ENZYME-MEDIATED IMMUNOASSAY

Edited by
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INTRODUCTION

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In 1959, Yalow and Berson used insulin labeled with radioactive iodine to develop a quantitative immunological method for determining the amount of insulin in human plasma. Their method depends upon a competition between insulin labeled with radioactive iodine (I^{131}) and unlabeled insulin from plasma for a fixed and limited number of specific binding sites on the antibody to insulin. The amount of the labeled insulin bound to the antibody is inversely proportional to the amount of insulin in the plasma sample. Their method, which is so elegantly simple in concept, is made possible by the ability to detect with ease extremely low levels of radioactivity, and by the exquisite specificity of an antibody capable of specifically binding the analyte. Such a combination of sensitivity and specificity is the basis of this versatile analytical tool called radioimmunoassay (RIA).

Twelve years later, Engvall and Perlmann (1971) and Van Weemen and Schuurs (1971) independently introduced the use of enzymes as another category of sensitive and even more versatile labels for use in immunoassays. Engvall and Perlmann (1971) coined the term ELISA, which stands for Enzyme Linked Immunosorbent Assay.

In both RIA and ELISA, the labeled and unlabeled antigens bound to the antibody must first be separated from the labeled and unlabeled antigens that are not bound before the activity of the label can be measured in either fraction. These methods are collectively called *separation-required immunoassays*. Because that separation inevitably involves the use of heterogeneous phases in the assay mixture, the term "heterogeneous"

immunoassay is interchangeably used for separation-required immunoassay.

In 1972, Rubenstein et al. developed a new immunoassay using an enzyme as the label which did not require the separation of the antigen bound to antibody from the unbound fraction before measuring the enzyme activity. The method depends on a change in the specific activity of the enzyme when the antibody binds to antigen labeled with enzyme. The enzyme activity in the unfractionated assay solution is proportional to amount of antigen labeled with enzyme that is not bound by the antibody. Because the assay does not require any heterogeneous phase to separate the bound and free antigens, it is called "homogeneous" immunoassay. We feel that the term "homogeneous" does not accurately describe the salient features of the assay and recommend that the more appropriate and more descriptive term "separation-free" to be used instead. Separation-free is a more accurate term to use, because some separation-free enzyme mediated immunoassays are not homogeneous and involve heterogeneous phases (e.g., see Chapters by Gibbons and Litman).

Since the first uses of enzymes as the label in immunoassays in 1971, there have appeared over 10,000 publications dealing with various aspects and aplications of enzyme-mediated immunoassays. The major aim of this volume is to collect state of the art reviews on this subject from scientists in the North American Continent. Reviews by European (Avrameas et al., 1983; Malvano, 1980) and Japanese (Ishikawa, et al., 1981) scientists have already been assembled.

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ENZYME MEDIATED IMMUNOASSAY: AN OVERVIEW

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INTRODUCTION

Since its introduction by Yalow and Berson (1959), radioimmunoassay (RIA) has played important roles in facilitating and accelerating basic discoveries in biomedical sciences and has become a standard tool in laboratory medicine. Millions of clinical tests are now routinely performed by using RIA. The rapid and wide acceptance of RIA as a routine clinical method can be attributed to (1) the general applicability of the method, i.e., any compound can be analyzed by a RIA so long as an antibody specific to that compound is available; (2) the selectivity and specificity of the method; (3) the sensitivity of a radioactive isotope as the label in RIA and (4) the minimal inference and ease of performing the test.

Notwithstanding the advantages of RIA, there are, however, several drawbacks in using RIA. They include (a) the relatively short half-life of gamma-ray emitting isotopes, (b) the health hazards involved in preparing the isotopic labels and in handling and performing the test, (c) radiation induced structural damage on the labeled molecules, (d) the need of separating antibody bound labeled and unlabeled antigens from unbound ones and (e) therefore the difficulty in automating RIA.

In attempts to replace the use of radioactive materials as labels and to develop separation-free immunoassays, a number of non-isotopic labels have been used. These include bacteriopha ges (Haimovich and Sela, 1969), free radicals (Leute et al, 1972), fluorescent groups (Soini and Hemmila, 1979),

Chemiluminescent and bioluminescent groups (Simpson et al., 1979; Kricka and Carter, 1982; Serio and Pazzagli, 1982), synthetic particles (Rembaum and Dreyer, 1980), red blood cells (Adler and Liu, 1971), electron dense materials (Singer and Schick, 1961 and Leuvering et al., 1980), liposome (Chan et al., 1978; Litchfield et al., 1984), metals (Cais et al., 1977), enzyme substrates (Burd et al., 1977; Wong et al., 1979; Ngo et al., 1979; Ngo et al., 1981; Ngo and Wong, 1985), prosthetic group (Ngo, 1985), enzyme modulators (Ngo and Lenhoff, 1980a; Ngo, 1983; Ngo, 1985) and enzymes (for a review of this subject, please read Schuurs and Van Weemen, 1977; Borrebaech and Mattiasson, 1979; Ngo and Lenhoff, 1981 and Ngo and Lenhoff, 1982).

ENZYME MEDIATED LABELS

Enzyme mediated labels (EML) are defined as compounds which can be quantitatively measured via enzymatic processes. They include enzyme substrates, enzyme cofactors, enzyme prosthetic groups, enzyme modulators (i.e., inhibitors or activators), enzyme fragments, apoenzymes and enzymes.

Advantages of EML

Advantages in using EML for immunoassay are: (1) long-term stability of most EML's, their half-lives at 4°C are generally longer than six months; (2) their concentrations can be determined rapidly by using instruments commonly available in most analytical laboratories; (3) no radiation hazards; (4) possibilities of developing rapid, separation-free (homogeneous) immunoassays which can be readily automated; (5) the development of qualitative visual tests for mass-screening and (6) amplification of detection signal by some EML's when used in separation-required (heterogeneous) mode.

Limitations of EML

Limitations of using EML in immunoassay include: (1) limited sensitivity of some separation-free systems; (2) carcinogenicity and instability of some enzyme substrates; (3) interference by some endogeneous EML's and other substances found in biological fluids and environmental samples; (4) long assay time for some EML immunoassays and (5) procedures for measuring enzyme activity can be more complicated and demanding than the counting of radioactivity of an isotope.

Sensitivity and versatility of enzyme label

Among EML's, enzymes appear to be the most sensitive and versatile labels because they are protein molecules endowed with an extraordinary efficient catalytic power. The presence of a minute amount of enzymes can be detected and quantified by measuring products of the reaction catalyzed by the enzyme. Most of the commonly used enzyme labels are capable of converting 10^6 molecules of substrate into products within one minute by one enzyme molecule at ambient temperature and pressure. The catalytic efficiency of an enzyme depends strongly on its three dimensional structure (conformation). The three dimensional structure of an enzyme or protein is maintained through numerous non-covalent interactions, such as hydrophopic and hydrogen bondings, ionic interactions and covalent linkages, such as disulfide bonds. The three-dimensional structure of an enzyme allows the juxtaposition of certain amino acid residues at spatially most strategic positions for bringing about catalysis. Non-covalent interactions, being weak chemical bonds are easily broken or altered by thermal energy or other non-covalent interactions such as by binding of ions, chaotropic agents, detergents, lipids, etc. It is known that the binding of a molecule (such as an allosteric effector) by an enzyme at a site far remote from its active site (i.e., catalytic site) can bring about conformational changes that alter the spatial position of active site amino acid residues. Alterations in the non-covalent interactions of an enzyme which lead to a new and different conformation may significantly alter the catalytic efficiency of the enzyme. This conformational flexibility of an enzyme is one of the drawbacks in using enzyme as a label. However, it can also be used to advantage for developing a separtion-free enzyme immunoassay which is based on an antibody induced changes in the conformation of a ligand conjugated enzyme. Another advantage of using enzyme as a label is the numerous functional groups in an enzyme molecule such as the amino, sulfhydryl, carboxyl, carboxamide and tyrosyl groups that are available for covalent linking to ligand molecules.

Separation-free (homogeneous) versus separation-required (heterogeneous) enzyme mediated immunoassays (EMI)

From an operational standpoint, enzyme mediated immunoassays (EMI) can be divided into two categories: (1) separation-free (homogeneous) or (2) separation-required (heterogeneous) systems. In the separation-free system, the enzyme activity of the assay solution is measured without a prior physical separation of the antibody-bound labeled ligands from the free, unbound ones. Such a procedure is made possible because the activity of the antibody-bound labeled ligands is

significantly different from the ones unbound by the antibody. In the separation-required assay, however, a procedure is required for separating the labeled ligand into antibody bound and free, unbound fractions. The enzyme activity of these fractions is then measured. A physical separation of the bound and unbound fractions is required, because the assay depends on the partitioning of the labeled conjugates into antibody bound and unbound fractions.

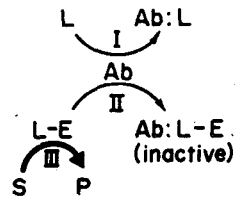

Fig. 1. Principle of separation-free EMI based on enzyme labeled ligand.

SEPARATION-FREE (HOMOGENEOUS) ENZYME MEDIATED IMMUNOASSAYS

Separation-free EMI using enzyme as the label

Rubenstein et al. (1972) described a separation-free EMI for morphine detection using lysozyme as the enzyme label. They coined the term "homogeneous" enzyme immunoassay to indicate that no physical separtion of the antibody bound enzyme labeled ligand from the unbound form was necessary. The principle of the assay is schematically shown in Fig. 1. The covalent enzyme labeled ligands (E-L) compete with ligands (L) from the sample

for a limited concentration of antibody (Ab) to that ligand to form the complex, E-L:Ab (reaction II). The resultant E-L:Ab complex exhibits very little enzyme activity because of either steric hindrance (Rubenstein et al., 1972) or allosteric inhibition (Rowley et al., 1975) caused by the bound antibody. In the absence of L, reaction I would not take place and E-L and Ab would form the enzymatically inactive E-L:Ab complex (reaction II). In the presence of L, however, there would be a competition for the antibody leaving more E-L uncomplexed and free to catalyze the conversion of substrates to products (reaction III). Thus the enzyme activity would be directly proportional to the amount of free L in the sample.

Separation-free EMI based on enzyme labeled ligand has been developed for a number of therapeutically important drugs and for hormones. The drugs include phenytoin, phenobarbital primidone, ethosuximide, carbamazepine, digoxin, gentamicin, valproic acid, and methotrexate. The assay is rapid. It takes less than 1 minute to perform one test. It is also very sensitive; substances at picomole levels can be detected with ease (Jaklitsch, 1985).

The precision and accuracy of separation-free EMI based on enzyme-ligand conjugates are comparable with other immunological (e.g., RIA) and non-immunological methods (e.g., GC, HPLC, and TLC) (Jaklitsch, 1985).

Gibbons et al. (1980) reported a separation-free EMI for proteins employing β-galactosidase labeled protein antigens. When the antibody to the antigen bound to the enzyme labeled antigen, it did not alter the catalytic action of the enzyme toward its small molecular weight substrate. The activity of the antibody bound enzyme labied antigen toward a macromolecular substrate, however, was inhibited up to 95 percent because of the steric exclusion of the substrate from the active site of the enzyme. As the concentration of antigen increased, more antibodies are tied up, leaving more antigen-enzyme conjugates free to hydrolyze the macromolecular substrate. The method developed by Gibbons et al. (1980) is very sensitive. With the prototypical system, they were able to assay human IgG down to 25 ng/ml with ease. A fluorometric macromolecular substrate for β-galactosidase has recently been developed. Using umbelliferyl-β-galactoside substituted dextran as the substrate, nanogram quantity of β-galactosidase can be quantified in less than 1 minute (Gibbon, 1985).

Separation-free EMI using enzyme modulator as the label

Enzyme modulator mediated immunoassay (EMMIA) is based on the ability of a ligand labeled enzyme modulator (M-L) to modify

the activity of an indicator enzyme (E). The M-L competed with free ligand (L), the analyte, for a limited amount of antibody to the ligand (Ab). The antibody bound M-L is unable to modulate the activity of the indicator enzyme. In the absence of analyte (L), reaction I (Figure 2) would not occur and the M-L and Ab to the analyte (L) would combine through reaction II, making M-L unavailable to modulate the enzyme activity. As the concentration of analyte increases, however, it would compete successfully for binding sites on Ab (reaction I) leaving more modulator free to complex with the indicator enzyme (reaction

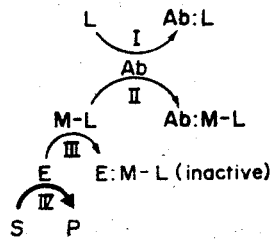

Fig. 2. Principle of separation-free EMI using enzyme modulator as the label.

III), thereby modulating its activity (Ngo and Lenhoff, 19801 Ngo, 1983; Ngo, 1985). Depending on the properties of the modulator, the enzyme activity will be increased by a positive modulator or it will be decreased by a negative modulator. Thus, for EMMIA with a positive modulator, the enzyme activity will be directly proportional to the concentration of the analyte. For EMMIA developed with a negative modulator, the activity will be inversely proportional to the concentration of the analyte.

Based on the principle of EMMIA, a practical assay for human serum thyroxine has been developed. The assay used an acetylcholinesterase as the indicator enzyme and a thyroxine labeled cholinesterase inhibitor as the enzyme modulator (Finley et al., 1980). Seventy-five samples were analyzed in one hour. The results were clear cut and showed excellent precision and accuracy. A theophylline assay based on the same principle has also been developed (Blecka et al., 1983).

EMMIA based on fragments of ribonuclease, such as S-peptide labeled ligand and S-protein, has been developed for thyroxine assay (Gonnelli et al., 1981 and Ngo, 1985).

Separation-free EMI using enzyme prosthetic group as the label

In this assay, an enzyme prosthetic group is covalently linked to a ligand and this conjugate is able to reconstitute an active holoenzyme from its apoenzyme. Flavin adenine dinucleotide (FAD) is a prosthetic group for glucose oxidase. A recombination of FAD and apoglucose oxidase, neither of which is active by itself, yield an active glucose oxidase. When FAD is used to covalently label a ligand (L) the resulting stable FAD-L conjugate serves two functinal roles: (a) FAD-L, as a ligand analog, competing with the analyte (L) for antibodies to L (reaction II, Fig. 3); or (b) as a modified prosthetic group that can bind to apoglucose oxidase (AG) through a high-affinity binding to form (reaction III) an enzymatically active hologlucose oxidase (HG) (Ngo and Lenhoff, 1980). On the other hand, once the FAD-L form FAD-L:Ab as in reaction II, the FAD moiety can no longer combine with the apoenzyme due to the steric hindrance imposed on FAD-L by the complexation with the antibody. In the absence of L, the analyte, reaction I would not occur; thus the antibody would combine the FAD-L instead (reaction II) making the FAD of the complex incapable of combining with AG (reaction III). Conversely, as the concentration of L increases, it would compete more successfully for Ab (reaction I) leaving more FAD-L free to combine with AG (reaction III), thereby increasing the amount of hologlucose oxidase to catalyze reaction IV (Morris et al., 1980).

Based on the principle shown in Fig. 3, a theophylline assay with a sensitivity of 2 µg/ml was developed. A novel application of FAD as label for macromolecule assay was first demonstrated by Ngo. Aminohexyl-FAD was covalently linked, via a bisimidate cross-linker, to human IgG to form a stable FAD-IgG conjugate. This conjugate can combine either with the antibody to IgG (Fig. 3, reaction II) or with apoglucose oxidase (Fig. 3, reaction III). In the absence of IgG, most of the FAD-IgG combined with the antibody to form FAD-IgG:Ab, rendering the FAD moiety of FAD-IgG incapable of serving as a prosthetic group for

apoglucose oxidase; hence no holoenzyme was formed and no enzyme activity was observed. As the concentration of IgG increased, more FAD-IgG's were uncomplexed from the FAD-Ig:Ab and became free to act as the prosthetic group of an apoglucose oxidase. Consequently, the enzyme activity formed is directly proportional to the concentration of free IgG present in the sample.

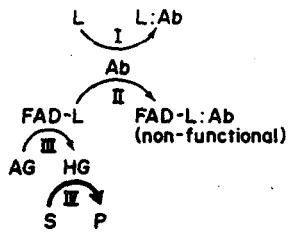

Fig. 3. Principle of separation-free EMI using enzyme prosthetic group as the label.

Nakane (1980) has recently described the development of an interesting separation-free enzyme immunoassay based on the above principle. The technique involves the reconstitution of an apoperoxidase with a heme labeled ligand that served as a prosthetic group for apoperoxidase.

Separation-free EMI using fluorogenic enzyme substrate as the label

Separation-free enzyme immunoassay using ligand-linked fluorogenic enzyme substrates were developed by Burd and Wong (1977; 1979). In this assay (Fig. 4), a ligand derivative was

covalently linked to a fluorogenic enzyme substrate to form a stable substrate-ligand (S-L) conjugate. The S-L competes with the analyte, L, for a limited concentration of antibody to L. Thus, through reactions I and II, L:Ab and S-L:Ab are formed. Because the free S-L conjugate is a fluorogenic substrate for the enzyme, E, whereas S-L bound to the antibody (S-L:Ab) is not, the absence of analyte (L) would allow the S-L to combine with the antibody to L so that the indicator reaction III would not take place. On the other hand, as the concentration of L increases, more S-L would remain free to act as substrates for E

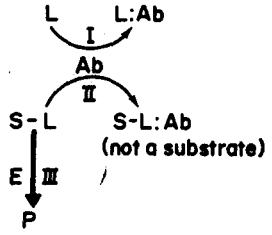

Fig. 4 Principle of separation-free EMI using fluorogenic enzyme substrate as the label.

and, hence, allowing more products to form by reaction III. Thus, the fluorogenic substrate-ligand conjugate (S-L) serves a dual role: (a) as a ligand analog which competes effectively for binding with antibody to the ligand, and (b) as an efficient substrate analog which allows the indicator reaction III to take place by forming products.

In this assay, rather than providing an amplification effect, the enzyme is used merely to distinguish and to quantify the proportion of free, unbound substrate-labeled ligand. The