LAH 264

Chapter 34
Solid-phase radioimmune assays

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Radioimmune assays (RIA) are some of the most sensitive, convenient and versatile methods for quantitative analysis of antigen–antibody reactions. RIAs provide rapid and accurate measurements of antigen or antibody concentration, even in relatively crude preparations with a detection sensitivity in the nanogram range. Many procedural variations have been adopted to meet specific research or clinical needs but the principle underlying all assays is the same: the amount of radiolabelled reagent (antibody or antigen) bound provides a measure of the amount of unlabelled antigen or antibody in a test sample.

The radiolabel, usually ^{125}I, can either be introduced directly on the antigen or antibody used for the primary reaction, or indirectly on a 'second step' reagent (e.g. a 'developing' antibody) that detects one of the unlabelled reactants in the primary antigen–antibody reaction. The final step of the assay separates unbound radiolabel prior to the determination of the radioactivity that has been bound.

In this chapter, the authors describe several variations of 'solid-phase' RIAs in which one of the reactants is immobilized on to wells of a flexible plastic microtitre plate. The use of these solid-phase assays facilitates washing (eliminating the need for centrifugation), especially in the final step when it is essential to remove all traces of unbound radiolabel. The solid-phase modification also substantially reduces the quantity of reagents and reactants consumed, thereby allowing large numbers of samples to be assayed rapidly. The examples presented illustrate the versatile applications of these RIA assay methods and the ways in which the assays can be manipulated to suit specific goals [3,4] (see also Chapter 26).

Solid-phase RIA—general steps

Coating the plate

Proteins and other macromolecules in solution tend to stick non-specifically to plastic. Since adsorption is virtually irreversible in PBS (pH 7.4), incubation of an antigen or antibody solution in microtitre wells produces a plate coat which remains intact through repeated washing.

The amount of antigen or antibody deposited depends on its concentration in solution, its inherent 'stickiness' and the amount of contaminating material capable of competing for sites on the plastic; therefore highly purified coating reagents are more efficient for coating. The optimal coating concentration must be determined empirically for each system since, in general, there is a coating concentration above which non-specific binding of radiolabelled reagents increases much more intensively than specific binding.

After the coat has been deposited, the plate must be 'blocked' to prevent non-specific adsorption of reagents added subsequently. This is effectively accomplished by several washes with a concentrated solution of an unrelated protein—1% bovine serum albumin (BSA) or gelatin (RIA buffer). Usually no incubation other than the washes is required. (Plates are washed by filling the wells with RIA buffer and then dumping the contents into the sink. The last drops of fluid are removed by throwing the plates, face down, on to absorbent paper.)

Binding the radiolabelled reagent

There are numerous variations of RIAs adapted for specific purposes (see Table 34.1). The three basic assay systems are as follows.

1 Direct binding (one-step) assays in which labelled reagent is added to the coated well and allowed to incubate.

2 Indirect (two-step) assays in which unlabelled 'first-step' antibody (or antigen) is incubated in the well for a period of time and the well is then washed with RIA buffer to remove all unbound material. Radiolabelled reagent specific for the first-step reagent is then added and incubated to reveal the amount of the first-step material that has been bound.

Table 34.1. Adaptation of solid-phase radioimmune assays for different purposes

Use	Well coat	First step	Second step
One-step assays:			
Measure reactivity of antigen or antibody	Ag	^{125}I-Ab	—
	Ab	^{125}I-Ag	—
Measure antigen, isotype and allotype levels	Ag	Ag + ^{125}I-Ab	—
	Ab	Ag + ^{125}I-Ag	—
Two-step assays:			
Measure reactivity of antibody or antigen; measure affinity of antibody; determine isotype or allotype of antibody	Ag	Ab	^{125}I-anti-Ig
Measure reactivity of antibody or antigen	Ab	Ag	^{125}I-Ab (to Ag—must not react with well-coat Ab)

3 Blocking or competition assays in which a solution containing material potentially similar to the radiolabelled antigen (or antibody) is mixed with the radiolabelled reagent. This is incubated in the well and an estimate made of any reduction of binding of the radiolabel to the antibody (or antigen) coated on the well.

In all protocols, the final incubation results in binding the radiolabelled reagent either to the coat antigen or to the first-step reagent bound to the coat antigen. Excess radiolabelled reagent is removed from the wells after incubation and the wells are washed with RIA buffer to clear any unbound label. Following the final washes, the wells are cut from each other, dropped into carrier tubes, and counted in a gamma counter. Wells of the soft plastic microtitre plates can be cut (with scissors or a knife) or the top of the tray can be severed with a hot wire to release the wells (after adhesive plate covers have been stuck to the plate bottom to retain the severed wells).

Converting 'counts bound' to quantitative data

Since ^{125}I decays with a half-life of 60 days, data expressed as counts per minute (c.p.m.) bound cannot be compared directly for a series of experiments conducted over a long period of time. Furthermore, even when corrected for ^{125}I decay, data expressed as c.p.m. bound may be misleading since the concentration of reactant being measured is not necessarily directly proportional to c.p.m. bound over the entire usable range of the assay. Therefore we always include a standard curve with each assay and express data in terms of standard equivalents (mg or μl) per millilitre of test serum. In general, data for these standard curves are plotted on linear scales (c.p.m. bound vs. amount of antibody or antigen added) and the range of the assay is defined as just slightly greater than the initial linear portion of the curve (see Fig. 34.1).

Preparation of radiolabelled reagents

Any of the standard iodination methods are suitable as long as most of the labelled protein in the preparation is active in the specific binding reaction. The iodogen method [1] is excellent for most preparations. The solubility of iodogen in ether allows 'plating' aliquots of any amount on the walls of the reaction tubes which can then be stored indefinitely in a desiccator. Iodinations are terminated by simply removing the reaction solution from the solid iodogen—there is no reducing agent used.

Preparation of iodogen tubes

Materials

12 × 75 mm glass test tubes.
Iodogen (Pierce Chemical Co.).
Ether.
200 μl pipetman.

Procedure

1 Dissolve 1 mg iodogen in 1 ml ether.
2 Dilute to a final concentration of 40 μg/ml by adding 24 ml of ether.
3 Deliver 50 μl of solution (2 ng) to the bottom of each test tube and evaporate the chloroform thoroughly by applying a stream of nitrogen at room temperature.
4 Store tubes desiccated at 4 °C.

Iodination

Materials

Iodogen tube.
Airfuge, air-driven ultracentrifuge (Beckman Instruments).
Geiger counter.
PD-10 column: Sephadex G-25A (Pharmacia Fine Chemicals), pre-equilibrated by rinsing with 25 ml RIA buffer.
Pipetmen, 20 μl and 200 μl.
Gamma counter.
Protein (antigen or antibody) to be radiolabelled: 50–100 μg in PBS.
Cotton-plugged Pasteur pipettes.
One λ capillary pipettes (Microcaps, Drummond).

Buffers and solutions

Sodium ^{125}I in 0.1 M-NaOH, carrier free: 10 mCi/0.1 ml.
Phosphate-buffered saline (PBS), 0.01 M-phosphate, 0.15 M-NaCl, pH 7.2–7.4.
RIA buffer: 1% (w/v) BSA in PBS containing 0.02% (w/v) sodium azide, pH 7.1.
Caution: sodium azide is extremely toxic.
15% Trichloroacetic acid (TCA).

Procedure

1 Deaggregate the protein in airfuge (100 000 *g*, for 10 min).
2 Dilute protein to the appropriate concentration and volume—usually 50 μg in 200 μl in PBS.
3 Add 0.4 mCi (4 μl) of ^{125}I and mix well.
4 Transfer the mixture to iodogen tube (on ice) with a Pasteur pipette and let the reaction proceed for 10 min. Agitate the tube every minute since iodination occurs at the walls of the iodogen coated tube.
5 Add 800 μl PBS to the mixture. The exact volume is critical for the PD-10 column calibration.
6 Transfer the mixture (1 ml) into the pre-treated PD-10 column. Add 1.3 ml of RIA buffer. Collect the first 2.3 ml effluent into one tube (this should not contain any radioactivity—check with Geiger counter before disposal). Add 2.8 ml more RIA buffer and collect the 'flow through' that contains the labelled protein.
7 Determine the percentage of TCA precipitable protein: take 1 μl of labelled protein with a capillary pipette and mix with 150 μl RIA buffer, and count (Count A). Add 150 μl of 15% TCA and mix well. Centrifuge to separate the precipitate. Carefully take a part of (75 μl) supernatant, then count (Count B).

$$\%\ \text{TCA precipitable counts} = \left(1 - \frac{\text{Count B} \times 4}{\text{Count A}}\right) \times 100$$

Most of the labelled protein shows $100\text{–}200 \times 10^3$ c.p.m./λ by this method and TCA precipitable counts should exceed 90% for RIA assay.
8 Store at 4°C.

Solid-phase radioimmune assay methods

General equipment and materials

Airfuge.
Microfuge.
Gamma counter.
Hot wire cutter (D. Lee, Inc.).
Microtitre plates—'U'-bottom, flexible (Cooke Laboratory Products).
Twenty microlitre and 200 μl pipetmen.
Parafilm.
Absorbent paper for drying plates.
Apparatus for vacuum aspiration.

Solutions

Phosphate-buffered saline (PBS), pH 7.4 (see 'Iodination').
RIA buffer (see 'Iodination').

Direct binding RIA: one-step solid-phase assay

Example: specificity testing of ^{125}I-labelled monoclonal antibodies to human immunoglobulins.

To determine the specificity of anti-allotype monoclonal antibodies, the authors usually radiolabel the antibodies and test them for binding to wells coated individually with a panel of myeloma proteins of each class and allotype (e.g. G1m f, G1m za, G2m n+, G2m n−, etc.). In general, there is either high level binding (> 5 times background) or background level binding (equivalent to the amount of radiolabel bound to a BSA-coated well). Thus positives are easily distinguished from negatives.

Occasionally, the authors see some binding that is above background level but is not high enough to allow clear classification as a positive. This problem appears to be due to plate coat contamination with inappropriate Ig. That is, purified myeloma proteins are often contaminated with small amounts of other Ig; thus too high a plating concentration may coat wells with enough contaminating Ig to cause binding of antibody to the contaminant. Further purification of the plate coat protein will cure this problem; however, in many cases, reducing the coating concentration will eliminate detection of binding to the

contaminant without interfering with the detection of binding to the 'correct' myeloma protein by reagents specific for it.

Reagents and samples

Myeloma proteins—purified from sera (for methods, see Chapters 13 and 18).
^{125}I-labelled anti-allotype monoclonal antibodies.

Procedure

1 Deaggregate the myeloma protein by centrifuging for 15 min in a microfuge.
2 Dilute myeloma protein in PBS to subsaturating coating concentrations (10–50 μg/ml).
3 Add 40 μl of protein solution to each well. All tests are performed in duplicate. Include a negative control of RIA buffer alone.
4 Cover the plate with parafilm (optional) and incubate for 1 h at room temperature or overnight at 4 °C.
5 Wash wells three times with RIA buffer.
6 After dumping the third wash, completely empty the plate by throwing it hard, face down, on to absorbent paper.
7 Add 20 μl of ^{125}I-labelled reagent. Cover plate and incubate for 1 h at room temperature.
8 Wash wells three times with RIA buffer—aspirate the first two washes into a radioactive waste vessel and discard appropriately. Dry as above.
9 Cut wells apart with a hot wire or scissors and count in a gamma counter for 1 min.

Sandwich RIA: two-step solid-phase RIA

In a two-step assay, an antigen or an antibody is coated to the well, the test sample is added and allowed to bind, and a radiolabelled second-step reagent is used to reveal the amount of the test sample bound. This type of assay is used routinely for measuring antibody responses to antigens (coated on the well), for determining relative affinities of antibody responses to haptens, and for 'in-well' purification assays where an antibody coated to the well is used to isolate a component from a test sample containing extraneous material that would interfere with second-step binding.

Example: RIA typing for isoallotypic determinants on human immunoglobulins.

Polymorphic determinants (allotypes) on immunoglobulins are usually found on a single Ig isotype (subclass) and are present on that isotype in some but not all individuals of the species being studied. Isotypic Ig determinants, in contrast, are present on a particular isotype in all individuals of the species. Either of these types of determinants can be measured in serum samples by relatively direct methods (e.g. the competitive inhibition assay described below). Isoallotypic determinants, however, pose a different problem since these determinants (by definition) are isotypic on one isotype and allotypic on a second isotype. Therefore all individuals have the determinant in serum and its presence or absence as an allotypic marker can only be detected by specifically isolating the isotype on which the determinant is expressed allotypically.

The authors use the 'sandwich' modification (RISA) of the RIA assay for this purpose, i.e. 'in-well' purification of an isotype to permit typing for an isoallotypic determinant on human immunoglobulins. To isolate the pertinent isotype, they coat the well with a monoclonal antibody that detects an isotypic determinant carried by the isotype. They then add an aliquot of test sera and incubate to allow the desired isotype to bind selectively to the plate coat. Finally, the authors incubate with an ^{125}I-labelled anti-allotype monoclonal reagent to reveal the presence (or absence) of the allotypic determinant on the bound isotype.

Reagents and samples

Deaggregated sera samples (spun for 15 min in a microfuge).
Anti-isotype monoclonal antibodies for subclass(es) of interest for plate coating.
^{125}I-labelled anti-allotype monoclonal antibodies.
^{125}I-labelled anti-isotype monoclonal antibody (different from plate coat antibody but specific for the same isotype).

Procedure

1 Dilute the plate coat anti-isotype monoclonal antibodies in PBS to subsaturating concentrations—usually 5–50 μg/ml but the optimal concentration must be determined empirically for each batch of antibody. If the concentration is too high, significant backgrounds will result.
2 Add 40 μl of protein solution to each well. All tests are performed in duplicate. Include a negative control of RIA buffer alone.
3 Cover the plate with parafilm and incubate for 1 h at room temperature or overnight at 4 °C.
4 Wash wells three times with RIA buffer.
5 After dumping the third wash, empty the plate by throwing it, face down, on to absorbent paper.
6 Add 20 μl of the appropriate dilution of test sera (diluted in RIA buffer)—usually three twofold dilutions of sera are tested (after an initial dilution of 1:50–1:100). As before, the appropriate dilutions

must be determined for each test system by prior titration with standard sera which are then run with each assay as internal controls. Incubate for 1 h at room temperature.

7 Wash as above.

8 Add 20 μl of ^{125}I-labelled anti-allotype or anti-isotype reagent. Cover plate and incubate for 1 h at room temperature.

9 Wash three times with RIA buffer—aspirate the first two washes into a radioactive waste vessel and discard appropriately. Dry as above.

10 Cut wells apart with a hot wire or scissors and count in a gamma counter for 1 min.

Notes and recommendations

There is significant individual variation in levels of total Ig as well as in concentrations of individual subclasses. Ideally, one wants to compare levels of allotype expression between individuals at similar isotype concentrations. By performing each test at several dilutions of sera, one can analyse data from the dilution corresponding to a designated concentration of isotype. Isotype concentration is easily determined in sandwich assay simply by substituting ^{125}I-labelled anti-isotype monoclonal reagent for the labelled anti-allotype reagent in the final incubation. For this purpose, it is usually necessary to use as the labelled reagent an antibody specific for a different isotypic determinant on the desired subclass, i.e. one that does not cross-block when tested in competition RIA—this is to avoid problems of competition between plate coat and developing reagent for the same site.

Special considerations for highly reproducible quantitative typing. For optimal results the following suggestions are made:

(1) centrifuge microtitre plates for 5 min after adding plate coat to ensure thorough coverage of the well;

(2) incubate plates overnight at 4 °C on plate coating step;

(3) perform both isotype and allotype assays for a particular individual in the same microtitre plate, in order to minimize any 'between plate' variation;

(4) take care in measuring the initial volume of sera to ensure accuracy—use an initial 1:10 dilution followed by the serial dilutions;

(5) for any particular batch of radiolabelled reagent, determine initially the dilution yielding 20 000 counts per 20 μl and use the same dilution of reagent thereafter, adjusting for radioactive decay—for best results and reproducibility, it is recommended that a particular batch be used for only 1 month after iodination;

(6) perform all wash steps, especially the final one after incubation of radiolabelled reagent with ice-cold RIA buffer.

Two-step solid-phase RIA: measurement of the amount and affinity of anti-hapten antibodies

Two-step solid-phase RIAs are widely used to measure antibody levels in sera and culture supernatants. These highly sensitive assays (1–10 ng/ml of specific antibody) will detect antibodies to virtually any antigenic determinant that can be immobilized on a plastic well. Furthermore, they allow independent measurement of the amount of antibody of each isotype or allotype represented in an antibody response and permit rough approximation of the average affinity in each of these components in individual anti-hapten antisera [2]. The assay for antibodies to the dinitrophenyl (DNP) hapten discussed here illustrates these capabilities.

For this assay, test sera at appropriate dilutions are incubated in two sets of wells, one coated with DNP_{10}–BSA and the other coated with DNP_{40}–BSA. The plates are then washed and the bound antibody revealed with radiolabelled anti-Ig second-step antibodies. The amount of antibody in the test sample is then determined by comparison with the binding obtained with a standard antiserum (or purified monoclonal antibody) tested on DNP_{10}–BSA. The affinity is estimated from the ratio of the amounts of antibody bound to DNP_{10}–BSA and DNP_{40}–BSA at a given dilution of the test sample.

Fig. 34.1 shows a representative standard curve from an assay in which the bound antibody was revealed with a monoclonal anti-allotype (anti-Igh-4^a) second-step reagent. The amount of radiolabel bound is directly proportional to the amount of first-step (Igh-4^a anti-DNP) antibody added to the well in the initial (linear) portion of this curve. This region constitutes the most sensitive and accurate range provided by the assay, even though substantially more radiolabel is usually bound at higher first-step levels (in the plateau region of the curve). Therefore the authors try to assay all test samples at dilutions such that the amount of radiolabel bound falls below the point at which the curve begins to depart markedly from linearity, and they treat the amount of antibody added at that point as the largest amount of antibody that is measurable in the assay.

The shape of the standard curve, particularly in the plateau region, appears to be largely defined by the properties (amount, affinity and heterogeneity) of the second-step reagent, since different second-step reagents give different curves when used on the same first-step anti-DNP antibody titration. In general, monoclonal second-step reagents tend to extend the usable (linear) range of the assay and to produce

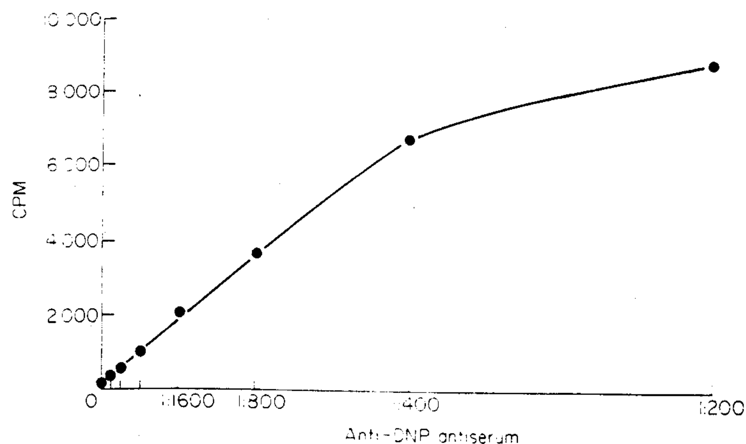

Fig. 34.1. Anti-DNP binding curve to DNP BSA-coated wells. Anti-DNP serum (from DNP-KLH immunized (SJL × Balb c)F_1 mice) were sequentially diluted and assayed for Igh-4^a (IgG_1) binding to the DNP_{10}–BSA-coated wells. Binding was revealed by incubation with radiolabelled anti-Igh-4^a monoclonal antibody (20–9.10).

higher plateau binding levels and lower background than conventional (affinity purified) reagents. This is not, however, a hard and fast rule.

The ratio of the amount of anti-hapten antibody bound to wells coated with high- and low-substituted hapten conjugates provides an empirical measure of the affinity of the antibodies in a test sample (Fig. 34.2). That is, low-affinity antibody binds better to highly substituted DNP-conjugates (DNP_{40}–BSA) while high-affinity antibody binds better to low-substituted conjugates (D_{10}–BSA); and, perhaps surprisingly, the D10/D40 binding ratios measured for a series of anti-DNP myeloma proteins and monoclonal antibodies are almost proportional to the log of the binding affinity constant (Ka) of the antibody [2]. Since this relationship holds throughout a 500-fold range in affinities and is valid for ratios between 0.3 and 1.4, it creates a reference curve that allows conversion of the D10/D40 ratio obtained for an anti-hapten antibody response to an approximation of the average affinity of the antibodies in that response.

Procedure

The following assay, using a combination of low- and high-substituted hapten conjugates, is essentially the same as a simple anti-hapten (or carrier) binding assay. However, it is crucial to keep incubation time constant (1 h) and to keep the concentration of radiolabelled second-step antibody (which should be cleared of aggregates by spinning before labelling—see 'Iodination', p. 34.3) constant to avoid variation in binding due to aggregation.

1 Preparation of low and high DNP-substituted BSA.

(i) Dissolve BSA (bovine serum albumin) in 0.5 M-$NaHCO_3$ at 10 mg/ml (500 mg/50 ml).

(ii) Add 100 μl of 1 M-DNFB (dinitrofluorobenzene, diluted in dioxane 1:8) (for low conjugate), or 1000 μl of 1 M-DNFB (for high conjugate) to 500 mg of BSA.

(iii) Rotate for 3 h at room temperature.

(iv) Spin out any precipitation that occurs.

(v) Extensively dialyse against 0.1 M-NH_4HCO_3 for

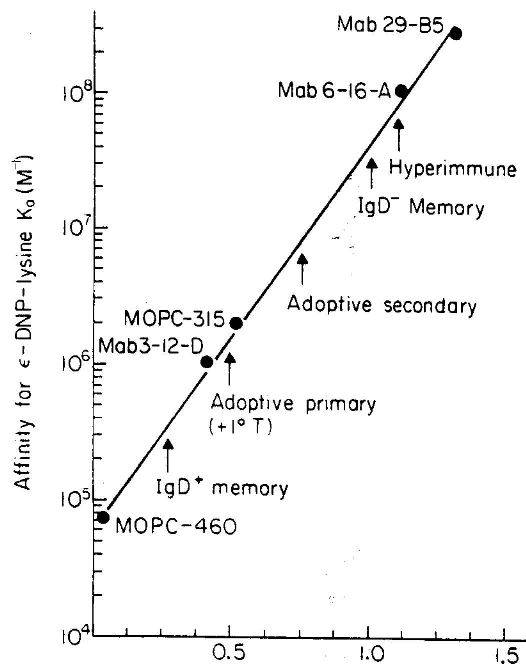

Fig. 34.2. Correlation of binding affinity constant (Ka) with the RIA binding DNP_{10}–BSA/DNP_{40}–BSA ratio. Each myeloma- or hybridoma-derived anti-DNP antibody was detected by the appropriate anti-isotype (or -allotype) radiolabelled antibody [2]. Ka values were determined by fluorescence quenching.

3 days in cold room (if the solution volume is small enough—around 2 ml); use a PD-10 column (p. 34.3) and dialyse overnight.

(vi) Lyophilize dialysed DNP-BSA completely.

(vii) Weigh lyophilized material, then resuspend in PBS to 5 mg/ml.

(viii) Estimate the molarity (mol) ratio:
$OD_{360}/1.74 \times 10^4$ = mol of DNP
5 (mg/ml)/6.8×10^4 = mol of BSA
Substitution of DNP to BSA = mol of DNP/mol of BSA.
DNP(5–10)–BSA and DNP(20–40)–BSA can be used as low and high conjugates respectively.

2 Coat wells with 50 μl of both kinds of (low and high) DNP–BSA at 50 μg/ml in PBS, incubate for 1 h at room temperature.

3 Wash wells with RIA buffer three times.

4 Add 20 μl of samples (appropriately diluted), incubate for 1 h at room temperature.

5 Wash wells as in step 3.

6 Add 20 μl of radiolabelled second-step antibody (around 2–3 ng/well; 1:100 dilution from stock antibody, see p. 34.4), incubate for 1 h at room temperature.

7 Wash wells as in step 3 (caution for radiolabelled waste, p. 34.3).

8 Count wells and calculate the amount of antibody using the binding curve for standard antibody (high affinity bearing antibody or antiserum) on DNP_{10} (low)–BSA. The amount is expressed by the maximum binding on either DNP(low)– or (high)–BSA. The ratio obtained from DNP(low)/DNP(high)–BSA binding typically covers the range of 0.3–0.4 (10^{-5} M Ka, primary response) through 1.0–1.4 (10^{-8} M Ka, hyperimmune).

Competitive inhibition (blocking) assay

Measurement of immunoglobulin levels

The quantification of immunoglobulin (Ig) levels can be performed by two kinds of blocking assays: competition between bound and soluble (sample) Ig for radiolabelled antibody, and competition between sample Ig and radiolabelled Ig for antibody bound to the plate. The latter method (competition between radiolabelled and test Ig) is discussed here since it tends to give results that are more reproducible.

In this assay, competitor and radiolabelled reagent are either pre-mixed or added in rapid sequence (competitor first) to antibody-coated wells. The wells are then incubated, washed and counted, and the amount of competitor (Ig) in the test sample is determined by comparison with the data obtained from a standard curve showing counts bound as a function of the amount of competitor (Ig) added.

Since this is a competition assay, there is a negative correlation between the amount of inhibitor added and the number of counts bound, i.e. the maximum number of counts are bound by wells that contain no competitor and the more competitor present the fewer the number of counts bound. Plotting the amount of competitor added against the reciprocal of the number of counts bound yields an index of competition that increases with increasing amounts of inhibitor. More important, however, plotting the data in this manner generally yields a straight-line standard curve that allows easier and more accurate interpolation of values than is possible with less regular forms.

There are straightforward reasons for expecting a linear relationship between the amount of inhibitor (unlabelled antigen) added to the well and the *reciprocal* of the amount of radiolabelled antigen bound to the antibody coated on the well. That is, in this assay, a constant number of antibody combining sites are put on the well and a constant amount of radiolabelled antigen, sufficient to saturate the available antibody combining sites, is placed in the well. Thus the amount of antigen bound is constant and the number of counts bound is proportional to the specific activity of the antigen.

Addition of unlabelled antigen to the well (pre-mixed with the radiolabelled antigen or added just prior to the labelled antigen) effectively reduces the specific activity of the antigen in the well. Therefore the number of counts bound decreases. This decrease reflects the dilution of the radiolabelled material with unlabelled material and is, therefore, inversely proportional to the amount of unlabelled material added.

Put into mathematical terms, if for each well
P = radioactivity bound
Ag^b = μg of antigen bound by the antibody used in coating the well
Ag = μg of labelled antigen used
Ag^* = number of counts/min (of Ag), and
μ = varying amount (in μg) of unlabelled (inhibitor) antigen added, then
Ag^*/Ag = specific radioactivity of the labelled antigen preparation, and
$Ag^*/(\mu + Ag)$ = specific radioactivity of total antigen in well when varying amounts of unlabelled antigen are added.
When no inhibitor is present,

$$P = \frac{Ag^b(Ag^*/Ag)}{Ag^*} = Ag^b/Ag =$$

maximum fraction of counts bound in the assay.

When varying amounts of inhibitor (μ) are present,

$$P = \frac{Ag^b(Ag^*/\mu + Ag)}{Ag^*} = Ag^b/(\mu + Ag)$$

and, taking the reciprocal,

$$1/P = \{(1/Ag^b)\mu\} + Ag/Ag^b$$

This is in the slope intercept form of the equation for a straight line when $1/P$ is plotted against μ. The inverse of the ordinate intercept of this line is equal to Ag^b/Ag, the maximum fraction of labelled antigen which is

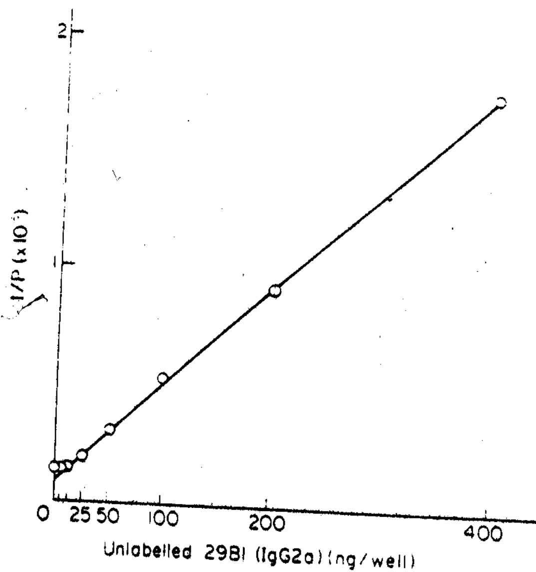

Fig. 34.3. Inhibition of binding of ^{125}I-labelled antigen by unlabelled antigen. P = radioactivity bound. Around 2 ng of labelled IgG_{2a} protein (from anti-DNP hybridoma, clone 29B1) and variable amounts of unlabelled homologous protein (as inhibitor) were incubated in each well (which had been coated with purified anti-IgG_{2a} antibody from the 20–8.3 hybridoma).

bound by the amount of antibody used. The slope, $1/Ag^b$, decreases as the absolute amount of antigen bound increases, hence as the amount of antibody used increases.

Data showing a typical reciprocal plot (linear) standard curve for an inhibition assay are presented in Fig. 34.3. Although the data in this figure is presented in terms of μg of inhibitor added, it is, of course, equally possible to use a standard unknown inhibitor concentration, e.g. serum, and express values for unknowns as μl of standard/μl unknown.

Procedure

1 Coat wells with 50 μl of antibody (10–50 μg/ml in PBS) at room temperature for 1 h.
2 Wash wells with RIA buffer three times.
3 Add samples (appropriately diluted in RIA buffer) 20 μl, and immediately add radiolabelled antigen in an equal volume (usually, 20×10^3 c.p.m./20 μl which contains around 2 ng of antigen; alternatively, add pre-mixed sample and radiolabelled antigen); then incubate for 1 h as above.
   Sequential dilution of known amounts of antigen (Ig) is used to give a standard inhibition curve in parallel with the sample assay.
4 Repeat step 2 and count wells.
5 Plot standard inhibition curve and calculate the amount of Ig in the sample.

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