RADIOASSAY IN CLINICAL MEDICINE

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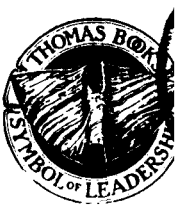

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PREFACE

THE NUMBER AND VARIETY of chemical compounds of clinical or investigative interest seems almost limitless. Further, many compounds of extraordinary physiologic potency may function in biologic material in extremely low concentrations. Both of these factors combine to confound the ordinary chemical laboratory seeking specific quantitative analyses of these compounds. Fortunately, the advent of radionuclides that can be measured in extremely low chemical concentrations and techniques to attach them to many compounds as trace labels have opened new approaches to quantitative study. However, in spite of a well-informed public regarding the properties of radionuclides and perhaps their use in immunoassay of some protein hormones, the editors of this volume have the impression that there is lack of appreciation of both the variety of approaches and the variety of classes of compounds that can be examined. Consequently, we gathered in this short volume a set of papers chosen to illustrate this variety, yet with each subject presented in sufficient detail to allow the reader to gain insight into the problems inherent to the individual systems.

CONTENTS

CHAPTER 1

PRINCIPLES UNDERLYING CURRENT RADIOIMMUNOASSAY TECHNIQUES

WILLIAM T. NEWTON AND BERNARD M. JAFFE

INTRODUCTION

MANY COMPLEX CHEMICAL compounds of biologic importance exist in such low concentrations in biologic fluids and tissues that they do not readily lend themselves to conventional chemical identification and analysis. Development of the techniques of radioimmunoassay in recent years has extended by several orders of magnitude the sensitivity of quantitative measurement of a rapidly growing list of these compounds. For many of these radioimmunoassays have replaced cumbersome and inexact bioassay techniques. The techniques of immunoassay require the production of serum antibodies to bind specifically the chemical complex being estimated. These antibodies can then be used by a variety of maneuvers to identify and quantitate the presence of the chemical complex in a reaction medium. Addition of a radiolabel to the chemical complex allows extension of the measurement of antibody-bound complexes to the very low concentrations that these compounds are found in biologic media.

Each individual radioimmunoassay technique presents individual problems as presented in subsequent chapters of this volume. However, contained principles underlie all of the techniques, and a common thread of empiric findings applicable to a variety of systems is emerging.

REAGENTS

Production of Specific Antibodies

Almost all Vertebrata of the animal kingdom react to the injection into their tissues of foreign proteins or polysaccharides by the generation of an immune response. One of the more frequent manifestations of this response is, of course, the appearance of specific antibodies in the globulin fractions of the serum. And, although the initiation of the immune response requires an immunogen of high molecular weight, the resultant antibodies can be shown to bind specifically only to small areas of the immunogen, no more than five or six amino acids in a chain or adjacent to one another stereoisometrically. Fortunately, for the purposes of immunoassay, chemical covalent conjugation of many chemical compounds to the side chains of an immunogen frequently induces the animal recipient to produce antibodies with specificities to the compound even though it is not itself a peptide or polysaccharide. For example, injection of human serum albumin into rabbits evokes the production of a population of antibody molecules with the capacity to bind to at least three separate amino acid chains of the HSA molecule. Chemical conjugation of 2, 4, dinitro-benzene sulfonic acid to the epsilon amino groups of lysine members of the protein chain of the serum albumin produces an immunogen which stimulates the production of antibodies specific not only for the usual three amino acid chains but also antibodies that bind dinitrophenyl lysine specifically. Production of defined immunogens and analysis of the reactions of the resultant antibodies with defined "antigenic determinants" have formed the basis of a large portion of modern immunochemistry.

Preparation of Immunogens

A variety of natural proteins and even chemically produced random copolymers of one to three amino acids have been used as "backbone" carrier molecules. In order to elicit specific antibodies animals are immunized with an immunogen in which the chemical complex to which antibodies are desired is conjugated

to side reactive groups of the carrier molecule. Of course, some important biologic molecules are large enough without conjugation to induce immune responses (growth hormone, insulin) or can be isolated from biologic sources already bound to immunogenic molecules (crude hog gastrin). If conjugation is necessary almost all immunogenic proteins contain reactive side groups available for conjugation. The gamma carboxyl group of glutamic acid and the epsilon amino group of lysine provide sites for peptide and azo linkages. The reader is referred to standard texts on amino acid and peptide chemistry for the variety of reactions available as well as to the individual techniques described in this volume. Choice of techniques will be dictated in large measure by the type of reactive group on the native biologic compound or the type of reactive group that can be conveniently introduced on this compound. Direct ethereal or peptide linkages are to be preferred, but use of intermediary linkage compounds such as imidoesters,[4] toluene diisocyanate[5] or bis diazobenzidine may be necessary. The most popular reaction recently has become the direct peptide linkage of either amino groups or carboxyl groups of the target compound or some derivative to the protein through the intermediary reaction with carbodiimide compounds.[6] The reader should be aware that all of these reactions introduce intermediary compounds and that antibodies specific for the intermediary will be found in resultant antiserum. Even the carbodiimides form immunogenic radicals, and binding of antibodies to these conjugates may be specific for the carbodiimide used as well as the compound conjugated. Fortunately, the two water soluble carbodiimides that are readily available do not produce cross-reactive antibodies.[7] Therefore, one carbodiimide can be used to synthesize the immunogen leaving the other available for synthesis of target compounds to be used in the reaction mixture if such a synthesis is needed.

In general, the more intrinsically immunogenic the carrier molecule is the more likely are conjugates of target compound to that molecule to induce antibodies to the conjugated compound. And, there appears to be little or no advantage to using a carrier molecule that excites few antibodies to its own structure. Although immunization with a conjugate of, say brady-

kinin molecules to a relatively poor immunogenic carrier such as polylysine may produce a more "pure" antibody population to the bradykinin groups, the total response is apt to be weak and demonstrable in only a few of the animals immunized.[8] As pointed out below the specificity of the reaction can usually be controlled precisely by manipulation of the reaction system and control of the target radiolabeled compound. The reader should realize that this point is controversial, and there can be envisaged circumstances where a host of interfering and cross-reacting substances in normal serum require the production of a more pure antibody population. Random copolymers of lysine, alanine and glutamic acid are available and are moderately immunogenic in themselves. They contain convenient side chains for conjugation reactions.

Another approach to the purity problem may be available by completely saturating the side chains of the immunogenic carrier molecule with the compound to be assayed. If bovine gammaglobulin, a highly immunogenic protein, is reacted with large excesses of dinitro-benzene sulfonic acid to the extent that sixty dinitrophenyl groups are conjugated per molecule BγG to the available lysine amino groups, the resultant conjugate is still highly immunogenic, but the antibodies produced are almost all specific for dinitrophenyl groups, and antibodies to the native BγG antigenic determinants may be absent or difficult to demonstrate.

Immunization Procedures

In general, the longer the time interval from initial injection to harvest of antiserum the broader will be the specificity of the antibody population. And, in the usual circumstance where the compound to be assayed forms only one of many antigenic determinants presented to the immunized animal it might be predicted that prolonged immunization times would be required. And, indeed this is the case with antibodies specific for the compound which are usually found only after three or four months and frequently longer. Further, intensive immunization schedules with several secondary immunization injections have usually been necessary. Most investigators have also used adjuvant tech-

niques such as Freund's incorporation of immunogen into a water-in-oil emulsion for injection or alum precipitation of immunogen.

Although a desirable immunization dosage might be in the range of one milligram immunogen per kilogram body weight per injection, these quantities are frequently out of the question due to the expense or rarity of the compound. In these circumstances it seems wisest to space the limited quantity available over several monthly injections rather than to commit all immunogen to a single injection.

The choice of animals to be immunized will depend to some extent on the volume of antiserum needed for the proposed investigations. A single guinea pig may provide enough antiserum for several years' work in some situation, since assays can frequently be done with 1:10,000 dilutions of antiserum (see below).

It would seem reasonable that the more disparate phylogenetically the animal is from the biologic source of the material to be assayed, the more foreign would be the immunogen and more likely that an immune response would ensue. When attempts to immunize rodents to human parathormone failed, chickens were found to be suitable responders to immunization.[9] Since the ability to respond to immunization is genetically determined, it would seem wise to use several mongrel animals rather than members of a given genetic strain. Of course, the animals chosen should be known to be capable of prolonged healthy survival in the facilities available.

REACTIONS

The Reaction Medium

Before attacking the problem of analyzing the reaction of antigen and antibodies *in vitro*, brief consideration should be given to the milieu in which the reaction takes place. Since the bonds of the complexes are relatively weak, they are sensitive to the alterations that affect hydrogen bonds. Alteration of pH from neutrality must be guarded against, and ionic strength

should be low, in the range of physiologic saline. Although reactions take place rapidly at 37° C, complexes are more stable in the cold.

Care must be taken that the medium does not allow chemical or physical alteration of the reactants. Many biologic fluids contain potent lytic enzymes for a variety of chemical bonds. Addition of epsilon amino caproic acid, an inhibitor of many proteolytic enzymes found in serum, to systems analyzing reagents with peptide bonds is frequently helpful in stabilizing these systems. Anti-oxidants or other reagents may be required. It may be necessary to separate the chemical to be estimated from its biologic source by one of the various chemical extraction procedures before a stable analysis system can be set up.

Many systems seem to require other large molecules in the medium to give most reproducible results. In an anti-peptide system the authors found egg albumin in concentrations of 2.5 mg per ml superior to ficoll, hydroxyethyl starch, dextrans and other proteins. Individualization of each assay system is part of the art of immunoassay and cannot be neglected.

Reaction of Antibodies with Antigens

The reactions, *in vitro*, of antibodies with antigens and particularly with defined chemical determinants, termed haptens, have been intensively studied. In a representative system antibodies raised to an immunogenic protein to which dinitrophenyl groups have been conjugated are separated and purified from the harvested antiserum by one of a variety of techniques. These antibodies are then examined in their reaction with the specific hapten, dinitrophenyl lysine, or other dinitrophenyl compounds. Techniques exist for the separation of bound and unbound hapten from the reaction systems and allow the construction of Sip's plots or other mathematic manipulations. The reader is referred to standard immunochemistry texts for more detailed treatment of these reactions.

Immunochemical experimentation has shown that the usual IgG antibody molecule of about 150,000 average molecular weight contains two binding sites. Thermodynamic treatment of the data shows that the binding forces of sites for specific haptens

have free energy exchanges in the order of those found for hydrogen bonds. Despite the low binding energies the intrinsic association constants reflect a high degree of completion of the reaction of antibodies with hapten to form complexes. These binding constants are developed from the law of mass action much as for any other chemical reaction and, in practical terms for this discussion, represent the molar concentration of hapten at which half saturation of the antibody binding sites occurs (the number of bound and unbound hapten molecules are equal). Values of 1×10^{-5} M are easily obtained, and population of antibodies with values of 5×10^{-9} M are not rare. Thus, serum antibodies are excellent reagents for the binding of many chemical groups in theory. For the purposes of assay it is now required that means be available to determine that this reaction has occurred and to what degree.

Measurement of Complex Formation—Indicator Techniques

Historically, the oldest immunoassay techniques employed a visual indicator of the reaction of antigen and antibodies. In these techniques the antigenic determinant or whole antigen was absorbed to or covalently joined to a visible particle such as the human erythrocyte. Since antibodies are multivalent, their union to antigenic determinants on separate red cells could lead to the complex process of hemagglutination with separation from the uniform erythrocyte suspension of large clumps of cells. Latex particles have also been used for this purpose. Quantitatively, the reaction is measured by titer, the highest dilution of antibodies that produces visible agglutination. Once the basic system is established, varying known quantities of unconjugated antigenic determinants could be added to the system to inhibit the agglutination by competing with the particle bound antigen for antibody binding sites. A standard inhibition curve with the titer of antibodies as a function of the amount of added inhibitor can be set up. Measurement of the antibody titer after the addition of an unknown sample of biologic material compared to the titers obtained after the addition of known amounts of the specific chemical allows determination of the amount of the specific chemical in the sample. The indicator techniques are cumbersome (pro-

duction of stable antigen-particle conjugates) and have been replaced almost entirely by the separation techniques discussed below.

Measurement of Complex Formation—Separation Techniques

Measurement of the amounts of antibody bound compound and the free unbound compound at equilibrium in a given reaction can be simplified markedly if the two reaction products can be separated physically and analyzed individually. A variety of techniques have been developed for this purpose and are discussed below. It is, of course, important that the separation process not alter the equilibrium established. Consequently, most separations are carried out in the cold. Very little attention has been paid to this point in the literature. For example, gel filtration columns have been extensively used to separate out the small chemical groups and allow the larger bound antibody-chemical complexes to percolate down the column. As the mixture of reactants proceeds down the column the concentration of unbound simple chemical might fall altering the equilibrium of the reactants and, theoretically at least, causing additional simple chemical groups to separate from the antibodies. In some studies with peptide hormones as the target chemical this dis-equilibrium has not seemed to be a significant problem, but this might not be the case for smaller molecules or different gels. Of course, the separation process should not produce chemical or physical alterations of either of the two reactants that affect their ultimate analysis. Mention of this requirement has been made above in describing the milieu of the reaction mixture; care should be taken that these precautions are carried through the separation process.

Of the various separation processes, the direct precipitation of antibody-bound complexes from the reaction medium is the simplest in theory. Most antibodies readily form precipitable complexes when their specific antigens are part of proteins or similar large molecules. A now classic example would be the precipitation of ovalbumin by rabbit antibodies. However, the conditions required for accurate and reproducible analyses are rigorous, slow and inconvenient for large scale work. Although specific

precipitation is not used widely today for immunoassays, the basic principles form the foundation for immuno diffusion analyses which have been widely used for estimation of concentrations of serum proteins. None of the direct precipitation methods allows analyses much below the microgram per milliliter level and are, therefore, not suited to the very low concentration of many important compounds found in biologic fluids.

On the other hand, a variety of coprecipitation processes have been used successfully for radioimmunoassays. The antibody target chemical complexes can be precipitated at neutral pH without disrupting the complex in high concentrations of sodium or ammonium sulfate salts. Although these processes may increase the sensitivity of measurements a thousand-fold over that obtained by direct precipitation, there is a slight solubility of the complexes that prevents most systems from reaching the picomole per milliliter level needed for many compounds. Of more value have been the so-called "double antibody" coprecipitations in which the complexes of antibodies and simple chemicals is precipitated by addition of antibodies to the serum globulins that form the antibodies of the complexes.[10] In a representative system antibodies are raised to human bradykinin in rabbits, and these antibodies are used to react with bradykinin in the test system. Addition of antibodies raised in goats by immunization with rabbit IgG globulin causes precipitation of the rabbit globulin antibodies and coprecipitation of the bradykinin. Frequently, it is necessary to add additional rabbit IgG globulin to the system before the goat antibodies, and to increase the amount of goat antibodies correspondingly (calculated from separate precipitation analysis of the reaction of goat antibodies with rabbit IgG). This additional precipitate need be only of the order of a few micrograms of rabbit IgG in order to form enough precipitate to insure quantitative precipitation of the immune complexes. The additional precipitate increases the nonspecific inclusion of unbound chemical in the total mass, and the proper addition that can be tolerated must be determined experimentally. Nonspecific binding of complexes of antibodies and target chemicals to charcoal particles coated with dextran has been used to separate bound and unbound chemical. In some cases simple binding of antibodies to