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PROPERTIES, VARIANTS, AND APPLICATIONS OF THE IMMUNORADIOMETRIC ASSAY METHOD

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For many years, fluorescent and radioactive antibodies have been used to locate and measure solid-phase antigens, but only recently has the labelled antibody been applied to measurement of antigens in solution. Two closely related methods have been described. Both convert the unknown antigen into a radioactive product. In the first method (introduced by Miles and Hales in 1968) the unknown antigen is reacted with soluble purified radioactive antibodies. The radioactive complex remains in solution while unused radioactive antibodies are removed by a second reaction with a solid-phase antigen. The amount of radioactivity remaining in solution is a direct function of the antigen concentration. This method is known as an immunoradiometric assay (IRMA)^{36, 37}, and was the first labelled antibody procedure to be generally applicable to the assay of soluble antigens. The second method involves the preliminary insolubilization of unknown antigen and *then* the reaction with soluble labelled antibody. In this case, the labelled complex is insoluble and unreacted labelled antibody can be washed away. As the amount of unknown antigen increases, the radioactivity in the solid-phase increases. Various methods for insolubilization of the antigen have been used including reaction with solid-phase antibodies^{3, 5, 18, 19, 22, 35, 54, 55}. The latter method has been variously termed '2-site IRMA'^{3, 5, 22, 35}, 'junction test'^{18, 19} and 'sandwich technique'^{54, 55}. Use of techniques such as 2-site IRMA is restricted to antigens which can either a) bind simultaneously to at least 2 antibodies, or b) bind to a labelled antibody after some non-immunological insolubilization procedure. Both IRMA and 2-site IRMA have been adapted to the use of labelled anti-IgG as an additional 'universal' reagent, thereby avoiding the necessity for the preparation of radioactive antibodies specific for the unknown antigen^{35, 57}.

Immunoradiometric methods have been the subject of several recent reviews^{1, 4, 6, 20, 34, 40, 57} and the techniques have been applied to the assay of a great variety of antigens. These methods differ from conventional radioimmunoassay (RIA) in that the antigen to be measured is assayed directly by reaction with excess labelled antibodies, rather than by competition between the unknown antigen and a labelled antigen derivative for a limited amount of antibody.

Key-words: 'High-dose hook effect'; Immunoabsorbent; Immunoradiometric assay (IRMA); Purified antibody; Radioactive antibody; Serum effect; 2-site IRMA; Solid-phase antibody; 'Spacer arm'; 'Universal reagent'.

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Preparation of antigen immunoadsorbent

The immunoadsorbent matrix (ImAd) used for preparation of the labelled antibody reagent and for the IRMA assay procedure, has usually been the m-aminobenzoxy-methyl cellulose (ABMC) suspension described by GURVICH et al.¹⁷. This matrix forms a finely dispersed, milky, highly reactive suspension which is difficult to filter or incorporate into a column, and is best manipulated by centrifugation and resuspension. It has a high content of solid-phase antigen, is efficient in extracting specific antibodies from antisera, has low non-specific protein binding, and the matrix itself is relatively unreactive during iodination^{1, 17, 20, 39}. Furthermore, the coupling procedures mediated via the diazo reaction can bond the matrix to the protein at a variety of aminoacid residues, thereby presenting the antigen in a variety of spatial orientations for reaction with the antibody, and any single antigenic site is unlikely to be systematically excluded. Immunoadsorbent containing highly purified antigen should be used for the preparation of the labelled antibodies; but an impure, multispecific antigen-ImAd can be used during the immunoradiometric assay.

The antigen content of the immunoadsorbents has varied from 1.7 μ moles/g cellulose (human IgG) to 33 μ moles/g cellulose (C-peptide of proinsulin)⁵⁷. One immunoadsorbent contained 105-185 mg of insulin/g of cellulose, accounting for 20 % of the titratable diazo groups.

The antibody-binding capacity of this solid-phase antigen also varies greatly. With increasing size of antigen, the ratio of moles of antibody bound, to moles of antigen fixed to the matrix, increases¹. There are three possible reasons for this. 1) With large antigens several antibodies may attach to each antigen; 2) greater numbers of low molecular weight antigens may be fixed since they can penetrate the cellulose to react with relatively inaccessible diazo groups, however a large percentage of these antigens may then be in a location inaccessible to the large antibody molecule; 3) the covalent bond between the small antigen and the matrix may occupy or inactivate one of its few antigenic sites. For these reasons, immunoadsorbents made with proteins with molecular weights varying as much as from 2,000 to 500,000 tend to contain (per mg of matrix), 50-200 μ g of antigen and 100-1,000 μ g of antibody, but the molar ratio of antibody to antigen increases from 0.26 to 13.2 over that molecular weight range¹.

AG-ImAd has been successfully stored for months and years either at 4 °C or frozen, and it is washed before use to discard the small accumulation of solubilized antigen. AG-ImAd that has been used in IRMA reaction 2 can be washed in dilute acid to discard antibody, and re-used.

Several other immunoadsorbent matrices have been used^{1, 16, 18, 21, 57}. So far, none have been found superior to the Gurvich amino-cellulose; some were unsatisfactory because the ImAd was unreactive in suspension, and others because the matrix itself reacted with radioactive iodine¹⁶. For the assay of high molecular weight antigens, immunoadsorbents may not be essential for the preparation of purified antibody. This can be carried out by first isolating and then dissociating an immunoprecipitate²⁹. It is also possible to dispense with the use of immunoadsorbents in the separation step of the assay should the antibody-antigen complex have markedly different physical properties from the free antibody (as in 2-site IRMA and as in the assay of very large or insoluble antigens)²³.

Preparation of purified radioactive antibodies

Purified radioactive antibodies have usually been prepared by iodination with ¹²⁵I after isolation on the solid-phase antigen-immunoadsorbent (AG-ImAd)^{37, 39, 42}. This serves to protect one antigen-binding site from the iodination reaction³⁹ and enables soluble non-antibody protein and iodination reagents to be simply discarded. Recovery

of the labelled antibody from the iodinated AB-AG-ImAd complex is carried out by extraction with successively more acidic solutions so that easily dissociated antibody can also be discarded^{36, 42}. Studies using extraction with pH gradients show that the best antibody is usually recovered by 0.001 N to 0.01 N HCl⁴². The labelled antibody is titrated to pH 7-8 and stored frozen in carrier protein, or stored frozen after being reabsorbed onto a limiting amount of fresh AG-ImAd⁴. Satisfactory preparations have provided effective assay reagents for up to six months. In trace labelling experiments, activity of free antibody has been found to be unimpaired by the incorporation of up to 2 atoms of iodine/molecule⁴⁶. Even when iodinating the antibody-antigen complex, the activity of the antibody is impaired at greater than 10 atoms/molecule^{4, 34}, so that in successful labelled antibody reagents specific activities are usually estimated in the region of 5-20 $\mu\text{Ci}/\mu\text{g}$, at 0.5-2 atoms of iodine/molecule.

Under some circumstances, univalent antibody fragments may provide a superior reagent^{34, 43}. In that case, an identical iodination procedure follows a preliminary papain digestion of the antiserum IgG and isolation of the Fab fragment on Sephadex G100⁴³. Soluble antibody has also been iodinated *after* recovery from the AB-AG-ImAd complex or from a simple immune precipitate²⁹, and others have iodinated soluble IgG prepared from a hyperimmune serum without isolating the specific antibody protein⁴⁴.

The specificity of the antibody can be improved. If the pure antigen is not significantly contaminated with apparently cross-reacting antigen, then saturation of a limiting amount of antigen-ImAd with crude antiserum will leave unreacted any 'contaminating' antibodies and most of the truly cross-reacting antibodies of low affinity for the prime antigen. In the same way, saturation of the AG-ImAd in the presence of soluble cross-reacting antigens will effectively prevent cross-reacting or contaminating antibodies from being isolated and purified. Both of these maneuvers can be monitored by the presence of a trace amount of previously prepared ¹²⁵I-AB. Low affinity antibodies are also discarded by extraction with acetate buffer after iodination³⁶. High affinity antibodies can be purified more gently by being isolated and recovered on a AG-ImAd made with a low affinity analogue of the antigen although the yield may be reduced⁴².

A variety of investigations have been used to characterize the immunochemical properties of the labelled antibody including dissociation rate experiments, tests of reaction time, temperature, concentration, volume, mixing, 'second site' cross-linking of soluble antigen and AG-ImAd, and chromatographic or electrophoretic separation analysis^{34, 39}. However, a simple series of incubations is sufficient to demonstrate that the radioactivity is bound to protein; that the labelled antibody and its complex with antigen is soluble; the extent of non-specific binding of labelled antibody to the tube wall and immunoabsorbent matrix; and the specific, inhibitable, binding of labelled antibody to the specific AG-ImAd⁴². Deterioration is first seen as a decrease in binding to AG-ImAd at zero dose (IRMA), and as an increase in the 'blank' (zero dose) of the 2-site IRMA, followed by a flattening and attenuation of the standard curve.

The radioactive antibody can be re-purified to discard low affinity antibodies and non-antibody radioactivity by readsorption and recovery from an AG-ImAd^{33, 34}. As the antibody is already labelled, the concentration of the AG-ImAd used can be adjusted so that only that percentage of the radioactivity found to reside in high affinity antibodies (determined by inspection of the standard curve) is bound³³. Physico-chemical re-purification can be carried out by ultrafiltration or by passage through a Sephadex G100 column to discard low molecular weight breakdown products. Incubation with charcoal very effectively reduces the zero dose in 2-site IRMA but often seems to discard useful antibody despite the presence of substantial amounts of carrier IgG.

Fig. 1 - Scheme for immunoradiometric assay (IRMA).

Cross-reacting or contaminating radioactive antibodies can also be discarded after iodination by reacting the radioactive antibody preparation with AG-ImAd made with the cross-reacting or contaminating antigen. The amount of the AG-ImAd used should be limited to that necessary to remove from solution only that percentage of the radioactivity found (in the IRMA or 2-site IRMA assay) to reside in high affinity cross-reacting or contaminating antibodies ³³.

Non-radioactive antibody labels can also be used ^{8, 30, 34, 36}.

IRMA procedure

The IRMA scheme is shown in fig. 1. Optimally, the first reaction should reach equilibrium, whereas the second reaction should aim for severe disequilibrium (titration) conditions. The amount of ^{125}I -antibody used, is that which has been found to provide optimal precision over the desired range of antigen concentrations. Higher antigen concentrations can be measured by simply increasing the concentration of ^{125}I -AB ^{34, 36}. Increasing the concentration of AG-ImAd reduces both the zero-dose response and the optimal duration for the second reaction ^{39, 53}. High concentrations of AG-ImAd will be a disadvantage if substantial re-equilibration occurs in reaction 2. The reactions occur more rapidly at room temperature than 4°C ³⁹, but an antigen in serum is usually more stable at 4°C and the complex has greater free energy of binding ¹¹. Incubation periods for the first reaction depend on the sensitivity required and the kinetics of the particular antigen/antibody system, but have varied from 1 h to 5 days. Optimal duration for reaction 2 depends mostly on the rate of dissociation of the antigen/ ^{125}I -AB complex, the concentration of AG-ImAd, and the rate of association of the unused 'free' ^{125}I -AB with the excess AG-ImAd ^{4, 33, 34, 53}. Extending the second reaction time reduces the dose-response most at high antigen concentration (due to the more rapid re-equilibration of lower affinity antibodies), while increasing the percentage of the total radioactivity bound to the AG-ImAd in the absence of added antigen ³³. This 'exchange' of low affinity antibodies explains how prolonged second reactions can sometimes result in a more specific assay ^{33, 34}. The optimal second reaction time varies from 5 min to 60 min, tending to be brief for both smaller antigens (because of their more rapid dissociation rate) and lower affinity antisera ^{4, 6, 14}. As with other solid-phase assay systems, the dose-response curve may be inhibited to a variable degree by the presence of serum so that it is advisable to assay serum unknowns using standards diluted in antigen-free serum. Cooperativity between the two binding sites of the ^{125}I -AB, and possible binding of the ^{125}I -AB to both soluble antigen

and AG-ImAd, can be prevented by using univalent ^{125}I -AB produced by papain digestion⁴³.

In some cases alternative separation procedures may be adopted, making use of a specific property of the antigen-antibody complex. Thus, for example, in the assay of anti-hemophilic factor, the antibody bound to antigen is precipitated in 25 % saturated ammonium sulphate, whereas the unreacted antibody remains soluble²³. This approach should obviously be easier if the small univalent ^{125}I -AB fragment is used.

2-site IRMA procedure

The scheme for 2-site IRMA is illustrated in fig. 2, and a typical standard curve is shown in fig. 3.

The most popular solid-phase antibody preparation is the antibody-coated tube prepared after the method of CATT and TREGAR¹³. This solid-phase antibody does not appear to be significantly dislocated from the tube wall throughout the assay⁴², however the antigen-binding properties will deteriorate if the tubes are stored dry at room temperature, 4 °C, or frozen, resulting in erratic replicates and a reduced dose-response. The coated tubes are most stable stored frozen and containing buffer^{41, 42, 50}. The tubes can be coated using solutions containing a wide range of antibody concentrations with little effect on the dose-response curve. The lowest effective concentration of solid-phase antibody must be determined experimentally for each antiserum. The coating is less effective if concentrations greater than 1:1,000 are required^{10, 42} possibly because of interference by albumin²⁷. Under these circumstances, an improved solid-phase antibody may be prepared by using purified antibodies¹⁰, by using an alternative matrix^{18, 21, 55}, or by using an anti-(IgG) system as the primary solid-phase antibody^{35, 41}.

Experiments using ^{125}I -labelled growth hormone show that in reaction 2 the antigen can dissociate from the solid-phase antibody. This dissociation depends on the affinity of the solid-phase antibody and may be influenced by the presence of serum³⁵. The phenomenon is probably responsible for the 'high-dose hook effect', a paradoxical fall in tube radioactivity which occurs when the antigen is in excess and the amount of ^{125}I -AB is limiting (fig. 3).

The dose-response is usually increased and the reactions proceed more rapidly at 25 °C as compared to 4 °C, but with some antigens the serum effect may be increased

Fig. 2 - Scheme for 2-site immunoradiometric assay (2-site IRMA).

Fig. 3 - A standard dose-response curve for the 2-site immunoradiometric assay of human serum ferritin. Tube radioactivity is plotted *versus* log dose. Sample volume was 200 μ l. At each point on the standard curve, the mean and SD of 4 replicates are shown. Zero-dose radioactivity (848 cpm) is included within brackets. Total radioactivity added to each tube was 30,702 cpm. The computer-fitted values for zero and 'infinite' dose-response were 868 cpm and 17,542 cpm respectively. The minimal detectable dose for a duplicate determination was 0.055 ng/ml and this occurred when the tube radioactivity increased to 954 cpm. The figure also shows the 'high-dose hook effect' (see text).

at 25 °C resulting in an inhibition of the dose-response curve at 25 °C compared to 4 °C^{35, 42}.

The dose-response increases as either reaction time is extended, at the cost of an occasional minor increase in zero-dose radioactivity. When using convenient total radioactivity (20,000 cpm) and 125 I-AB of usual specific activity, the effect is more pronounced in reaction 2. Eventual total re-equilibration occurs only very slowly, and with second reaction times longer than 7 days, the first indication being an accentuation of the 'high-dose hook effect'.

Increasing the concentration of 125 I-AB increases the dose-response, extends the assay range, decreases the optimal duration of reaction 2, and tends to suppress the 'high-dose hook effect'; all at the cost of a variable increase in zero-dose radioactivity.

A non-specific inhibitory effect of serum appears to vary in severity according to the type of antigen being assayed (ferritin more severe than growth hormone), and the animal species of the serum (more severe in human and rabbit, minimal in guinea-pig and rat). For serum assays, it is therefore advisable to dilute the standards in an antigen-free serum of the same species as the unknowns. Normal rabbit serum diluted 1:20 was a suitable diluent when assaying human serum ferritin at a dilution of 1:20⁴². This effect is not mimicked by albumin and may be due to IgM or α_2 -macroglobulins⁵¹. The effect can sometimes be minimized by lengthening the spacer arm between the solid-phase antibody and the matrix^{35, 41}. I have not been able to confirm reports that this serum effect can be modified by the use of detergents or other reagents³¹, but it is likely that the effect can be avoided by extraction of the serum or immunological removal of high molecular weight serum components⁵¹. Washing of the solid-phase after reaction 1 ensures that the serum effect does not directly influence the uptake of 125 I-AB, but carry-over of as little as 10 μ l of human serum into the 200 μ l second reaction can significantly reduce the binding of 125 I-antibody to the solid-phase. Electrolytes, urea and other components can influence solid-phase assays.

The 2-site IRMA of Moore's S-100 protein⁴⁵ is dramatically effected by the concentration of calcium, being virtually obliterated in the presence of EDTA²⁶.

Repeatedly washing the solid-phase following reaction 1 causes a small reduction in the dose-response, while a marked and progressive decrease in the dose-response was observed with multiple washing after reaction 2^{35, 42}. Washing after reaction 1 may reduce the assay dose-response by causing loss of insolubilized antigen and it does minimize the 'high-dose hook effect' by ensuring that rapidly dissociating antigen and free antigen is discarded and therefore not available for forming soluble complexes with ¹²⁵I-antibody in reaction 2. Experiments with automatic washing devices showed that quite small variations in washing technique after reaction 2 make a major impact on the assay dose-response.

Variants of 2-site IRMA

Repeated extractions of unknown antigen. The assay can be made more sensitive if several successive aliquots of the unknown solution are incubated with the solid-phase. By this means, antigen is 'extracted' from a large aliquot of the unknown solution and concentrated into a very small volume on the solid-phase. Using this method, 10 pg/ml of human growth hormone has been detected⁵.

Specificity. In addition to manipulations of specificity described earlier, in 2-site IRMA the solid-phase antibody can be made specific for 1-site on an antigen, while the ¹²⁵I-AB can be directed against a second antigenically-distinct site on the same antigen. The assay will therefore recognize antigens with both sites but will not recognize antigens with either site alone. Using this technique it was possible to assay intact parathyroid hormone. Separate antibodies were directed specifically to the C-terminal (53-84) or N-terminal (1-29) peptides, yet neither peptide was recognized in the complete assay⁴⁸.

A non-specific antiserum or a mixture of antisera can be used in the solid-phase to insolubilize a variety of antigens. The assay specificity then relies upon the specificity of the ¹²⁵I-AB and the same solid-phase preparation can be used to assay several antigens. This technique has been used in the hepatitis-B assay to identify subtypes 'ay' and 'ad'²⁸ and is analogous to the use of a multispecific antigen-ImAd in IRMA³³.

Several of these techniques may prove to be useful in the assay of the two subunits of pituitary glycoprotein hormones⁵⁰.

Alternative solid-phase antibody preparations. Antibodies can be covalently or physically bound to a great variety of solid-phase matrices or surfaces including cellulose, Sephadex, agarose, polystyrene, polypropylene, polyethylene, teflon and glass. The preparation can also have a variety of configurations (e.g. suspensions, discs, tape, tubes, tubing, balls). The linking 'spacer arm' between the antibody and the matrix is often important. In one experiment, polystyrene tubes were coated with non-immune guinea-pig immunoglobulin (GP IgG) or rabbit anti-(GP IgG) [R-anti-(GP IgG)], and immunoglobulin 'arms' of various lengths were built up by alternate reactions with GP IgG and R-anti-(GP IgG) leaving a final coat of the latter. Guinea-pig antiserum to glial fibrillary acidic protein [GP-anti-(GFAP)] was then added and was immunologically bound into the solid-phase. This placed specific solid-phase antibody at various distances from the matrix, and was similar to the preparations described by DRIZLIKH and GURVICH¹⁵. Assays were then carried-out using ¹²⁵I-rabbit-anti-(GFAP) in reaction 2, because ¹²⁵I-guinea-pig-anti-(GFAP) might be bound by any unreacted R-anti-(GP IgG). In both serum and buffer, the slope of the standard curve became increasingly steeper and progressively displaced to a lower concentration range with increasing 'arm' length. The precision of the dose-response variable (radioactivity) was also improved. More relevantly, the serum effect was abolished with 'arm' lengths longer than two. These effects did not appear to be due to an increased concentration of

solid-phase antibody, since tubes prepared using anti-(GFAP) serum [trace-labelled with ^{125}I -anti-(GFAP)], show less antibody retained in the solid-phase of 5 'arm' tubes (2.5 % of the radioactivity) than 1 'arm' tube (10 %). In addition, increasing the concentration of the antiserum solution used for tube-coating did not alter the serum effect. The 'high-dose hook effect' seen in the buffer 4 'arm' and 5 'arm' systems was not present in the serum 4 'arm' and 5 'arm' systems, possibly due to inhibition by serum of the binding of antigen to low-affinity solid-phase antibody. Use of non-immunological 'spacer' arms may have similar effects ⁴⁷.

Other methods of insolubilizing the antigen. The unknown antigen can sometimes be insolubilized without using solid-phase antibodies. This usually is done by taking advantage of some special property of the antigen (e.g. the ability of TSH to bind to receptor proteins in thyroid nuclei ³⁰; the ability of IgE to bind to solid-phase allergen ⁵⁶) but direct covalent bonding or physical trapping of antigen in a matrix is also possible ⁸.

Use of ^{125}I -anti-(IgG) as 'universal reagent'. Two methods have been described for the use of ^{125}I -anti-(IgG) as a general assay reagent. The first was described by MILES et al. ³⁵ and applied to the 2-site IRMA of human ferritin. In this system the tubes were coated with guinea-pig anti-(ferritin) and in reaction 2 the tubes were reacted with *non-radioactive* rabbit anti-(ferritin). At the end of reaction 2 the tubes were washed and they then underwent a *third* reaction with ^{125}I -guinea-pig-anti-(rabbit IgG). The use of 2 species of anti-(ferritin) prevents the ^{125}I -anti-(IgG) being bound non-specifically into the solid-phase. The same reagents can be equally used in 'mirror image' (that is, sequences culminating with either rabbit *or* guinea-pig labelled-antibody as the final reagent). Exactly the same technique, and the same radioactive antibody should be able to be applied to the assay of any antigen for which there are 2 species of antisera. We have used a single ^{125}I -GP-anti-(R-IgG) to assay ferritin, GFAP, luteinizing hormone and parathyroid hormone. The reagent can also be used to label the residue AB-AG-ImAd complex in IRMA.

The second method was described by WOODHEAD et al. ⁵⁷. In this procedure, the ^{125}I -anti-(IgG) is used to label the specific antibody-AG-ImAd complex. The joint antibody reagent is then dissociated from the AG-ImAd by acid elution, allowed to recombine, and used as the labelled-antibody preparation in IRMA ⁵⁷.

The latter technique still requires purification of the specific antibody, however the first technique optimally requires an additional assay reaction. A systematic comparison of the two techniques is not available. Present experience indicates that the second method may be more suitable for IRMA and that the first technique may be more suitable for 2-site IRMA, especially when assay sequences are automated.

Data processing and assay optimization

Mathematical analyses of IRMA and 2-site IRMA have been published ^{52, 53, 58}, indicating how concentrations of ^{125}I -AB, AG-ImAd, and the second reaction time can be optimized. It appears that identical data processing methods can be applied to IRMA and 2-site IRMA. Usually, the dose-response curve approximates a sigmoid curve when the radioactivity in the supernatant (IRMA) or solid-phase (2-site IRMA) is plotted against the log of the dose of added antigen (fig. 3). It is then most suitably analyzed by weighted fitting of the 4 descriptive parameters (zero-dose response, infinite-dose response, slope, and midpoint of dose-response curve), and carrying out a linear transformation by logit/log plot ⁵². Infinite-dose is difficult to approximate experimentally and is most usefully fitted ^{35, 42}. Any dose-response showing the 'high-dose hook effect' is omitted. Not infrequently, however, the curve is not closely sigmoidal over the otherwise validly extensive range, and the computer program will only fit the experimental points satisfactorily by discarding the high-dose response

values and sometimes by 'fixing' a very high 'infinite-dose' response (e.g. 500,000 cpm). Standard curves of this shape may be more common when reaction 2 is abbreviated. At present, our assay data are read from punched paper tape into an IBM-360 computer and analyzed by a Fortran version of the 4 parameters, weighted, logistic, curve fitting program of ROBBARD and HUTT⁵², with modifications to allow the calculation of minimal detectable dose and the mean coefficient of variation of the unknowns over 5 selected dose ranges.

Assay evaluation

In evaluating any assay system one must assess sensitivity, precision, accuracy, range, economy, convenience (including possibilities for automation) and specificity. In many assays, stability and microheterogeneity of the specific antigen may also need to be carefully established.

Sensitivity. The minimal detectable dose (MDD) is usually expressed as that dose which provides a response two standard deviations away from the zero-dose response. It is easiest calculated from the standard curve but should be fully qualified, e.g. the MDD of human ferritin (molecular weight = 450,000) when assayed in duplicate in 200 μ l incubation volume, in normal rabbit serum diluted 1:100 with BSA buffer, was 15.7 ± 6.3 pg (established from 5 successive 2-site IRMA standard curves). In idealized theory, the MDD of IRMA has been calculated to be less than the MDD of RIA³³, but there has been no rigorous comparison using the same antisera and standard antigen. An additional increase in sensitivity is possible in 2-site IRMA because the antigen can be 'recycled' and 'extracted' into a smaller volume for reaction 2⁵. Note that specific activities (mCi/ μ g or mCi/ μ mole) are low in the labelled-antibody method compared with the specific activities commonly used in RIA.

Precision. In order to calculate between-assay variance, a single unknown solution is frozen away in many aliquots. One aliquot is thawed freshly for inclusion in each assay. The 'quality control' unknowns are often chosen to represent the high, low, and midpoints of the dose-response curve. For example, one quality control sample (human serum B) which was assayed in triplicate in 20 consecutive assays over a 2-month period, had a mean ferritin curve of 142.7 ng/ml, the standard deviation (SD) was 11 ng/ml and coefficient of variation (CV) was 7.7 %⁴².

Within-assay variance can also be calculated from the quality control data or, more suitably, as the mean CV of the actual unknowns at various dose ranges (outliers with greater than 50 % CV being noted but excluded). The variance throughout the 2-site IRMA standard curve is unlike that in radioimmunoassay, the CV of the response variable being nearly constant over a 500-fold range of antigen concentrations^{35, 52}. For example, using data from 4 successive HGH assays, the mean CV of 294 duplicate unknown sera in the range 0.4-4 ng/ml was 8 % whereas the mean CV of 312 duplicate unknown sera in the range 4-80 ng/ml was 6.8 % (no outliers excluded). From 5 successive ferritin assays the mean CV of 47 duplicate unknown sera in the range 100-200 ng/ml was 6.6 %; of 588 unknowns (200-400 ng/ml) was 5.14 %; and of 448 unknowns (400-800 ng/ml) was 5.6 % (4 outliers excluded).

Another type of within-assay variation can be assessed by assaying the quality control samples at the beginning and at the end of the unknowns, or by including a second dose-response curve after the unknowns. In large assays, this variance can equal or even exceed the between-assay variance. This demonstrates that highly-standardized and automated techniques are important.

Accuracy. The word 'accuracy' is often used to categorize the results of recovery experiments. In recovery experiments, an unknown is assayed before and after the addition of an increment of known standard antigen.

Fig. 4 - Serum growth hormone in a traumatically-blinded subject (HTD). Continuous blood sampling with 2-site immunoradiometric assay of alternate 6-min aliquots throughout nocturnal sleep and the oral administration of L-Dopa. Mean and duplicate values are shown at each point. Serum samples were all assayed at the same dilution and results extended over a 150-fold range (0.1-15 ng/ml), with mean coefficient of variations (CV) of 4.3 % (one outlier discarded). The CV showed no trend over this dose range.

Good 'recovery' of the added antigen depends on the identical nature of the standard and unknown antigens, the absence of non-specific interference, and the assay precision. Excellent 'recovery' has been reported in the serum ferritin 2-site IRMA⁴².

Assay range. This indicates the range of antigen concentrations over which a single dose-response curve provides satisfactory precision (e.g. CV of less than 15 %). Because of the low zero-dose response, the range is unusually extensive in 2-site IRMA, enabling the measurement of 100-500-fold fluctuations in serum HGH (fig. 4).

Economy. IRMA is relatively uneconomical with AG and antisera whereas 2-site IRMA uses AG only for the preparation of labelled antibodies⁴. ¹²⁵I-anti-(IgG) (universal reagent) systems are very economical in specific antibody and require only 1, easily obtained, radioactive reagent for the assay of a variety of antigens^{35, 57}.

Convenience. As IRMA and 2-site IRMA are disequilibrium assays they do require at least 2 incubations, however the use of solid-phase reactants and the sensitivity to washing (in 2-site IRMA) make these systems ideally suited for automation. Up to 600 tubes have been processed manually/day.

Specificity. The ability to produce specific assays is one of the great advantages of IRMA and 2-site IRMA. Several possible techniques have already been described. The recent recognition of the relevance of immunochemical heterogeneity of polypeptide hormones has complicated the interpretation of assay data⁵⁹. An antibody might regard all sub-populations of a heterogeneous antigen as immunologically identical, and the standard antigen might be different or differently heterogeneous to the unknown. The best check as to the validity of relative changes in the concentration

of the unknown is to assay a single serum sample at several dilutions (using a hormone-free serum as diluent). If the estimated antigen content is systematically inappropriate to the dilution then either the standard and unknown antigens are dissimilar, the unknown contains a cross-reacting or contaminating antigen, or a non-specific 'serum' effect is present. Changes in the unknown antigen with storage may also require study. This may be identified as a change in the potency of quality control sera, and should be distinguished from a deterioration in the stock source of standard antigen. Fragile antigen may be protected from some forms of serum damage by being rapidly bound to the excess antibody. In 2-site IRMA, non-specific serum components are removed and cannot perturb the binding of ^{125}I -AB in reaction 2. The two-site assay may be effected by other means of linking the solid-phase and free antibodies. This problem is likely to occur in unknown serum containing antibodies to gammaglobulin. This artifact has already been observed in the hepatitis-B assay⁴⁹, and can be dealt with by adding excess non-immune gammaglobulin of the same species as the labelled antibody to the second incubation, by using antibody from 2 species, or by using labelled antibody obtained from the same species as the unknown antigen⁹.

Comparison of IRMA, 2-site IRMA and RIA

Comparison of the features of IRMA and 2-site IRMA variants, and conventional radioimmunoassay are found in the original publication of the IRMA method³⁶ and in subsequent reviews^{1, 4, 6, 20, 34, 40, 57}. The assay of small haptens and large molecular weight antigens involve different considerations, but no systematic comparison has been carried out using a single antiserum and the same standard antigen.

Assay applications

Antigens assayed by IRMA include angiotensin²⁵, calcitonin¹⁴, ACTH⁶, insulin³⁷, parathyroid hormone⁷, HGH³⁸, FSH³³, LH^{21, 33}, tetanus toxin¹⁸, IgG⁴, ferritin², and anti-hemophilic factor²³.

Antigens assayed by 2-site IRMA include parathyroid hormone⁴⁸, nerve growth factor²², HGH^{3, 5, 35}, LH²¹, FSH⁵⁰, HCG^{54, 55}, ovalbumin, tetanus toxin and albumin^{18, 19}, IgE⁵⁶, hepatitis-B antigen²⁹, TSH³⁰, X-antigen⁴⁴, ferritin^{35, 42}, GFAP^{35, 41}, S-100^{26, 41}, α -fetoprotein²⁴, and anti-thymocyte globulin¹².

SUMMARY

The immunoradiometric assay (IRMA) and 2-site IRMA techniques employ purified radioactive antibodies to convert soluble antigens into a directly detectable complex. Unreacted labelled antibody is discarded by reaction with solid-phase antigen (IRMA), or by a preliminary insolubilization of the unknown antigen using solid-phase antibody (2-site IRMA). The preparation and properties of antigen-immunoabsorbent, solid-phase antibody, and purified radioactive antibodies are discussed. Analysis of the dose-response curve and problems in dose-interpolation are similar in IRMA and 2-site IRMA, and different from radioimmunoassay and other competitive-binding assay systems. The most significant assay variables include: exchange of radioactive antibody in IRMA reaction 2, stability and reactivity of the solid-phase antibody, washing the solid-phase, non-specific interference by serum proteins, and a paradoxical fall in tube radioactivity at high dose (2-site IRMA) (the 'high-dose hook effect'). 2-site IRMA has advantages in specificity (by using separate antibodies directed to 2 different antigen sites), sensitivity and assay range (because of the low zero dose-response). Assay variants include: alternative antibody labels, various solid-phase antibody preparations (including immunological 'spacer-arms'), non-immunological insolubilization of antigen, univalent radioactive antibodies, repeated immunological extractions of unknown antigen, and non-immunological separations in IRMA reaction 2. Both IRMA and 2-site IRMA have been adapted to the use of labelled anti-IgG as an additional 'universal' reagent, thereby avoiding the necessity for the preparation of radioactive antibodies specific for the unknown antigen. These techniques have been applied to the assay of a great variety of antigens and will be especially appropriate for automation.

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