CLINICAL IMMUNOLOGY OF THE HEART

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Preface

Within the past decade, there has been a virtual explosion of knowledge in immunology, at both the basic and clinical levels, and its association with many disease states. Many of the books and reviews have dealt with specific topics in immunology in which experts have presented the most recent concepts in their fields of investigation. However, these works often require an extensive background in the principles of immunology and sometimes a detailed knowledge of a particular immunological vocabulary. In view of the enormous reading commitments within their own field of cardiology, many clinical cardiologists find it difficult to understand or appreciate the immunological approaches used in the study of cardiac diseases today.

With this fact in mind, the editors felt that a book devoted to immunology of the heart should be aimed primarily at the clinical cardiologist and those individuals interested in cardiac disease. It is hoped that the reader will derive from this book a sense of where the field of clinical immunology of the heart is today and what new immunological approaches might be forthcoming in the study of cardiovascular diseases.

JOHN B. ZABRISKIE, M.D.

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Immunologic Methods in Cardiology

Howard Fillit

During the past decade there has been a veritable explosion in the field of immunology, particularly in the area of immunologic methods of diagnosis, cellular immunity, and immunogenetics. It is the purpose of this chapter to acquaint the practicing cardiologist with a number of immunologic tests now commonly in use. Particular emphasis will be placed on those techniques that are described in later chapters of this book.

ANTIGEN–ANTIBODY INTERACTIONS: IMMUNE COMPLEXES

Many forms of disease are thought to be associated with immune complexes. This section defines immune complexes, describes some of their properties, and then discusses some methods that are used to detect the presence of immune complexes in the serum of patients with various disease states. Although many of the methods were devised for renal diseases, the general principles are also applicable to cardiac diseases of presumed immunologic origin.

An immune complex is defined as the *association of antibody with antigen* and constitutes one of the most fundamental reactions in immunology. The antigen–antibody reaction is similar to an enzyme–substrate reaction in many respects. One of the important features of this interaction is its specificity. Each immune complex contains a particular antigen and a corresponding antibody. Thus, some of the properties of an immune complex will be determined by the properties of the particular antibody and the particular antigen in the immune complex. For example, the antibody may be any one of the particular classes of immunoglobulins (such as IgG, IgM, IgA, and IgE), as well as subclasses of the immunoglobulin types such as IgG1 or IgG2. The antigen may be a small, soluble hapten or a large, insoluble macromolecule. The size of the immune complex is partly determined by the size of the antigen and the antibody involved, and the number of valences in the antigen for each antibody molecule. The biologic properties of any given antigen or antibody may also determine certain characteristics of the immune complex. For example, certain

streptococcal antigens which are known to activate the alternate pathway of complement could be part of a given immune complex (1). Similarly, an immune complex may be composed of antibody that does (IgG1) or does not (IgG2) activate complement (2). In addition, since antibodies and antigens have varying affinities for one another, the stability of an immune complex is partly determined by the amount of attraction between the antibody and the antigen. The particular biologic properties of a given immune complex are important with regard to its pathogenicity. Thus, during antigen excess with the soluble antigens, immune complexes are small and soluble. These complexes tend to persist in the circulation longer and are more pathogenic. When there is antibody excess, large, generally insoluble immune complexes are formed, which are easily removed from the circulation. Animal models have been important in defining the characteristics of pathologic immune complexes (3,4).

With regard to function, it is probable that circulating immune complexes are part of the normal immune response to a circulating soluble antigen. Many cells have receptors for immune complexes, and the interaction of immune complexes with lymphocytes is an important part of the normal immune response (5–7). Elevated levels of circulating immune complexes are detected in many disease states unrelated to classical clinical immune-complex disease; for example, during infections (8), pregnancy (9), and in neoplastic states (10). In addition, the presence of circulating immune complexes is not always associated with complement activation (11). Thus, elevated titers of circulating immune complexes are not necessarily pathologic.

It is apparent that immune complexes are not a homogeneous and simple class of compounds. In fact, they are quite varied with respect to their components, their size, and their pathologic properties. Thus, the measurement of pathologic immune complexes in the serum is not a simple task.

A number of methods have been devised for the measurement and detection of circulating immune complexes. All the types of assays used to date are limited by the particular property they measure. Because of the great variability in immune complexes, no one method can detect all types. However, a number of methods have a good measure of generality (for a general review of the methods mentioned below, see reference 12).

Among the many methods used for the detection of circulating immune complexes, one commonly favored by many investigators is the method using the C1q component of complement. Several methods are available for the isolation of C1q. In our laboratory, we have used a method that takes advantage of the particular ionic properties of C1q for its isolation (12). We then use the C1q in a modified solid-phase assay for the detection of the immune complexes in serum (13). Briefly, C1q in buffer is placed in 1-ml aliquots in standard polystyrene tubes and allowed to incubate overnight at 4°C. During this incubation, the C1q binds noncovalently to the sides of the polystyrene tubes. The next day the tubes are briefly washed with phosphate-buffered saline (PBS) to remove any C1q that has not become bound. The tubes are then incubated with gelatin dissolved in PBS to eliminate the remaining nonspecific binding sites on the tube. After this incubation, 50μl of serum is added to the tubes and brought up to 1 ml of volume with PBS containing Tween 20, a nonionic detergent that also helps to decrease nonspecific binding. After a

one-hour incubation at 37°C and a 30-minute incubation on ice, the tubes are again washed with PBS–Tween solution. ^{125}I-radiolabeled protein A, which binds to IgG, is used to detect immune complexes that have become bound to the C1q on the tubes. After another hour of incubation at 37 C and 30 minutes on ice, the tubes are finally washed with buffer and counted. A standard curve is established using aggregated IgG. Simply stated, the aggregated IgG is a fraction of human IgG which has been prepared in a uniform way and has been heat aggregated to form complexes of IgG that have similar functional properties to IgG present in immune complexes. These aggregates are stored at −70°C and are used as a standard for the test. Once a standard curve is established, the unknown serums and control serums may be compared with the standard curve, and the amount of circulating immune complexes in the serum can be estimated on a quantitative basis. Using this system, we have demonstrated (13) that patients with rheumatic fever or glomerulonephritis have moderately elevated titers during the acute phase. These data give direct evidence for the presence of circulating immune complexes during acute poststreptococcal glomerulonephritis and rheumatic fever.

There are disadvantages to employing C1q for the detection of immune complexes, despite the fact that it is likely that C1q is the in vivo binding component of complement to immune complexes. The main drawback is that many substances other than complexes of immunoglobulin react with C1q. These substances include many polyanionic molecules such as DNA, ribonucleotides, endotoxin lipopolysaccharide, meningococcal group A polysaccharides, heparin, and other low-molecular-weight molecules found in the sera of patients with SLE (15,16).

A more recent method for the detection of immune complexes has employed bovine conglutinin (17), a molecule found normally in the sera of certain members of the bovida family that is known to bind to fixed complement (especially C3d). Few, if any, other known molecules will bind to bovine conglutinin, and it is easily obtained from the serum of cows in fairly large quantities. Conglutinin has been used in solid-phase immunoassays as well as in radioimmunoassays and results have generally been similar to other assays used concomitantly, such as the Raji cell immunoassay or the C1q-binding assay (10,17). Specificity for aggregated IgG has also been well demonstrated, the approximate size of the aggregates detected has been investigated, and the standard curve with aggregated IgG is well within the range of other assays. The advantage of conglutinin compared to C1q is that it binds to immune complexes that already have bound complement; therefore it may be detecting immune complexes that have pathologic effects. DNA, heparin, and gram-negative endotoxins do not appear to inhibit the binding of conglutinin to C3d. In general, the conglutinin assay seems to detect large immune complexes as well as the Raji cell assay. Another advantage of conglutinin is its exceptional stability, allowing for long-term storage. The easy reversibility of the binding between C3d and conglutinin has also made conglutinin attractive for the isolation of immune complexes and the subsequent important isolation of the immunoglobulin and antigen in the immune complexes. This development is essential for the identification of the antigens involved in many immune-complex diseases.

The other representative assay that is currently used for the detection of immune complexes is the Raji cell radioimmunoassay (18). This method employs a human lymphoblastoid cell line that has receptors for the Fc receptor of immunoglobulin C3b, C3d, and C1q. In this method, Raji cells are grown in media containing fetal calf serum. For each test sample, about 2 to 4 million Raji cells are incubated in media with a small amount of serum to be tested diluted in saline. After an incubation period, the cells are washed and an optimum amount of ^{125}I-radiolabeled rabbit antihuman IgG in media is added. The cells are then washed again after an incubation period, and the radioactivity remaining in the cell pellet is determined in a gamma counter. The amount of radioactive uptake by the cell is then referred to a standard curve that has been established using aggregated IgG as described above. In general, the Raji cell assay detects larger immune complexes, and the normal values for this assay agree well with those employing conglutinin and C1q. The method has been used by many workers with generally equivalent results; further, the results often, but not always, correlate well with other assays employed (10,19,20). In general, the complexes measured by this assay are limited to those types containing IgG. In addition, it has been demonstrated that immune complexes made near equivalence bind best to cellular component receptors. Thus we see again that the Raji cell detects a particular type of immune complex. A major disadvantage to this assay is that because viable cells must be employed, the technology and expertise necessary for the use of viable tumor cells must be at hand, and the problems generally encountered with tumor cell cultures must be dealt with (21).

Serology

An understanding of immune-complex formation necessarily involves the understanding of antigen–antibody reactions. Such reactions can be used for the detection and quantitation of antigen or antibody in human tissue, including serum. Methods for the observation of antigen–antibody reactions were first described near the turn of the century and have since been improved upon. The value of serologic techniques in medicine has been enormous. The measurement of antiviral antibody (AVA) titers (24), antistreptolysin O antibodies (ASO) (25), and numerous other microbial components, as well as the measurement of serum immunoglobulins (26), complement (27), and a large variety of other serum components has obvious importance to the practicing physician. A brief outline of the basis of the methods used in serology is presented here. Specific reviews are referred to in the text for more detailed explanations of the various methods.

Perhaps the simplest technique for the observation of antigen–antibody reactions is double diffusion in agar gel, or the Ouchterlony method (28). In this method, agar gelatin is dissolved in a neutral buffer such as PBS, pH 7.4, by mild heating. The solution is poured onto a microscope slide and allowed to cool, forming a gel. Small holes (2 to 3 mm) are punched in the gel, one in the center and several others at a constant radius from the center. A small (5 μl) volume of antibody (or antigen) is placed in the center well, and antigen (or antibody) is placed in the peripheral wells. The solutions are then allowed to diffuse into the gel. They do so randomly from the well. If the antigen and

antibody have specificity for one another, they will react in the gel to form immune complexes. These complexes will form large lattices that can be visualized as a precipitin line in the gel. Specificity can be determined by observing the patterns of lines between differing wells. When such lines merge, they are called lines of identity; this merging implies that the reagents employed have immunologic identity. When the lines cross each other, nonidentity is said to be present, indicating that the antigen–antibody reactions differ in specificity.

Variations in this concept have led to a number of other, more sensitive techniques. One is immunoelectrophoresis (29). In immunoelectrophoresis, advantage is taken of the fact that most antigens and antibody have an overall charge. Applying an electric current to the gel will cause these molecules to migrate in the electric field, and not randomly as in double diffusion. Immunoelectrophoresis helps to separate a number of components in a single sample, so that reactivity to a single antigen can be seen. This method is used commonly on a qualitative basis to identify the normal components of human serum, as well as the presence of large abnormal precipitin lines, such as a monoclonal "spike" seen in immunoproliferative states (30), or the absence of normally present precipitin lines, such as in agammaglobulinemia (31). A further refinement of this technique is counterimmunoelectrophoresis, in which the pH of the gel is adjusted so that the antigen and antibody, usually having different isoelectric points, will actively migrate toward each other. This technique is more sensitive and rapid than simple double diffusion, and has been used for detecting hepatitis B viral antigens (32). Other variations include rocket electrophoresis and two-dimensional electrophoresis (29).

Another useful variation of simple double diffusion is the Mancini technique, or radial immunodiffusion (33). In this method, antiserum is diluted into the liquid agar, which is then poured onto a slide. A uniform amount of antiserum is distributed throughout the gel. Antigen is placed in a well in the gel and allowed to diffuse. As the antigen diffuses, it forms a circle of precipitation. The area of diffusion is proportional to the concentration of antigen added. By measuring the radius of the precipitin circle finally formed, one can calculate the concentration of antigen added. This technique is commonly employed for measuring serum complement and other components (34).

Agglutination reactions constitute another type of commonly employed assay used in the immunology laboratory to detect the presence of antigens and antibodies. The antigen–antibody reaction is again the basis of these tests. However, unlike the diffusion techniques in which components are soluble, an insoluble phase is specifically used in order to allow observation of the reaction. A number of insoluble particles are used. Most commonly, red blood cells *(hemagglutination)* or latex particles *(latex agglutination)* that have been coated with antigen are employed. Normally, in a test tube or on a microscope slide, these particles form a fine, uniform suspension. However, when antibodies are added, which are polyvalent and bind to more than one particle (antigen) at once, the particles become "clumped" or agglutinated. The reaction is rapid and usually requires only small amounts of antibody. By varying the dilution of antibody being tested, one can obtain a semiquantitative estimation of the minimum amount of antibody necessary to obtain agglutination, reflecting the "titer" or quantity of the antibody in the solution.

Examples of agglutination methods commonly employed include the latex fixation test for rheumatoid factor (35), an IgM molecule that agglutinates IgG-coated latex particles; and the Coombs' test, which indicates the presence of circulating antibodies to red blood cells (RBC) (36). In the Coombs' test, RBCs are the actual targets of the antibody. Passive hemagglutination refers to the use of RBCs as particles that have been coated with antigens. The direct hemagglutination assay results in agglutination directly upon the addition of serum to the insoluble particles, while the indirect assays require the additional step of adding an antiglobulin reagent in order to obtain agglutination. By using passive hemagglutination-inhibition assays, one may detect the presence of circulating antigen in an unknown serum. This is accomplished by observing the inhibition of agglutination by an unknown serum in the presence of a known agglutinating serum. Semiquantitative titers of circulating antigen are thus obtainable. Detailed discussions of these methods are available (37,38).

Immunofluorescence

As will be seen in subsequent chapters in this book, the use of immunofluorescent techniques has provided an invaluable tool for the study of immunologically mediated diseases of the heart. In general, these studies have been limited to the detection of antibodies that bind to heart and their cross-reactions with various parasites (39,40). The widest application of immunofluorescent methods has been in the study of renal diseases, where it has been possible to detect the deposition of immune complexes, complement components, antigens, and antibody in human renal disease and in experimental animal models (41,42). Part of the difficulty in studying the nature and deposition of complexes in heart disease is that, unlike the kidney where many of the lesions are concentrated in the glomeruli, cardiac lesions are often diffuse and not easily subjected to studies involving elution of antibody or detection of specific antigen in the lesions (43).

The concept of immunofluorescence was initially conceived by Coons as a method to study the possibility that streptococcal antigens were present in the Aschoff lesions, the pathologic hallmark of rheumatic fever. Though the original question has never been completely resolved, the technique has provided a powerful tool for the study of basic immunology as well as various disease states.

While it is not the purpose of this section to discuss in depth the use of the technique of immunofluorescence in a research setting [these details are available elsewhere (43,44)], a brief description of the concepts behind immunofluorescence is warranted. Fluorescent dyes are compounds that absorb the radiation of ultraviolet light, enter an excited state, and emit radiation, which ceases almost immediately after withdrawal of the exciting radiation. The main fluorochrome dye employed is fluorescein, which is excited by ultraviolet blue light to emit visible yellow green light. These dyes are easily conjugated with various chemicals that facilitate coupling with protein, thus labeling the protein with fluorescent dye. In this way, antibodies that have been conjugated with fluorescein, while retaining their immunologic activity, can be used as tracers on a tissue section for the presence of the antigen with which these antibodies

combine. Rhodamine is another fluorochrome dye less commonly employed in the study of tissues; it is often used, however, in studies of particular cell populations.

The general method of immunofluorescence using the indirect technique may be described as follows. The direct method differs only in the fact that a directly labeled antibody is used instead of the "piggyback" anti-antibody technique. Briefly, pieces of human heart obtained within 12 hours after death are quick-frozen in tissue-tek using standard techniques for embedding tissues (40). Four-micron sections of this tissue are then placed on glass slides and allowed to dry overnight in a desiccator. The next day the slides are fixed in acetone for a few minutes and washed two times in PBS. The excess fluid is removed and the serum to be tested is then layered over the section. If the serum contains antibodies that bind to heart, they will fix to the heart sections. Sera lacking antibodies that fix these sections will be washed off during the subsequent washes in PBS. Following washes in PBS, a fluorescent-labeled anti-antibody (prepared by immunizing another animal species with the appropriate immunoglobulin) is layered over the section. The fluorescent-labeled antibodies then bind to antibody fixed on the heart section. Excess antibody is once again washed off the section and the application of an appropriate mounting fluid and coverslip to the section completes the procedure. The slide is then placed on a U-V microscope and read for the degree of immunofluorescence. Obviously, positive fluorescence will be detected only with those sera containing antibody that binds to heart.

While in principle the indirect technique is quite simple, many factors enter into the successful attainment of reproducible results. For example, the reagents (i.e., immunoglobulins) used for the preparation of anti-antibody must be of sufficient purity to ensure the production of a specific anti-antibody. Contamination of the preparation with other serum proteins may result in false-positive test results. The antibody to be labeled must be of high titer in order to permit detection of the fixed molecules in the sections. Another pitfall has been the use of antibodies that nonspecifically bind to the sections even in the absence of the antigen in question. Therefore, appropriate controls such as known positive and negative sera should be run with each test, as well as controls for the counter-staining antibody. A final area of potential hazard to the researcher is the recent observation that many tissues contain receptors for the Fc portion of the immunoglobulin (45). Thus, antisera will bind to these receptors, giving a false-positive result. Appropriate control sera, as well as digested antibody molecules that have lost their Fc portions of the immunoglobulin molecule, will help in evaluating the specificity of the reaction. Similar problems arise when antigens are used for absorption studies. A number of bacterial antigens (protein A is the best example) are now known to contain receptors for the Fc portion of antibody molecules. It is appropriate to institute proper controls in order to be sure that the antigen does not nonspecifically absorb the antibody that binds to the tissue.

The indirect method is more sensitive than the direct method for the detection of antigens in tissue. This is probably related to an amplification effect of the two layers of applied antiserums. Fluorescence on the tissue after washing indirectly reflects the binding of the first layer of antisera to specific

tissue antigens. The use of the indirect method is helpful in two ways. One is that sera of unknown specificity may be layered onto normal tissue for the detection of antibodies in the sera that are directed against the tissue. For example, sera having antibodies directed against the glomerular basement membrane may be tested by layering this serum on normal human kidney. Subsequent linear staining, seen by using a second layer of fluorescein-conjugated rabbit immunoglobulin directed against human IgG will confirm the presence of glomerular basement membrane antibodies (43). A second application, one that is more research oriented, is best exemplified by the search for streptococcal antigens in the glomeruli of patients with acute glomerulonephritis (46). In this case, rabbit antisera from rabbits immunized with whole streptococci are layered on the glomeruli. The slide is washed and the glomeruli are then stained with, for example, fluorescein-conjugated goat antirabbit IgG antiserum. If any rabbit immunoglobulin directed against streptococcal components has attached to the tissue section, then the goat antisera, which is directed against the rabbit IgG, will bind to the rabbit immunoglobulin that has bound to the streptococcal antigen in the tissue section; and positive immunofluorescence will be seen.

Two fluorescent dyes are readily available for use in this technique. One is fluorescein, which is conjugated with isothiocyanate and is easily coupled to various protein antigens or antibodies. The apple-green color of fluorescein is readily distinguished from the blue autofluorescence that may be seen in tissues. An alternative to fluorescein is rhodamine, which imparts an orange color to tissue. The fluorescence of rhodamine and that of fluorescein occur at different wavelengths, so they can both be used simultaneously on the same tissue section. Rhodamine is available as tetramethyl rhodamine isothiocyanate and can be used for conjugation to proteins. The various methods for conjugation of fluorescein dyes with antigen and antibody will not be discussed here. The methodology is described in detail elsewhere (41,47,48). In general, most preparations of fluorescent conjugated proteins require exchange chromatography or gel filtration for their purification. A simpler method to remove nonspecific staining while retaining activity for the tissue is absorption. Absorption is a relatively simple procedure, especially useful for crude conjugates.

The specificity of the fluorescent reaction can be determined by using control antiserums. Pretreating the tissue with unconjugated antiserum against the specific antigen should result in a marked decrease in the specific staining of the fluorescein-conjugated antiserum. Direct staining of the tissue with a fluoresceinated antiserum of unrelated specificity from the same species will also help in determining the specificity of the unknown antiserum.

The final aspect of immunofluorescence methodology pertains to the fluorescent microscope and its accessories, including the filters, source of illumination, and optics. The purpose of a primary filter is to allow light with a wavelength necessary for excitation of the fluorochrome to pass through, while excluding light at the wavelength of emission by the fluorochrome, decreasing nonspecific light interference from the source of illumination. Similarly, a secondary filter passes maximal light at the emission wavelength and excludes light at the excitation wavelength. High-pressure mercury vapor arc and iodine-quartz lamps are most commonly used as light sources, having a high

energy output at the excitation wavelength (495 nm) of fluorescein. Further discussions of the details of various microscopes, optics, and detailed information regarding other facets of fluorescent microscopy are available (49,50).

Radioimmunoassay

This section will briefly outline the principles of the radioimmunoassay (RIA). Detailed discussions of the principles and methodology of the RIA are available and are discussed elsewhere in this book (see Chap. 3). The understanding of the principles of the technique is of value to the practicing cardiologist not only because new research may widen its applicability in heart disease but also because diagnostic kits for compounds such as digoxin and quinidine, which are currently commercially available, are important for the patient with heart disease.

The principal advantages of RIA are its sensitivity and specificity. Once the standards for the reagents used in the RIA are met, the assay becomes a highly accurate and reproducible method for determining very small amounts of a substance in human fluids. Indeed, once the assay has been established in the research laboratory and the reagents have been made available, the actual performance of the technique is simple enough to make diagnostic kits commercially important.

The basic principle of RIA is the antigen–antibody reaction, which has been described in detail in the section on immune complexes in this chapter. If the reader understands the basic information on antigen–antibody reactions in that section, then the RIA becomes a simple application of that understanding. In essence, the RIA requires a purified antigen, a purified antibody, the ability to label the purified antigen with a radioisotope, information on the kinetics of the particular antigen–antibody system employed, and a method for eventually separating the immune complexes formed from the unbound radiolabeled antigen so that the amount of bound radiolabeled antigen can be measured.

Establishing an RIA system begins with the purification of the antigen and antibody reagents to be used. Many years may have to be spent purifying a component of blood, such as insulin, before it can be used as an antigen in an RIA. Purity of the antigen is essential to the specificity and sensitivity of the RIA; it is also of primary importance in the production of reagent antibody to be used in the RIA, since the antibody is made by injection of the antigen into an animal. Problems with antigen isolation include the immunogenicity of the antigen (some hormones or drugs are not immunogenic and need to be covalently bound to immunogenic molecules or carriers in order to induce antibodies in animals), the ability of the antigen to be radiolabeled, and the stability of antigen after radiolabeling since radioactivity per se can alter the immunogenicity of antigen.

Problems also exist in isolating antibody with specificity for only the antigen of importance. Since even purified antigens often possess moieties that cross-react with irrelevant antigens, elimination of unwanted cross-reactive antibodies from the reagent antibody is important in the specificity of an RIA. In addition, the particular affinity of the reagent antibody is often of extreme importance in the kinetics of the assay.