Enzyme-Immunoassay

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Director
Technical Development and Planning
Scripps-Miles, Inc.
and
Adjunct Associate Member
Department of Pathology
Scripps Clinic and Research Foundation
La Jolla, California

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PREFACE

Novus Ordo Seclorum. Perhaps! With radioimmunoassay (RIA) only 20 years old, the new order is already rapidly emerging.

In the broad field of nonisotopic immunoassays, enzyme-immunoassay is today the most rapidly growing segment, growing not just in applications, but also (very interestingly) in basic concepts. The potential applications of enzyme-immunoassay clearly exceed those of RIA, since its theoretical applications are equally as broad as those of RIA while its practical uses are not restricted by the stringent safeguards and special handling requirements.

It should be noted that enzyme-immunoassay is not alone in the field of practical nonisotopic immunoassay methods. Recently there has been a resurgence of interest in the historically older field of fluorescence immunoassay. This renewed interest is undoubtedly derived in large part from the infusion of new ideas and perhaps the recognition of some of the shortcomings arising out of developments in the enzyme-immunoassay field.

The molecular complexity and varied capabilities of enzymes make them more interesting than radionuclides as immunochemical labels, at least from a conceptual standpoint. Recent developments in enzyme-immunoassay span a broad spectrum of ideas and concepts ranging from direct signal-modulation (homogeneous assays), to passive solid-phase separation (heterogeneous assays), to interactive solid-phase measurements (enzyme-immunoelectrodes).

The purpose of this book is to focus attention on some of these ideas and concepts. In doing so, it has captured a glimpse of the past and it attempts a projection of the future, but mostly it reveals an overview of the field as it exists as the present time. Hopefully it will serve to spawn further growth in ideas and encourage applications to increasingly broader segments of both the clinical and general analytical chemistry fields.

Edward T. Maggio
La Jolla, Calif.
November 1979

THE EDITOR

Edward T. Maggio, Ph.D., is Director of Technical Development and Planning, Scripps-Miles, Inc., and Adjunct Associate Member of the Department of Pathology, Scripps Clinic and Research Foundation, La Jolla, California. He received his B.S. degree from the Polytechnic Institute of Brooklyn, New York in 1968 and his M.S. and Ph.D. degrees from the University of Michigan in 1969 and 1973, respectively.

Dr. Maggio is a member of the American Chemical Society, the American Association for Clinical Chemistry, the New York Academy of Sciences and the American Society for Microbiology. His research interests include new concepts in nonisotopic immunoassay as applied to serology, virology, and therapeutic drug monitoring, and the mechanistic and applied aspects of clinical enzymology.

CONTRIBUTORS

Carl Borrebaeck, Ph.D.
Doctor of Science
Department of Pure and
Applied Biochemistry
Chemical Center
University of Lund
Lund, Sweden

John E. Butler, Ph.D.
Associate Professor of Microbiology
University of Iowa Medical School
Iowa City, Iowa

Luis A. Cantarero, Ph.D.
Postdoctoral Fellow
Immunology Division
Duke University
Durham, North Carolina

Brian R. Clark, Ph. D.
Assistant Research Scientist
Division of Immunology
City of Hope National Medical Center
Duarte, California

Eva Engvall, Ph.D.
Assistant Research Scientist
Division of Immunology
City of Hope National Medical Center
Duarte, California

Robert S. Galen, M.D.
Assistant Professor of
Clinical Pathology
Columbia University
New York, New York
Associate Director of Laboratories
Overlook Hospital
Summit, New Jersey

David S. Kabakoff, Ph.D.
Manager of Drug Assay Development
Syva Company
Palo Alto, California

Edward T. Maggio, Ph.D.
Director, Technical Development
and Planning
Scripps-Miles, Inc.
La Jolla, California

Bo Mattiasson, Ph.D.
Associate Professor
Department of Pure and Applied
Biochemistry
Chemical Center
University of Lund
Lund, Sweden

Patricia McGivern
Research Assistant
Department of Microbiology
University of Iowa
Iowa City, Iowa

Richard M. Rodgers, Ph.D.
Research Group Leader
Syva Research Institute
Palo Alto, California

Pricilla Swanson
Research Assistant
Department of Microbiology
University of Iowa
Iowa City, Iowa

Edwin F. Ullman, Ph.D.
Vice President
Director of Research
Syva Company
Palo Alto, California

Frederick Van Lente, Ph.D.
Associate in Pathology
Columbia University College of
Physicians and Surgeons
New York, New York
Chief Laboratory Scientist
Overlook Hospital
Summit, New Jersey

Alister Voller, Ph.D.
Reader in Immunology
Department of Clinical Tropical Medicine
London School of Hygiene and Tropical Medicine
London, England
Research Associate
Nuffield Laboratories of Comparative Medicine
Zoological Society of London
London, England

David Wellington
Scientific Programmer
Syva Company
Palo Alto, California

Dedicated in Loving Appreciation to

My Wife Georjean

and to my children

Christine and Eric

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Chapter 1

INTRODUCTION

Edward T. Maggio

Advances in molecular biology, specifically the rapidly evolving understanding of the molecular basis of disease, generated a need for new assay methods which are quantitative, specific, and ever more sensitive. Early studies on antibody-antigen interactions using radiolabeled proteins[1] helped to lay the groundwork for the first such method able to respond to this need. The development of competitive binding assays using radiolabeled ligands as first described by Berson and Yallow[2,3] in 1958 unleashed an enthusiastic proliferation of applications of this new technique, particularly in the fields of biomedical research and clinical chemistry.

Most radioimmunoassays utilize the competition of labeled ligand which may be either a hapten or a macromolecular antigen (Ag*) with the corresponding unlabeled ligand or analyte (Ag) from sample for a limited number of antibody binding sites (Ab) as shown below:

$$\begin{array}{ccccc} Ab & + & Ag^* & \rightleftarrows & Ab{\cdot}Ag^* \\ + & & & & \\ Ag & & & & \\ \uparrow\downarrow & & & & \\ Ab{\cdot}Ag & & & & \end{array} \qquad (1)$$

The concentration of antibody binding sites available to bind the radiolabeled ligand is inversely related to the concentration of analyte present in the sample.

The excellent performance characteristics of radioimmunoassay, most notably its sensitivity (down to 10^{-17} mol), specificity, and relative insensitivity to variations in the chemical composition of sample, have resulted in its adoption as a primary analytical tool not just in the clinical field, where it receives its most frequent use, but in many other basic and applied scientific fields as well.

Nevertheless, the use of radionuclides as immunochemical labels does have certain inherent drawbacks. The relatively short half-life of the gamma-emitting isotope most commonly used in highly sensitive assays limits the useful shelf-life of the reagents. The potential health hazards associated with the routine use of radioactive materials and problems associated with disposal and release of radioactivity into the environment are perhaps of even greater concern.

Partly in response to the challenge posed by these apparent drawbacks, a wide variety of nonisotopic immunoassay techniques has arisen. Included in this category are quantitative fluoroimmunoassay,[4] fluorescence polarization immunoassay,[5] free-radical immunoassay,[6] viroimmunoassay,[7] hemeagglutination inhibition,[8] and, of course, enzyme-immunoassay.[9,10]

Two of these techniques have been shown to compete favorably with radioimmunoassay in many areas of performance. These are fluorescence immunoassay and enzyme-immunoassay.

While the use of fluorescent dyes as immunochemical labels predates the use of enzymes,[11] research in the use of fluorophores as immunochemical labels has been greatly overshadowed by the current interest in the use of enzymes in quantitative immunoassay procedures. The broad range of application of enzyme-immunoassay to

the determination of serum proteins and hormone levels, therapeutic and illicit drug levels, carcinofetal proteins, immune status, and viral and bacterial antigens will attest to this. Interestingly, while the applications of enzyme-immunoassay have continued to expand dramatically in the less than 10 years since they were first described,[12, 13] there has been a resurgence in interest in fluoroimmunoassay as an alternate nonisotopic methodology.[14-22]

Since all of the types of determinations mentioned in the preceding paragraph may be and have been made using radioimmunoassay, clearly there must be a strong impetus generating interest and effort applied to enzyme-immunoassays on so large a scale.

The drawbacks associated with the routine clinical use of radioactive reagents cited above undoubtedly contribute to this impetus; however one may question to what extent. Where equal performance can be demonstrated, the practical concerns such as reagent cost, technician time required for the assay, simplicity of protocol, availability of suitable instrumentation, and adaptability to automation, are likely to be the overriding factors in the selection of a new methodology.

In this regard it is probably the ability of enzyme-immunoassay to address itself to these concerns, rather than the drawbacks associated with the use of radioactivity, which provides much of the current driving force behind research efforts in the enzyme-immunoassay field.

Early comparative studies of enzyme-immunoassay and radioimmunoassay spawned a short-lived debate concerning the relative sensitivity capabilities of the two techniques. The very elegant study by Rotman[23] demonstrating the measurement of activity of single molecules of β-galactosidase, while not concerning itself with enzyme-immunoassay, certainly demonstrates that there is no inherent lack of sensitivity associated with the detection of enzymes. Since there are now many reports in the literature demonstrating the superb sensitivity of both enzyme-immunoassay and radioimmunoassay, some alternately claiming the relative superiority of each method for a number of similar applications, it seems reasonable to conclude that there is no significant difference in the sensitivity of enzyme-immunoassay and radioimmunoassay as far as practical clinical determinations are concerned. When both methodologies are optimized, limitations on sensitivity and specificity appear to reflect primarily the properties of the antiserum employed rather than the nature of the immunochemical label.

It has been in the areas of instrumentation and convenience of protocol that enzyme-immunoassay has greatly surpassed radioimmunoassay. In the case of some solid-phase (heterogeneous) enzyme-immunoassays, the use of visually read endpoints eliminates the need for an instrument altogether. When an instrument is needed, many of the heterogeneous procedures allow the use of inexpensive and generally available colorimeters and spectrophotometers.

Homogeneous enzyme-immunoassays generally employ spectrophotometers with enzyme rate-analyzer capability. Since the determination of enzyme activity by rate measurement is a common procedure in the clinical laboratory, the availability of instrumentation suitable for homogeneous enzyme-immunoassays is usually not a problem. Very significant simplification of assay protocol is accomplished by elimination of the separation step required in radioimmunoassay. Elimination of the separation step in turn greatly simplifies the automation of homogeneous enzyme-immunoassays on existing automated enzyme rate-analyzers. The use of automated equipment is one means of reducing the actual "hands on" time for the laboratory technician. This latter aspect of enzyme-immunoassay along with the generally longer reagent shelf-life (i.e., less frequent outdating) compared with radioimmunoassay tends to reduce the cost per determination.

The future of enzyme-immunoassay will undoubtedly bring more simplified protocols, more rapid test results, and wider clinical application. In addition, one may an-

ticipate extension of the basic technology to encompass a much broader user-base consisting of increasingly larger numbers of potential users. In a lateral sense this broader base will transcend the boundaries of technical disciplines, just as radioimmunoassay has done, extending into a host of nonclinical areas. Unlike radioimmunoassay, the nonisotopic nature of enzyme-immunoassay will allow a vertical growth in its user-base to encompass more simplified laboratory facilities and increasingly less sophisticated users.

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Chapter 2

ANTIBODY-ANTIGEN AND ANTIBODY-HAPTEN REACTIONS

J. E. Butler

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I. INTRODUCTION

The presence of "humoral factors" in blood capable of neutralizing and presumably combining with toxins or microorganisms, was first recognized by von Behring. The exclusive nature of these "anti-bodies" in protecting the host remained a controversial issue between the German immunological school and the French school,[1] the latter of the belief that the phagocytic cells provided such protection. By 1903 the importance of both antibodies and cells had been demonstrated. The two subdisciplines of immunology, cellular immunology and immunochemistry, still remain today. Whether cells or antibodies are the attackers, the target of the host's attack is always the "antigen", some of the attacking cells armed with antibodies.[2] Investigators in both subdisciplines and nonimmunologists who use immunological techniques as tools will benefit from an understanding of the interactions between antibodies and antigens. This chapter discusses such interactions as well as many immunochemical assays that depend on these interactions.

In present day terminology, an antibody is a member of the family of mildly glycosylated proteins called immunoglobulins, which can specifically combine with an antigen. Hence, the term antibody is a functional term. Immunoglobulins are a diverse group of proteins sharing a number of important and diagnostic structural features. While elegantly reviewed elsewhere,[3,4] it will suffice to mention that each immunoglobulin is composed of equal molar concentrations of heavy (50,000 to 75,000 mol wt) and light (22,500 mol wt) polypeptide chains. The N-terminal 110 amino acid sequence of each is referred to as the variable region. The term variable was coined because sequence analyses of this region in different proteins revealed an extremely low probability of finding two alike. Within the intact molecule, variable region sequences of both heavy and light chains are structurally associated with each other and form the antibody combining site (that region of the molecule which binds to the antigen and is responsible for antibody specificity). IgG immunoglobulins occur as monomers and possess a pair of identical heavy and light chains. Unless enzymatically degraded, each monomeric antibody molecule has two identical combining sites. Polymeric antibodies have multiples of this bivalency. The combining site is of course important to a discussion of antibody-antigen reactions and will be treated in more detail later. The remaining regions of both heavy and light chains constitute the constant region sequence, and contain antigenic markers which determine the isotype (i.e. class, subclass, or light chain type) of an immunoglobulin or antibody. In humans and many common experimental animals, two light chain isotypes, kappa and lambda, and five heavy chain class isotypes, IgG, IgM, IgA, IgD, and IgE, have been identified. While the constant region of an antibody molecule is not involved in the combination with antigen, this region is important in the discussion of antibody-antigen reactions because (1) certain secondary antigen-antibody reactions are restricted to certain isotypes, (2) antibodies of certain classes are much more effective in certain immunochemical assays than oth-

ers, and (3) the interaction of an antigen with antibodies of different isotypes, and the distribution of antibody activity among isotypes in the immune response to different antigens are currently being studied to elucidate the functional significance of antibody-isotype diversity. The diversity among isotypes is almost certainly functionally related. Constant region sequences determine such functional properties among isotypes as selective membrane transport, the ability to attach to neutrophils, macrophages, lymphocytes, epithelial and mast cells, as well as to bind complement components and bacterial proteins.

The term antigen has a more complex terminology than does antibody. Simplistically, an antigen is merely the substance to which the antibody binds. As initially shown by Landsteiner, antibodies can bind and have specificity for relatively small chemical groupings.[5] It has been estimated that this chemical group may be as large as a pentasaccharide[6] or a tetrapeptide.[7] An antigen can be an entire protein molecule, a microorganism, or a mammalian cell. Hence, the actual site of antibody attachment is best referred to as an antigenic determinant[8] or an epitope.[9] Antigens therefore may be multivalent, such as proteins and bacteria, or may be univalent such as haptens or very small hormones. The term hapten has additional meaning. A hapten may indeed combine with an antibody, but is incapable, without attachment to a larger "carrier" protein, of eliciting an immune response. Despite their lack of immunogenicity, (failure to elicit an antibody response) the univalency of haptens makes them extremely valuable for studies of antibody affinity, reaction kinetics, and the nature of the antibody combining site. The use of haptens has perhaps been the most important approach to understanding the molecular basis of antigen-antibody reactions, as these small chemical compounds may also be studied when attached to proteins or cells; in such situations acting as the epitopes of multivalent antigens.

Because antibodies are at least bivalent, combination with multivalent antigens often leads to aggregates or clusters. Such clusters are important in secondary reaction phenomena, and will be discussed in Sections IV and V. As mentioned above, some types of antibody molecules have in their constant region specific attachment sites for complement or certain cell membranes, and accordingly produce yet other types of secondary antigen-antibody reactions. Secondary reactions may be manifest in vitro or may produce an in vivo effect. Finally, most secondary antigen-antibody reactions can also be inhibited with haptenic antigens or other multivalent antigens, thus giving rise to an array of inhibition- and competition-type antigen-antibody reactions.

Interactions between antibodies and their antigens involve noncovalent bonds. Conformational changes in antibodies and antigens have been known to occur after combination, although these changes are not known to produce denaturing effects. Hence, at least primary antigen-antibody reactions and many secondary reactions are readily reversible. Secondary in vivo effects, such as those which involve cell lysis, are, of course, nonreversible.

The study of antigen-antibody reactions has three discrete and equally important applications. First, the study of antigen-antibody reactions is the key to understanding the specificity of antibodies, the size and nature of the combining site, the forces involved in the combination, and the kinetics of the reaction. Secondly, because the immune response of the host to an antigen is affected by many variables, the comparative study of antigen-antibody reactions from animals treated differently, sampled at different times, or with different genetic constitution, provides a valuable tool for probing the immune response. For example, much valuable information about the immune response has been obtained by measuring the affinity of antibodies produced after different times and treatments. The third important application of the study of antigen-antibody reactions involves their use as biochemical, research, or clinical diagnostic tools for the quantitation of antigenic substances or antibodies themselves.

Radioimmunoassay for example, is in use in clinical and research laboratories throughout the world, many of which have no connection with immunology except for the methodology involved. A similar case can be made for immunohistochemical techniques, agar-gel precipitin assays, and more recently, enzyme-linked immunoassays.

This chapter will concentrate on: (1) primary binding and competition assays, (2) the precipitin reaction as an example of a secondary reaction with only cursory mention of other common secondary serological assays, and (3) multiple antibody binding assays.

II. CLASSIFICATION OF ANTIGEN-ANTIBODY REACTIONS

The valency of the antigen, as well as the immunoglobulin class of the antibody, influences the type of reaction that occurs between the reactants. This fact together with other considerations has led to classification of antigen-antibody reactions. It is generally agreed among immunologists that the initial combination of antigen with antibody constitutes the primary reaction. Accordingly, all reactions between antibodies and antigens begin with a primary reaction. The equilibrium relationships involved in primary reactions can readily be studied using haptens, as will be discussed in Section III. Primary reactions occur rapidly (milliseconds), are macroscopically invisible, and are a property of that portion of the antibody molecule which contains the antibody combining site.

Secondary reactions are the result of antigen multivalency and require a longer time to develop. For example, while the combination of precipitating antibody and antigen occurs in milliseconds, the measurable or visible precipitation usually requires minutes to hours to develop. Hence, a molecular change, visible microscopically or to the unaided or partially aided eye, is also a characteristic of the secondary-reaction phase of the precipitin reaction.

Some investigators further classify immunochemical reactions as tertiary, etc., when additional components other than antibodies and antigens are involved. For example, the binding of complement components to soluble complexes of antibody and antigen or the release of histamine by basophils which contain bound IgE-antigen complexes, are examples of such tertiary reactions. Such terminology becomes cumbersome when one studies multistep reactions (where multiple antibodies or antiglobulins are involved) and especially in in vivo reactions which often involve a poorly understood "wave" of cellular processes. For simplicity, all reactions, other than those invoiving the primary interaction of antigen and antibody, will be classified in this chapter as secondary reactions.

Antigen-antibody interactions are sometimes classified according to their univalent vs. multivalent nature. Functionally, such a classification has approximately the same boundaries as the terms primary vs. secondary reactions. Where antigen multivalency is known to be involved, additional nomenclature is often used in discussing cross-reactivity (i.e., a distinction is made between Type I and Type II reactions). The former is used in connection with the heterogeneity of a population of antibodies for a single antigenic determinant while the latter refers to heterogeneity in an antiserum resulting from specificity for two or more epitopes on the same antigen. The topic of antibody heterogeneity will be discussed in Section III.

Antigen-antibody reactions may also be differently classified. In vitro reactions may be distinguished from in vivo reactions. Although not always demonstrated experimentally, in vitro reactions also occur in vivo. While precipitin reactions very likely do not progress to the precipitate stage in vivo, the complexes typical of early stages of the in vitro reaction do form and depending on their makeup, are involved in more complex types of in vivo reactions. Based on our present knowledge, the differences between