheumatoid arthritis and systemic lupus erythematosus. It is also of value in monitoring the effects of plasma exchange therapy. It may also have a prognostic value in some malignancies such as acute leukemia.

In all conditions where an IC disease is suspected, a direct analysis of tissue samples (e.g., kidney, skin) should be done, when possible. Such examinations cannot be replaced by the detection of circulating IC.

AUTOANTIBODIES BY INDIRECT IMMUNOFLUORESCENCE

The most widely used method for detection of autoantibodies directed against tissue antigens is indirect immunofluorescence (IIF). However, many other methods in common use provide diagnostic information by employing defined antigens. In the future, more procedures using purified antigen can be expected.

Methodological Considerations and Pitfalls

The IIF procedure involves the application of a patient's serum to a section of appropriate human or animal tissue, removal of unbound globulin by repeated washing, and subsequent addition of antiserum to human immunoglobulin (prepared by immunization of an experimental animal) which has been conjugated with a fluorescent tag. The site of antibody fixation can be visualized with fluorescence microscopy. Rather than a fluorescent dye, antibody can be labeled with an enzyme such as peroxidase and appropriate cytochemical methods used to trace antibody localization. The most important variables involved in a reproducible technique are: (1) the type of substrate employed including source, method of

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fixation, storage, and preparation; (2) duration of incubation and washing of the patient's serum; and (3) specificity and sensitivity of the antiglobulin conjugate. An essential part of each test is the incorporation of known positive and negative sera as controls.

The four groups of autoantibodies that are most requested are antibodies to nuclei, to thyroid, to mitochondria, and to smooth muscle. It is possible to prepare composite blocks of several tissues processed at one time.

To test for *antinuclear antibodies* (ANA), appropriate substrates are cryostat sections of rodent liver or kidney, but human leucocytes are used in special cases. Fixed tissue culture cells are available commercially, but they are visually less satisfactory than tissue sections because they give more nonspecific fluorescence.

Different patients' sera may produce different patterns of nuclear staining. Antibodies producing the homogeneous pattern are mainly directed against nucleohistones. The peripheral pattern is probably due to antibodies against native DNA. The antibodies associated with speckled staining are directed against soluble nuclear antigens such as the Sm or ribonucleoprotein antigens. The nucleolar patterns are due to reaction with RNA.

The substrate for demonstrating *thyroid autoantibodies* consists of frozen human or monkey thyroid tissue and the procedure is the same as described for ANA. At least two distinct autoantibodies can be differentiated by IIF. They are directed against the thyroid epithelial cells or colloid, respectively. A positive test of patient's serum on unfixed slides appears as bright fluorescence of the epithelial cells. The autoantibody responsible for this reaction is directed to a microsomal lipoprotein of the epithelial cell. Autoantibodies reacting to colloid can be seen only when using methanol fixed slides. These autoantibodies can also be demonstrated effectively using hemagglutination tests with red blood cells coated with respective antigens.

For the demonstration of *mitochondria antibodies*, rat kidney is usually employed as substrate and immunofluorescence is seen in the cytoplasm of epithelial cells lining the ducts. *Smooth muscle antibodies* are generally tested with rat stomach sections as substrate.

Clinical Indications

Requests for unspecified screening for autoantibodies should be discouraged. Clinicians should rather ask for precise autoantibody tests appropriate to the clinical context.

Tests for ANA are ESSENTIAL for the diagnosis of systemic lupus erythematosus (SLE). The occurrence of ANA in low titers is relatively common and is associated with a variety of disorders. Even sera from normal individuals show a low incidence of ANA, especially in aged populations. Therefore the greatest use of the ANA is to exclude the diagnosis of SLE, since the vast majority of all active SLE cases are positive. Further confirmation of the diagnosis of active SLE requires the demonstration of antibodies to native (double-stranded) DNA which can be demonstrated by IIF (with Crithidia lucilleae kinetoplast) or by other techniques; the demonstration of antibodies to Sm antigen is also of great diagnostic value in this condition.

Tests for ANA are USEFUL in the diagnosis of "mixed connective tissue disease" (speckled pattern associated with antibodies to RNP), and the autoimmune form of chronic active hepatitis. They are also helpful in many cases of drug-induced SLE and a characteristic pattern of nucleolar strain occurs in progressive systemic sclerosis. ANA is sometimes of value in the study of family members of patients with SLE, because it may lead to earlier detection of this disease.

Tests for *thyroid autoantibodies* are ESSENTIAL for the diagnosis of chronic thyroiditis and spontaneous adult myxedema. Over 90% of thyroiditis patients have autoantibodies directed against either cell microsomal antigen, thyroglobulin, or both. A positive test, however, does not eliminate the diagnosis of such conditions as adenocarcinoma or Graves' disease, since 20% of these patients have antibodies to thyroid antigen, although titers are generally lower than in those patients with thyroiditis.

Antibodies to mitochondria are characteristic but not specific for primary biliary cirrhosis and antibodies to *smooth muscle* are frequently found in high titer in the sera of patients with chronic active hepatitis. Both of these groups of autoantibody are found in many other conditions, but the tests may become more useful when purified antigens become available.

Other autoantibodies of clinical interest are found in certain uncommon diseases. For instance, antibodies to the intercellular substance of stratified aqueous epithelium are present in pemphigus, while a different fluorescent pattern involving the basement membrane of stratified epithelium is characteristic of pemphigoid.

Antibodies to muscle striation are often detected in the sera of patients with myasthenia gravis. However, in this condition, a more useful test is the detection of antibodies to the acetylcholine receptor, which can be detected by radioimmunoassay.

Autoantibodies to adrenal cortex found in chronic cases of idiopathic adrenal insufficiency or to pancreatic islets in some cases of insulin-dependent diabetis mellitus are not frequent enough to be of diagnostic value, but are useful for clinical research.

B- AND T-CELL DETERMINATION

A major advance in the study of the lymphoid populations was made when it was shown that they could be characterized by certain cell surface markers. This has since generated a considerable number of studies of the enumeration of T and B cells in health and disease. Although these studies have been disappointing for most clinical purposes, they have helped in the characterization of cellular markers and in our understanding of human lymphocyte physiology.

Methodological Considerations and Pitfalls

Lymphoid Cell Separation

Most of the studies of human T and B cells are performed on human peripheral blood and use the Ficoll–Isopaque method for mononuclear cell separation. Such preparations contain a variable number of monocytes which it is important to

distinguish from lymphocytes. This can be most easily achieved by either latex particle ingestion or peroxidase staining.

It is advisable to carry out study of cell markers on freshly drawn samples of blood and to check the viability of the cells since this may influence cell surface characteristics.

T-Cell Markers

At the present time, two types of methods are recommended to detect all peripheral T cells; they are the formation of sheep red cell rosettes (E rosettes) and the use of T-cell-specific monoclonal antibodies.

E rosettes is the most commonly employed and recommended assay for enumerating T cells. Different laboratories have reported great variability in the percentage of E rosettes in the normal population; these variations are still frequent although they have been mostly overcome as a result of better standardization of techniques. The source of sheep red blood cells, their conservation, the presence of small amounts of serum (fetal calf serum or human AB serum) as well as the careful handling of the rosette preparations, are important factors. Serum factors (like antibodies to cell surface components, or lipoproteins) may interfere with rosette formation in certain conditions by coating the cells and competing with the sheep red blood cells for their binding sites. In such situations, short-term culture (1–18 hr) of the cells is usually very helpful in removing or shedding these substances.

Specific anti-T-cell antisera are now used increasingly for detection of all T cells in peripheral blood lymphocytes and in the lymphoid organs. Such reagents are directed against the E receptor or against other common T-cell membrane determinants. The most promising and reliable reagents are monoclonal antibodies. The preferred method for the use of such antibodies is by indirect fluorescent labeling rather than by cytotoxicity, which is less accurate.

T-cell subsets were defined initially by the presence of receptors for the Fc of IgM or IgG. However, recently defined monoclonal antibodies are more reliable and accurate reagents for defining T-cell subsets.

B-Cell Markers

Surface membrane Ig is the most reliable B-cell marker if properly carried out. Membrane immunoglobulin (SmIg) is most commonly identified by fluorochrome-labeled anti-immunoglobulin antisera. The recommended reagents for the enumeration of B cells are antisera raised against the Fab portion and/or mixture of anti-κ and λ light chain. They are commercially available but should be very carefully checked for specificity. Monospecific reagents to the various Ig chains are used for characterization of the heavy and light chains on the cell membrane and in the cytoplasm.

The following *pitfalls* should be emphasized: (1) involvement of Fc receptors that may bind autologous as well as the reagent's Ig. This is largely overcome through incubation and the use of labeled $F(ab)_2$ reagents for immunofluorescence; (2) specificity and potency of reagents; (3) inadequacy of monocyte identification; (4) potential interference by autologously reacting antilymphocyte

antibodies, conferring positive surface staining to an otherwise SmIg-negative cell.

Anti-immunoglobulins have also been labeled by enzymes, isotopes, or red cells for the determination of SmIg.

Using a similar approach to that employed for T cells, antisera and/or monoclonal *antibodies reacting specifically with all B cells,* have been recently described. Antibodies to B-cell subsets have also been reported and await further characterization.

A group of *other markers* present on B-cell membranes have been described. Some, such as the complement receptor and the receptor for Fc, are not specific for B cells. Therefore, procedures such as the EAC rosettes are not recommended at present for routine enumeration of B cells. However, studies of these receptors as well as that of the Epstein–Barr virus and mouse red blood cell receptors to differentiate B cells and B-cell subsets could be used for research purposes.

In summary, the recommended basic methods for T- and B-cell determination are at present the SmIg, E rosettes, and, where available, suitable monoclonal antibodies.

Clinical Indications

Enumeration of T and B cells is: ESSENTIAL is the assessment and monitoring of primary immunodeficiencies and USEFUL in the diagnosis of secondary immunodeficiency and for the classification of lymphoproliferative disorders. It should include where possible a larger number of reagents including monospecific anti-Ig and monoclonal antibodies to lymphoid populations.

In addition, study of T- and B-cell subsets may be useful in selected patients and mainly for RESEARCH PURPOSES since so far enumeration of B and T cells has not proved to be of clinical value in infectious, antoimmune, or nonlymphoid malignant diseases.

LYMPHOCYTE RESPONSE TO MITOGENS IN THE EVALUATION OF CELL-MEDIATED IMMUNITY

The investigation of cell-mediated immunity (CMI) is important in the evaluation of the host immunological competence. For this purpose, a group of *in vivo* and *in vitro* procedures are commonly used.

It is essential that these assays should be employed in an orderly fashion so as to obtain pertinent information, minimize abuse, and overcome pitfalls.

Delayed hypersensitivity skin testing using two or more common recall antigens (streptokinase-streptodornase, PPD, Candida, trycophyton, mumps) should be the first assay to perform.[2] It is only following this initial stage, if the results obtained suggest possible alterations of CMI, that cell function should be explored *in vitro*. In addition to mitogen responses, lymphocyte response to foreign antigens and alloantigens should also be investigated.

The following remarks will deal exclusively with proliferative response to mitogens.

[2] Sensitization with 1-chloro-2,4-dinitrobenzene (DNCB) is at present the only way to explore the primary response *in vivo* but should be performed only in selected patients.

Methodological Considerations and Pitfalls

Proliferative response of lymphocytes to several *mitogens* is best measured by radioactive thymidine uptake. Mononuclear cells separated by Ficoll–Hypaque from peripheral blood in the micromethod should be used. Results are commonly expressed as total radioactivity uptake.

In order to optimize the assay, it is essential to define culture conditions, standardize the biological and commercial reagents, and control the number of cells in the culture (including concentrations of monocytes). In addition, due to the great variability inherent in the systems, the regular use of normal controls is critical. These should consist of both controls matched for the patients as well as controls for computing the daily variation of the laboratory. Results could then be expressed as relative proliferative response index which takes into consideration the above-mentioned factors. Dose–response curves are also of importance to select for the optimal response while suboptimal concentrations of mitogens may be of advantage in the study of certain disease states such as some immunodeficiencies.

In the evaluation of the proliferative response, the level of background should be taken into account since it may clearly affect the final results. The use of overnight culture prior to the addition of the mitogen may help to explain depressed proliferative responses secondary to inhibitory factors.

The most commonly used mitogens are phytohemagglutinin, concanavalin A, and pokeweed extract; the first two are mainly T-cell mitogens whereas the latter is a T and B stimulator. It is, however, likely that these as well as some other mitogens stimulate poorly defined subpopulations of T and B cells.

Clinical Indications

The assessment of the lymphocyte response to mitogens is NOT INDICATED for routine use and should be used rather selectively. Abnormal results from single isolated CMI assays are clinically meaningless and will not necessarily indicate abnormalities of CMI in the patients.

Evaluation of cell-mediated immunity is ESSENTIAL in assessing a suspected or proven primary immunodeficiency. Evaluation of CMI is USEFUL in (a) assessment of secondary immunodeficiencies, including those associated with chronic infections; (b) monitoring and evaluating the application of immunostimulatory therapy. It may be helpful for RESEARCH purposes in diseases with possible impairment of immune function, such as autoimmune processes, cancer, and in evaluation of the effect of immunosuppressive drugs.

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Received September 24, 1981.