Raciloimmunoassay: Principle and Technique

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Introduction

Radioimmunoassay allows for the measurement of wide range of materials of clinical and biological importance. This technique has a significant impact on medical diagnosis due to the ease with which the tests can be carried out, while assuring precision, specificity and sensitivity. The radioimmunoassay technique, as the name implies, achieves sensitivity through the use of radionuclides and specificity that is uniquely associated with immunochemical reactions. Yalow and Berson were awarded the nobel prize for poineering radioimmunoassay when they evolved radioimmunological assay for insulin[1]. At about the same time Roger Ekin[2] also published his work on determination of serum thyroxine using a competitive binding assay. In the latter system, the naturally occurring thyroxine binding globulin (TBG) was used as binding protein and radioactive labelled thyroxine (T4) was used as tracer. Though not strictly an immunological test, the principle of competitive binding of compound under test to a specific protein remains the same. The radioimmunological determination of thyroid stimulating hormone (TSH) was demonstrated by Odell[3] and thyroxine by Murphy[4]. The discovery that haptens coupled to carrier proteins could also invoke an antibody production[5] accelerated the development of radioimmunoassays for steroids and other low molecular weight substances as well.

Principle of Radioimmunoassay

The radioimmunoassay technique is based on the isotope dilution principle, alongwith the use of a specific antibody to bind to a portion of the substance to be measured. If an antigen (for example, a hormone) is mixed with a specific antibody to that substance, an interaction will occur, forming an antigen/antibody complex that is chemically different from either the antigen or the antibody. If there is insufficient antibody to complex all the antigen present, mixing of the antibody with a known amount of isotopically labeled antigen along with an unknown amount of labeled antigen allows quantitation of the unlabeled antigen.

Antigen + Antibody -. Complex + Leftover antigen
Antigen* + Antibody — Complex* + Leftover antigen
then
Antigen + Antigen* + Antibody Complex + Complex +
left over antigen +
left over antigen *

If the amount of antigen* present is fixed, then by competitive binding the amount of antigen/antibody complex* formed will be inversely proportional to the amount of unlabeled antigen present. Since the reactions are reversible, the interaction between an antigen and an antibody to form the antigen-antibody complex is equated as:

Ag + Ab AgAb

Hence the rate constant of the forward reaction (association of antigen and antibody to form a complex) is denoted by ki and the rate constant of the reverse reaction (dissociation of the antigen-antibody) is denoted by k2. It should be noted that ki and k2 are constants, in other words they describe the fraction of the available molecules which will react within a unit time. The absolute rate - the number of molecules which react in unit time is obviously dependent on the concentration of molecules. Thus when the reaction begins, with the addition of antigen and antibody, the forward reaction rate is high and the reverse reaction rate correspondingly low.

Ag + Ab = AgAb

As the reaction proceeds, the concentration of free antigen and antibody decreases, and so too will the forward reaction rate. At the same time the concentration Of the antigen- antibody complex increases, and with it the rate of the reverse reaction. Eventually the stage is reached where the number of free Ag and Ab molecules reacting in unit time to form AgAb is identical with the number of AgAb molecules which dissociate in this time:

Ag + An == AgAb

At this stage of equilibrium there will be no further net change in the concentration of the molecules on the two sides of the equation. The exact concentration which is reached at equilibrium depends on the energy with which the binder and ligand react. The situation can be described by the law of mass action which states that, at equilibrium, the ratio of the products of the concentrations on the two sides of the equation will be a constant, designated as K:

(AgAb) — K (Ag)(Ab) where Ag , Ab and Ag Ab are the concentration of free an-igen, free antibody and antigen-antibody complex respectively, and K is the affinity constant.

Because of the equilibrium established in this reaction, the addition of some quantity of unlabeled antigen Ag will compete with Ag* for the binding sites on Ab, yielding AgAb and increasing the concentration of Ag* in the medium. The ratio between the bound fraction B of labeled antigen Ag*Ab, and the free antigen, which is called F, decreases with increasing concentration of the unlabeled antigen Ag. The ratio B/F can be determined by separating the bound and free fractions and measure ing their activity. Prior to testing unknowns it is necessary to prepare a series of solutions of increasing antigen concentration. These solutions of known antigen concentration are referred to as standard solutions. To these solutions is added a fixed amount of labeled antigen and antibody insufficient to bind all the antigen present. The complex is permitted to form, and the bound antigen is separated from the free antigen. By relating the count from the separated complex to the control, one can establish a standard curve. Unknown samples can then be quantified from this curve.

The Standard Curve

A plot of the distribution of radioactivity as a function of the amount of unlabeled antigen present is known as the standard or dose response curve. Standard curves can be plotted in a variety of ways. The most commonly used response curves are the bound-free (B/F), free-bound (F/B), fraction bound (B) of occasionally the percent bound B/Bo ratios. The dose can be plotted on either the arithmatic or logarithmic scale. To obtain response curves that are essentially linear over a large part of the antigen concentration, the logit function defined as follows has been used:

Logit(y) =

where

y = either B/Bo or (B/F)/(Bo/Fo)

When logit (y) is plotted against the log concentration of the antigen, a linear dose-response curve results for most of the assay system[6,7]. Computer programms are now available not only to analyze the data and calculate results, but also quantify various associated errors.

Requirements of RIA

Radioimmunoassay involves three components: pure antigen, radiolabeled antigen, and antiserum (antibody). In addition a separation technique is essential to estimate the distribution of radioactivity in the free and bound fractions. The sensitivity of an assay depends to a large extent on the quality of these components and choice of a suitable separation technique.

Pure Antigen

The preparation of standards and tracer and the production of antibodies depends on the availability of pure antigen. Several procedures, such as electrophoresis, chromatoelectrophoresis, gel filteration, and ion exchange chromatography are available for the extraction and purification of hormones from biologic samples. A pure synthetic preparation, if available, can be substituted for the natural preparation. A number of hormones produced synthetically are now available with apurityto match the best materials isolated from natural sources. In any case, before the antigen can be used as a standard,

the specificity between this antigen and the antigen in the test sample toward the antibody binding sites must be clearly established.

Radiolabeling of Antigen

The labeled antigen used as a tracer must generally be present in low concentrations because the quantity of substance to be measured is usually small. The concentration of Ag* must not be greater than the least quantity of Ag to be measured. It is thus necessary to have labeled antigen of high specific activity, and once labeled, it must maintain the same characteristics of the unlabeled antigen to react qualitatively and quantitatively with the antibody. C-14 and H-3 have the principal draw back of longhalflives(5740 and 12.3 years respectively) and they are also more difficult to measure, requiring cumbersome liquid scintillation counting. They are almost exclusively used in those systems in which the addition of a larger molecule may alter the immunoreactivity of the system[8]. Radioiodine generally fuffills the label requirements; 1-125 is preferred over 1-13 1 because of its longer half life, ease of handling, and higher counting efficiency. A method of producing radioiodinated proteins with high specific activity was described by Hunter and Greenwood[9-10] using chloramine-T. Other methods have been developed, such as the use of iodine monochloride and Electrolyte labeling[11] Lactoperoxidase[12,13] polyacrylamide-coupled lacto-peroxidase[14], conjugation method[15], but chloramine-T continues to be the most commonly used technique. To ensure good sensitivity, the radioiodine-labeled compound used should have a specific activity of 100 to 300 mCi/mg. Chloramine-T method is easy and consists simply of adding the protein and the chloramine-T to a solution of sodium iodide. The mechanism of reaction is not clearly understood, but itis believed that the chloramine T forms hypochlorites in water and thus acts as a mild oxidizing agent. The oxidative process is essential for producing the radioiodide ion with positive charge for substitution onto the tyrosine fraction of the protein molecule. The amount of chloramine-T required is dictated by the quantity and nature of the protein that is being iodinated. If excess quantity of the oxidizing agent is used, the protein might be damaged. To prevent such damage it is advisable to limit both the reaction time and the amount of the oxidizing agent. Hunter and Greenwood[9] reported that one molecule of chloramine-T is required for every one of iodide. The effect of pH on iodination is also very pronounced and there is a fairly narrow range of pH optimum around 7.5 for most of the proteins. After labeling, the reaction medium contains labeled and unlabeled proteins, denatured protein reactants, and unbound iodide. Purification is therefore essential and may be accomplished by a variety of techniques, including dialysis, gel filteration, adsorption chromatography, ion exchange chromatography or gel electrophoresis[16,17]. Gel filteration, using a molecuir sieve such as sephadex, has been widely used because of the simplicity and efficiency it offers. The labeled antigen is filtered through a properly prepared column and several fractions collected. The particular fraction that possess both high specific activity and high immunoreactivity is chosen for the RIA. The immunoreactivity of the labeled antigen can be assessed by obtaining a set of antiserum titration curves. The simplest and most direct method of testing immunoreactivity is by reacting a small amount of labeled antigen with excess antibody. If a significant portion of the label is not bound to the antibody, it denotes the presence of nonimmunoreactive elements[8]. A labeled and purified antigen can generally be kept in solution for a week at 4°C without suffering appreciable loss in activity. It can be fractionated and rapidly frozen and kept in a freezer at -23°C or lyophilized and stored at 4°C for a period of 60 to 90 days. Once thawed, it is important to use the antigen soon thereafter. Refreezing is generally not recommended.

Antibody

The sensitivity and specificity of RIA depend on the affinity of the antigen-antibody reaction and the highly specific binding sites on the antibodies used. The production of a good antiserum is essential for a satisfactory assay. In order to produce a highly specific antiserum, the antigen should have a unique antigenic determinant. Production of antiserum invloves rather a complex procedure. Arnibodies are relatively easily raised against protein compounds of molecular weight in excess of 4,000 to 6,000. For

smaller proteins and non-immunogenic substances such as thyroid and steroid hormones, cyclic nucleotides and drugs, it is necessary to conjugate the compound to a larger polypeptide or a protein such as albumin, thyroglobulin or polysine prior to immunization[18-21]. It is customary to couple the happen in such a way as to expose any functional groups characteristic of the molecule, so that the likelihood of production of specific antibodies is enhanced. Antibody formation is generally augmented by adding an adjuvant to the antigen, which retards its absorption and increase the antigenic stimuli[8,19,20]. These substances includes aluminium hydroxide, gelatin, mineral oil and Freund\'s adjuvant. The latter is most commonly used and consists of a neutral detergent, paraffin oil, and killed becteria. The general method of inducing antibody formation is to inject into a number of animals the pure antigen mixed with Freund's adjuvant. Based on the view that impurities may have an adjuvant effect, some investigators prefer to immunize with relatively impure material, rather than a highly purified or synthetic equivalent[8]. The type of animal used is still some what empirical. Rabbits and guinea pigs are most often selected because they are small enough to be handled easily and large enough to withstand bleeding of upto 10 cc. Other animals such as sheep, goat, chicken or monkey may occassionally be used. The immune response is systemic rather than local, and this may explain why the results of immunization by different routes are very similar. Nevertheless, a number of different injection sites such as intradermal, subcutaneous, intramuscular, intraperitoneal, intravenous, intranodal and footpad may be used. In general, 0.2 to 2 mg of the antigenic preperation is injected as a suspension, although in certain cases it may be necessary to use larger quantities. In other cases as little as 20 to 100 ug of the immunogen is sufficient. Antiserum is usually collected 1 to 2 weeks after a booster injection. In some animals the titre is maintained for months or years in the absence of further booster injections; in others it shows a progressive fall. Once harvested, the antisera is tested for antibody titre, sensitivity and specificity to determine its suitability for RIA. The antiserum after appropriate testing should be divided into aliquotes and stored at -20 C to minimize the potential damaging effects of freezing and thawing. The size of each aliquote will depend both on the total volume and the rate at which the antiserum is used.

Standards

In MA reference standards are necessary in order to interpolate values of samples to be measured. A material intended as a standard should have certain characteristics, it should be available in large quantities, it should bestable, it should not contain substances which can interfere with assays, it should be highly purified and it should be available in a form which allows convenient and accurate preparation for radioimmunoassay. There are several types of RIA standard: International standards distributed by WHO, reference preparations distributed by National Institute of Arthritis and Metabolic Disease and laboratory preparations of restricted use prepared by the investigator or a manufacturer and not having international validity.

Separation Techniques

Once the primary reaction is complete it is necessary to determine the distribution of the tracer between the free and the bound form. Usually this requires the bound fraction be physically separated from the free fraction. A variety of techniques described for this purpose exploit physico-chemical differences between the tracer in its free and bound form. The operative criteria for a separation procedure are efficiency and practicality. The efficiency of a procedure can be defined as the completeness with which the bound and free phases are separated. The practicality includes speed, simplicity, applicability and cost. Various methods include electrophoresis , gel ifiteration, solid-phase adsorption of antigen, solid -phase absorption of antibody, immunoprecipitation and fractional precipitation[22-24].

RIA procedure

The general procedure of RIA consists of adding suitable quantities of standards, unknown, labeled antigen and antibodies to a buffer solution and allowing the reaction to reach equilibrium. Most of the competitive binding assays are of equilibrium type, in which all the reagents are added at the same time

and the reaction is allowed to proceed until equilibrium is established. In some cases it may be necessary to add tracer at a later stage to improve the sensitivity. This type of assay is known as sequential saturation assay. At the end of the incubation period the free and bound fractions are separated using a suitable technique. The distribution of radioactivity in each sample is determined by counting either free or bound or both with the use of suitable counting equipment. The estimation of antigen concentration is made by comparing the inhibition observed in the unknown with that produced by standard solutions of known antigen concentration. For this purpose, the dose response curves are plotted using the data from the standards, and the unknown antigen concentration is read directly from the graph.

Quality Control

Comprehensive QC is essential to ensure reliability and to reduce the so called grey area between definite pathological values and values which are normal. The purpose of regional and international quality control programmes is to allow a comparison of results between different participating laboratories. Quality control assures that a laboratory continuously produces highly reliable assay results on clinical samples. RIA is a technique with many variables and without any agreed 'best way' to set up an assay, imprecisions may occur at every step and the finalprecision will be effected by the sum of all the random errors. The principal determinants affecting RIA precision are experimental errors (pipetting and other manipulations, variation in quality and
concentration of reagents), Counting errors (specific activity of label, counting error, tube geometry, data handling and calculations) and sensitivity of the assay (slope of the standard curve and avidity of the antibody for the ligand). In routine sample analysis using RIA kits, problems do crop up at times. A systematic analysis of the procedure helps in sorting them out. In general, strict adherence to the kit protocol, adequate training in handling the kits and use of good quality precision pipettes and other instruments ward off most of these problems. Some of the common problems met within RIA are:

1. Changes in zero binding, blank and Q.C. samples.
2. Poor precision.
3. Non- linearization of standard curve and flat standard curves.
4. Non- agreement of RIA results with clinical diagnosis.

A strict QC programme ensures minimization of then errors and ensures reliable and good quality results.

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