In Vitro Methods for Detection of Circulating Immune Complexes*

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ABSTRACT

The possible pathologic consequences of the presence of circulating immune complexes in certain disease states has caused considerable attention to be given to the development of laboratory procedures capable of quantitatively measuring the presence of immune complexes in serum. Many assays are available which utilize differing properties of immune complexes for their detection. Examples of assays which depend on different biologic properties of circulating immune complexes are presented and discussed. Included are the Raji cell assay, Clq binding assay, inhibition of rheumatoid agglutination, and nephelometric assays for determination of circulating immune complexes.

Introduction

Over the past several years, a considerable amount of interest has been generated in the development of methods for the detection of circulating immune complexes (CIC) in patients exhibiting a variety of clinical conditions. Several different methods have emerged designed to give quantitative information on the level of circulating immune complexes in patient's serum. Utilizing these methods, immune complexes were reported to be elevated in infectious diseases (both acute and chronic), cancer, and a variety of autoimmune syndromes. The appearance of immune complexes during the course of acute infectious diseases is transient and of no particular pathologic consequence. However, immune complexes present in the circulation of patients with chronic illnesses may contribute significantly to the pathogenesis of the diseases.

The methods developed to detect immune complexes have utilized certain chemical and physical characteristics of CIC which allow them to be differ-
IMMUNE COMPLEX ASSAYS

entiated from free immunoglobulins; included are their size, their ability to bind complement, their interaction with rheumatoid factor, and their ability to bind to Fc and complement receptors. Immunoglobulin classes and subclasses differ in their molecular weight, complement binding activity, and the properties of the Fc portion of the molecule. Additionally, immune complexes vary in the size of the aggregate. For these reasons, the various methods described are likewise heterogenous in what they detect, and thus they do not always correlate with one another when directly compared.11

The number of different assays for detecting immune complexes is well over 20, and it would be impossible to describe them all in a review of this scope. The assays can be generally categorized in a manner listed in table I. Several methods depend on the ability of immune complexes to bind to receptors on mammalian cells; among these are the Raji cell assay22,23 and inhibition of erythrocyte-antibody-complement complex (EAC) rosette assay7 which detect complexes which bind to C3 receptors on these cells. Receptors for the Fc fragment of immunoglobulins are present on B-cells, macrophages, platelets and neutrophils, and a variety of assays have been developed which detect binding of immune complexes to this receptor.15,16,19,20 Rheumatoid factor (RF), both monoclonal and polyclonal, binds to the Fc receptor of immunoglobulins if the immunoglobulin is either complexed with antigen or heat aggregated. Therefore, RF has been useful in the development of assays which detect C1q binding.18,21

Certain immunoglobulin subclasses interact with complement allowing detection of immune complexes by the interaction. Complement protein (C1q) binds to immune complexes and, at the same time, decreases their solubility. Assays utilizing this property of CIC either detect the binding of C1q directly or inhibition of binding of C1q.9,13,27 Conglutinin, a protein found in the serum of cattle, binds to C3 when it is fixed to immune complexes and has also been useful in the detection of CIC.4 More recently, procedures have been developed based on the differential solubility of immune complexes and immunoglobulins in solutions of polyethylene glycol.10,25 These procedures allow rapid detection of complexes with nephelometry and may become clinically useful owing to their ease of performance.

Assays for Immune Complexes

Four of the assays for CIC will be discussed in detail. Each of the assays depends on a different property of immune complexes for detection. These assays are presented as examples of one of many assays which have been devised for a particular CIC property. In general, most of the advantages or disadvantages of an assay which depends on the same property of CIC for its performance will also apply to other assays which take advan-
tage of the same CIC characteristic. Certain special considerations for specimen handling apply to all assays for CIC. Both heating above 56° C and freezing of serum can cause immunoglobulins to aggregate and, therefore, these manipulations should be avoided. If freezing is necessary, the specimen should be frozen one time only and thawed one time only. Freezer temperatures of −70° C should be maintained.

RAJI CELL ASSAY

Theofilopoulos and coworkers\textsuperscript{22,23} described an assay for immune complexes which utilized the ability of complement receptors on lymphoid cells to bind complexes which had fixed complement. They choose to use a cell line in continuous culture since the cell preparations would be relatively homogeneous and readily available. The Raji cell line derived from a Burkett lymphoma was chosen because it was devoid of surface immunoglobulin, yet it did possess both Fc and complement receptors. The Fc receptors on this cell line are of low avidity, and the majority of binding of CIC by the cells can be attributed to its complement receptors. The amount of CIC in serum is determined by uptake of radioactive anti-human IgG by washed cells following incubation with patient sera. By comparison to a standard curve constructed by incubating Raji cells with known amounts of aggregated human gamma globulin, the concentration of CIC in the test serum can be determined. Raji cells were first incubated with test serum or with control serum containing known amounts of aggregated human gamma globulin (HGG). After washing, the cells were incubated with \textsuperscript{125}I-labeled anti-HGG. The cell pellets were counted following three washes, and the amount of radioactivity in the test samples was compared to that bound when known amounts of aggregated HGG were present.

One advantage of the Raji cell assay is its sensitivity, as it detects six to 12 \textmu g of aggregated HGG per ml of serum. Additionally, it requires small amounts of serum. It may be difficult to keep tissue culture lines of Raji cells available in some clinical laboratory settings, and a recent modification which uses glutaraldehyde fixed cells may be more desirable, since a large batch of cells could be prepared and stored for long periods of time. The glutaraldehyde modification may partially eliminate difficulties which can be encountered with tissue cultured cells, which may change their expression of complement receptors. Disadvantages of the assay are the possible interference of anti-lymphocyte antibodies which could bind to the Raji cells and result in false positives. The aggregated HGG used as a standard in this assay must be rigorously controlled in its preparation. It is particularly important to use aggregates of uniform size. Variations in this reagent can be a major source of error in this assay and all others which use aggregated HGG a standard reagent.

\textsuperscript{125}I-C1Q BINDING ASSAY

The assay described by Zubler and Lambert\textsuperscript{27} utilized the binding properties of C1q to immune complexes as a measure of soluble immune complexes. The laboratory must first purify C1q because this reagent is not commercially available. Additionally, iodination of C1q must be performed locally. On the day of the test, labelled C1q is added to buffer containing 1 percent heat inactivated normal serum. After centrifugation at 18,000 g for 40 minutes, the aggregate free supernate is used in the assay itself. This step is necessary to remove aggregated C1q which is less soluble in polyethylene glycol (PEG) and, if present, can result in false positive results. Test serum is mixed with ethylene diamine tetraacetic acid (EDTA) solution
and incubated for 30 minutes at 37°C. This step will prevent the incorporation of \(^{125}\text{I}\) Clq into intrinsic Clqrs complexes. Negative controls consist of normal human serum run in parallel while positive controls contain various amounts of heat aggregated human gamma globulin mixed with heat inactivated normal human serum. At this juncture, \(^{125}\text{I}\) Clq is added to each tube followed immediately by five percent polyethylene glycol (PEG) solution. The tubes are incubated on ice for one hour. After centrifugation at 1500 g for 20 minutes, the supernates are discarded and the radioactivity in the precipitate determined. One hundred percent binding is determined by precipitating \(^{125}\text{I}\) Clq in serum with trichloroacetic acid (TCA), followed by counting the radioactivity in the precipitate. Results are expressed in percent binding as related to the TCA precipitate.

Values are compared to the percent binding of Clq by a range established for normals. Although it is possible to relate the binding to a standard curve for aggregated HGG, this method of interpretation could be erroneous since the Clq binding varies with both the size and nature of the immune complexes. The Clq binding assay will not detect complexes which should contain mostly IgA or non-complement binding IgG subclasses. False positives may result owing to binding of Clq to desoxyribonucleic acid (DNA) or various other polyanionic or polycationic biological substances. Heparinized plasma should be avoided, since addition of heparin to normal human serum has resulted in increased Clq binding.

INHIBITION OF RHEUMATOID FACTOR AGGLUTINATION

Lurhuma et al\(^{13}\) described a very simple technique for the detection of CIC. The procedure takes advantage of the fact that immune complexes will inhibit the agglutination of IgG-coated latex particles by rheumatoid factor (RF). Reagents are commercially available for the detection of rheumatoid factor and, therefore, this test becomes an attractive procedure for the routine clinical laboratory.

The RF reagent used in the assay was titered and the lowest concentration of RF which results in strong agglutination of the particles was used in the assay. An equal volume of test serum and RF were mixed on a slide, followed by addition of IgG-latex particles. The slide was rocked for several minutes and inspected to determine if agglutination occurred as compared to a control where normal human serum was used instead of patient serum. If inhibitory activity was found, it was quantitated by testing two-fold dilutions of patient serum.

The presence of RF in the serum could interfere with the assay, however, intrinsic RF could be removed by passing 0.1 ml of serum over a 0.2 ml column of IgG-agarose. Purified Clq could also be used as the agglutinating reagent in this assay and if samples were tested with both Clq and RF, a wider range of CIC was detected.

Disadvantages of the assay are that immune complexes containing less than seven IgG are probably not detected and that endogenous RF may interfere with the test. If Clq is the precipitating agent, single stranded DNA can interfere. Additionally, the CIC may already be saturated with the patient’s endogenous Cl. With this assay system, inhibiting factors can be found in approximately 22 percent of normals (at titers as high as 1:2). Titers of 1:64 may be reached during the course of mild illnesses such as sore throat, etc. These titers usually disappear within one to seven days.

Nephelometric Assay

In the past year, at least two different groups\(^{10,25}\) have described a rapid, non-
radioactive assay for the detection of CIC. The procedures utilize nephelometry to detect immune complexes which have been precipitated from the serum by the addition of PEG solution.

The procedure to be described was published by Virella and coworkers in 1979. The patient serum was first diluted 1:5 and 1:10 in saline which was suitable for nephelometry. Twenty-five μl of the specimen were added to one ml of saline and to one ml of a 3.5 percent PEG solution in saline. The saline only sample served as a negative control. The PEG sample had CIC precipitated. After incubation of the blank and test samples for one hr at room temperature, the relative light scatter of each sample was determined on a laser nephelometer.

Known positive and negative controls were included with each patient specimen collected. The most important drawback to the procedure resulted from increased light scatter in the serum containing low density lipoproteins. The light scatter by lipids was increased upon the addition of PEG. Since methods which might be used to remove lipids from the specimen also remove CIC, the serum was always collected from fasting patients. Freezing and thawing of specimens even one time, also produced an increase in the relative light scatter and, therefore, was avoided. The nephelometric method detected aggregated HGG in concentrations as low as 75 μg per ml and was comparable in sensitivity to the Clq binding assay. While this procedure may not be any better than the others previously described, in a laboratory where a nephelometer is available, its ease of performance may make it useful as a screening assay.

World Health Organization Collaborative Study of Methods Detecting Immune Complexes

Eighteen different methods for detection of immune complexes were compared in 11 different laboratories in a WHO collaborative study which was published in 1978. Each laboratory used different assays to evaluate a panel of reference and control sera. Because the highest incidence of positive results in the various disease states under study were frequently reached with different assay procedures, it was concluded that each disease may exhibit different types of CIC. Thus, one assay was preferable to another depending on the disease.

Until a determination of the best assay is made for a particular disease (if indeed, this is possible), it was suggested that evaluations be done by several assays which depend upon detection of CIC according to different principles. This would allow determination of both quantitative and qualitative information on the immune complexes present. It was further suggested that there was a need for the development of certain reference reagents to be used in CIC assays. Included were stable preparations of heat aggregated immunoglobulin of known and differing sizes, stable antigen-antibody complexes, and/or materials to be used to prepare antigen-antibody complexes.

The participants in the WHO study strongly suggested that investigators try to develop procedures for isolation of complexes and for identification of the antigens present in the immune complexes. It was the opinion of this group that future availability of antigen specific assays for immune complexes may be more clinically useful than are nonspecific procedures, since such methods could help in diagnosis as well as in the treatment of diseases where CIC are pathologic.

Clinical Significance of the Detection of CIC

Over the past several years, it has become clear that the detection of CIC may be clinically important in some disease states and not in others. In some diseases,
such as systemic lupus erythematosi
(sLE), the presence of CIC can certainly
elicit pathologic manifestations and the
detection of CIC may be used as a monitor
of therapy. Similar observations have
been applied to rheumatoid arthritis. The
finding of CIC in infectious diseases
which exhibit a more chronic course, in-
cluding chronic hepatitis B, leprosy, and
coccidiodomycosis, has pathological
consequences. The presence of CIC in
parasitic diseases, such as dengue fever, malaria, and schistosomiasis, may help
the parasites escape immune destruction
by the host. Monitoring of CIC levels in
some types of neoplastic diseases has
proven to be of prognostic significance.
Indeed, CIC levels may help the clinician
to monitor the effect of anti-cancer treat-
ment and may result in earlier detection of recurrent disease.

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