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RADIOIMMUNOASSAY

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Immunoassays utilize the binding specificity of an antibody for its specific antigen to measure either the antigen or antibody (1). To aid in following the reaction, either the antigen or the antibody is labeled. In theory any suitable label permitting sensitive measurements may be used but as a rule the label is a radioactive isotope, and the term radioimmunoassay then becomes applicable. In most radioimmunoassays it is the antigen that is labeled. The measurement depends on the ability of the unlabeled antigen (Ag) (the unknown) to inhibit binding of the radioactive antigen (Ag*) by antibody (Ab). To maximize sensitivity the assay is carried out in the presence of only enough Ab to achieve substantial Ag* binding. As a rule dilutions of Ab are selected that give about 50% binding of Ag* when unlabeled Ag is absent. The process is a simple competition in which Ag occupies a portion of the antibody-combining sites reducing the free Ab available to Ag*:

$$\text{Ag.Ab} \leftrightarrow \text{Ag} + \text{Ab} + \text{Ag}^* \leftrightarrow \text{Ag}^*.\text{Ab}.$$

In performing the assay, fixed concentrations of Ab and Ag* are incubated in the absence and presence of the unknown samples containing Ag. Once adequate Ag* binding has been established, free and antibody-bound Ag* are separated, and one or the other is measured by radioactive counting. The antigen concentration in an unknown sample is determined by comparing the decrease of Ag* binding it produces with a standard curve (Figure 1) obtained by adding known quantities of Ag to the assay system. The standard must be the same as the unknown, but the iodinated antigen and unlabeled antigen need not be identical.

Radioimmunoassays have provided a sensitive, precise, convenient, and

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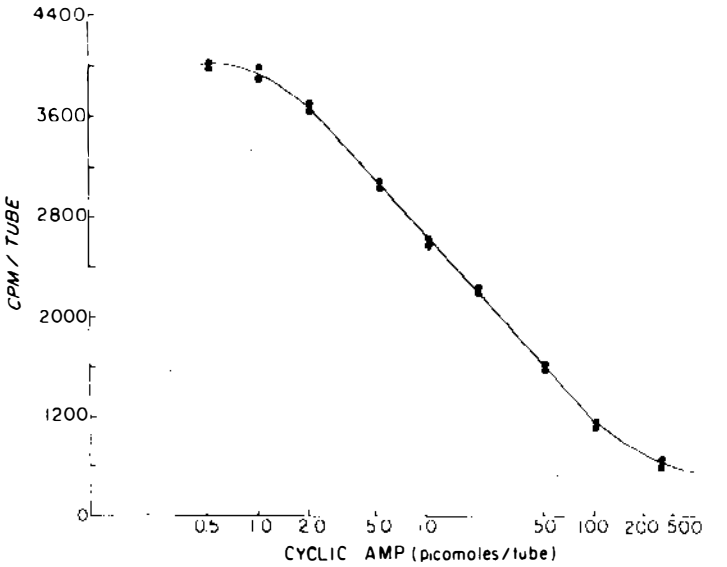

Figure 1 Typical standard immunoassay curve for cyclic AMP. Each tube contained 0.1 pmole ^{125}I-succinyl-cyclic AMP-tyrosine methyl ester and a final anticyclic AMP-antibody dilution of 1:2000. [From ref. (9).]

broadly applicable approach to the measurement of molecules of biologic interest. In sensitive assay systems measurements of femtomole quantities of antigen may be possible. The potential value of radioimmunoassays for analytical purposes was first pointed out by Berson & Yalow in their studies with insulin (2). Radioimmunoassays were soon developed for growth hormone (3) and a variety of other polypeptide and protein hormones. My colleagues and I extended the radioimmunoassay concept to low molecular weight drugs and metabolites that must be chemically coupled to proteins in order to produce antibodies (reviewed in 1) and pointed out the remarkable degree of specificity that was possible even with relatively small determinants. Assays were developed for digitalis (with G. C. Oliver and B. Parker) (4–6), morphine (with Sidney S. Spector) (7), cyclic AMP and cyclic GMP (with A. Steiner, R. Utiger and D. Kipnis) (8, 9), and prostaglandins (with J. Smith and B. Jaffe) (10, 11). A representative standard inhibition curve showing the degree of discrimination possible in the cyclic AMP system is shown in Figure 2. As with the polypeptide and protein hormones, immunoassay applications involving drugs and other low molecular weight substances soon became extensive. The subject of immunoassay as applied to drugs was last reviewed in these volumes by Spector in 1973 (12). A detailed monograph entitled *Radioimmunoassay of Biologically Active Compounds* by Parker was published in 1976 (1). Since

that time, the number of applications of immunoassays has continued to increase rapidly. Rather than review these recent applications individually, this review emphasizes certain of the principles that are particularly important in the performance of immunoassays. In addition, the important technique recently introduced by Milstein and his colleagues for generating monoclonal antibodies is discussed in some detail.

IMMUNIZATION AND IMMUNOLOGIC RECOGNITION

Considerations in regard to antigen purity, selection of a procedure for conjugating a hapten to a protein, mode of immunization, and choice of an animal species for immunization have been reviewed in detail previously as has the physicochemical basis for antibody recognition, the thermodynamics of antigen-antibody reactions, and antibody heterogeneity (1).

INDICATOR MOLECULES

The importance of the specific activity of the indicator molecule in developing a highly sensitive assay has been discussed in detail in the past (1) but deserves continuing emphasis. Among the commonly available radioactive isotopes for labeling proteins the half-lives range from 8 days (^{131}I) and 56

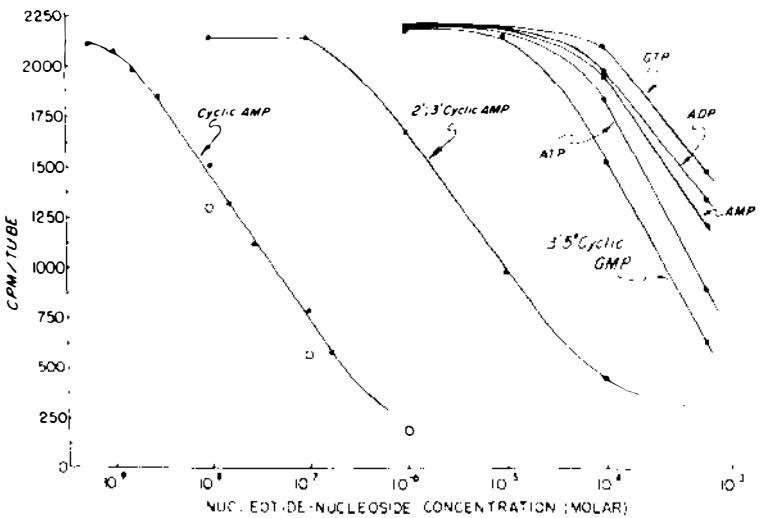

Figure 2 The inhibition of ^{125}I-succinyl-cyclic AMP-tyrosine methyl ester binding to cyclic AMP antibody by various nucleotides. o is 2'-deoxy 3'5'-cyclic AMP. [From ref. (9).]

days (^{125}I) to 5730 years (^{14}C) and 12.3 years (^{3}H). These differences in radioactive decay rates largely explain the great popularity of ^{125}I and ^{131}I as radioindicator molecules for proteins. If the indicator molecule does not have a high specific activity, substantial concentrations of Ag* (and therefore Ab) are needed to obtain statistically adequate counts within an acceptable time period, and the sensitivity of the assay is limited. The introduction of a single atom of ^{131}I into a molecule of bovine insulin provides approximately 1000 times the number of radioactive disintegrations per unit time as would be provided by an insulin molecule with all 263 carbon atoms labeled with ^{14}C (13). ^{125}I and ^{131}I have other advantages, including the ease of their introduction into proteins and the applicability of well-type γ scintillation spectrometers for counting, which avoids the use of scintillation fluid and special counting vials. Based on specific activity considerations alone ^{131}I is theoretically preferable to ^{125}I. However, when other factors are considered such as the rapid loss of ^{131}I radioactivity, differences in counting efficiency, relative isotope abundance, and external radioation hazards, ^{125}I is almost always preferable to ^{131}I.

A variety of methods are available for labeling low molecular weight haptens for radioimmunoassays (1). As with proteins iodine is often the preferred radioindicator molecule. With some haptens the iodination is easily accomplished by introducing a residue that is easily iodinated such as a phenolic hydroxyl group into the immunologically noncritical area of the hapten molecule. The same methods are then used for iodination that are successful with proteins. This approach was used to label the aglycone of digitoxin and digoxin (4–6) which were succinylated at the 3 position (where the glycone is normally attached), coupled with tyrosine methyl ester and iodinated. By using these markers the first practical immunoassays for a drug present in low concentrations in body fluids were developed (4, 5). A similar procedure was used later in the development of sensitive assays for cyclic GMP, cyclic AMP, and testosterone (8, 9, 14). With some haptens the iodophenol moiety may create unfavorable nonspecific binding problems or interference with normal antigenic reactivity, and the use of a ^{3}H or even a ^{14}C label may be preferable. However, with the use of increased quantities of albumin to decrease absorption to glassware, the nonspecific binding problem can usually be controlled. Lipids, polysaccharides, and nucleic acids can also be labeled exogenously with radioiodine or other isotopes although more frequently they are labeled by biosynthesis in the presence of radiolabeled precursors (1). This is an area where further investigation is desirable.

When the antigen to be iodinated is not unduly subject to inactivation by the iodination reagents, the time-honored chloramine T method first described by Hunter & Greenwood in 1962 is often satisfactory (15). Chlora-

mine T introduces iodine onto tyrosyl and to a lesser extent other amino acid residues, probably through a cationic iodine intermediate. The advantages of the chloramine T method include its reproducibility, rapidity, efficiency of the iodination in the absence of carrier nonradioactive iodine, and avoidance of extremes of pH or organic solvents which might denature the protein. However, some proteins undergo substantial denaturation during iodination with chloromine T. Although a careful adjustment of the iodination conditions may minimize the iodination damage, it may be preferable to iodinate with lactoperoxidase (16) or use a two-stage iodination procedure in which the iodine is introduced into the protein through a low molecular weight carrier such as 3-(*p*-hydroxyphenyl)propionic acid-*N*-hydroxysuccinimide (HPPS) (TAGGIT®, Pierce Chemical Co.). Lactoperoxidase is a 90,000 molecular weight protein with high peroxidizing activity obtained from unpasteurized bovine milk and is available commercially from several biochemical suppliers. In the presence of hydrogen peroxide and inorganic iodide it promotes the iodination of proteins, particularly at tyrosyl residues that are easily accessible sterically. Since the iodinating species is generated in the active site of the enzyme which is in turn bound to the protein being iodinated, oxidation damage to the protein as a whole is minimized. Comparative studies have indicated that the lactoperoxidase method is as satisfactory as and sometimes preferable to the chloramine T method for labeling of commonly measured hormones (glucagon, insulin, growth hormone, LH, TSH, and FSH) (17, 18), and certainly there are some proteins for which only the lactoperoxidase method is satisfactory. However, the lactoperoxidase method sometimes fails to provide effective iodination, presumably because the only available tyrosyl residues are inaccessible to the enzyme.

In the two-stage labeling method a low molecular weight carrier is iodinated in the absence of the protein and then combined with the protein via a stable bond. The standard procedure utilizes HPPS which was introduced as an iodine carrier by Bolton & Hunter in 1973 (19). This reagent has both an easily iodinatable phenolic hydroxyl group and an active ester group through which it can be attached to nucleophilic functional groups on proteins, particularly ϵ-ammonium groups of lysyl residues. By comparison with the other two commonly used iodination methods, the major drawback to the Bolton and Hunter procedure is the need for relatively large amounts of radioactive iodine and the somewhat lower specific activities of the final iodinated product. On the other hand, there are proteins that can be labeled with the indirect method that cannot be labeled directly. For example, HPPS has been recently used to label isozymes of creatine phosphokinase in the development of an immunoassay for the form of the enzyme present in cardiac tissue (20, 21). This procedure was chosen be-

cause the direct iodination of these proteins destroys their antigenic reactivity, presumably because essential sulfhydryl groups are oxidized. This assay system has permitted the more rapid and specific diagnosis of acute myocardial infarction. The indirect procedure can also be used to iodinate polypeptides, proteins, and low molecular weight chemicals that have no tyrosine but do contain amino groups available for conjugation. Other carriers such as histamine may be used instead of HPPS to attach the radioiodine molecule to the ligand (22).

A variety of other convenient iodination procedures are available. For example, 1,3,4,6-tetrachloro-3a-6a-diphenylglycoluril (Iodo-Gen®, Pierce Chemical Co.) can be used to transfer radioiodine to proteins (23). It is insoluble in water and can be prepared as a thin film in the iodination vessel. When an aqueous solution of $^{125}I^-$ and protein is added, iodination is rapidly initiated. The iodination reaction can be terminated immediately by decantation.

Regardless of the iodination procedure, it is important that protein antigens not be labeled too heavily. High levels of iodination, although theoretically desirable for assay sensitivity, often result in antigen denaturation, decreasing immune reactivity and stability and increasing the variability in the immunoassay (1). Even if a protein can be labeled more heavily, antibody affinity will often place a practical limit on assay sensitivity in any case. As a practical rule of thumb, in the direct iodination methods, it is ordinarily satisfactory to achieve an average substitution of a little less than one atom of iodine per molecule of antigen. This normally will ensure that most of the antigen molecules are iodinated (1). However, each antigen-antibody system is something of a law unto itself, and suitable levels of iodination have to be determined empirically.

Once the iodination reaction is completed the labeled antigen is rapidly purified to remove unconjugated radioactivity (and in some systems damaged antigen), usually on a short molecular sieve or ion exchange column. Each new labeled antigen preparation must be evaluated both initially and in subsequent assays for its suitability as a radioligand in the immunoassay, as indicated both by its overall reactivity with antibody and susceptibility to inhibition by standard amounts of antigen. In some antigen systems repurification of Ag* may be necessary on a weekly or even daily basis. Even with iodinated antigens which are normally stable for many weeks, individual preparations may be obtained which deteriorate much faster. Antibody selection may be very important here because antibodies to the same antigen may differ markedly in their ability to recognize the iodinated antigen, particularly if the iodinated antigen is readily denatured.

Instead of labeling the antigen the label may be introduced onto the antibody (1). There are now many immunoassays in which labeled antibod-

ies are used. This may be particularly desirable when the antigen is unusually labile to iodination. Fluorescent, electron spin resonance, and enzymatic labels also have broad applicability to immunologic measurements, although some of these methods suffer from lack of precision or sensitivity. Enzyme immunoassay measurements that utilize fluorogenic substrates appear to be potentially equal in their sensitivity to radioimmunoassay measurements, but as ordinarily performed their sensitivity is not as high. Fluorescent immunoassays may be of particular value if radioactivity-counting equipment is not readily available, but at their present stage of development they do not appear to offer clear-cut advantages in sensitivity or reproducibility, and the preparation of a suitable enzyme conjugate may present problems due to denaturation or steric hindrance to the antigen-antibody reaction. Nonetheless, because the use of a radioactive marker is avoided such systems are likely to see increasingly widespread use. A microbiologic immunoassay in which an antibody affects the attachment, replication, or metabolism of a microorganism conjugated to the antigen is normally too tedious or imprecise to warrant their use for routine immunoassay purposes. However, phage immunoassays are potentially more sensitive than radioimmunoassays (24) and may find a limited application.

KINETIC AND THERMODYNAMIC CONSIDERATIONS

The sensitivity obtainable in an immunoassay depends not only on the specific activity of the marker but also on antigen valence, the association constant for the antigen-antibody reaction (K_a), and the precise conditions of the assay (reviewed in 1). The stability of antigen-antibody complexes is increased if the antigen as well as the antibody is multivalent, permitting cross linking and the formation of lattices. In this case, the overall antigen-antibody reaction is best described by an overall avidity constant (K_{av}) rather than the association constant of the antibody-combining sites for their individual antigenic determinants (1). The effect of antigen valence on complex formation probably is the major explanation for the generally greater sensitivity of immunoassays for proteins and polypeptides than for low molecular weight (haptenic) antigens. As a rough rule of thumb, the practical sensitivity of an assay is equal to $1/K_a$ or $1/K_{av}$. K_a's for antibody hapten interactions usually range between 10^6 to 10^{10} per mole whereas K_{av}'s for antibody protein interactions may be as high as 10^{12} to 10^{13}. Coulombic, hydrophobic, and hydrogen binding interactions all appear to contribute to the binding of an antibody to its specific antigen. Small, relatively hydrophilic haptens tend to have a lower affinity for their specific

antibodies than larger or more hydrophobic haptens, placing a practical limit on the sensitivity of the immunoassay. In haptenic systems structural differences between the marker, the unlabeled hapten under study, and the form of the hapten on the original immunizing protein can very significantly affect assay sensitivity. The antibodies, of course, best recognize the hapten as it appears on the immunizing protein. Under favorable circumstances the unknown antigen on biologic samples can be chemically altered in situ to increase its structural resemblance to the actual immunogen. This approach was used by Cailla et al (25) to increase the sensitivity of immunoassays for cyclic AMP and cyclic GMP. The cyclic nucleotides in unknown tissue samples were acetylated at the 2'0 position, increasing their resemblance to the 2'0-succinyl cyclic AMP used in preparing the immunizing conjugate. This resulted in an improvement of several orders of magnitude in the practical sensitivity of the immunoassay.

When assay sensitivity needs to be maximized, empirical screening may be desirable to determine the usefulness of an initial incubation of the nonradioactive antigen with antibody before adding Ag*. While some assays are not appreciably improved in regard to their sensitivity by the delayed addition of radioactive antigen, others are quite substantially affected (26).

SEPARATION SYSTEMS

Methods for separation of free and bound Ag* are many, and include the use of a second antibody, salt precipitation, absorption onto charcoal or another nonspecific absorbent, or two-phase systems in which the antigen or the antibody is attached to a solid phase. One very recent approach makes use of the binding affinity of staphylococcal protein A for immunoglobulin molecules. In one such application killed but otherwise intact staphylococci from strains that produce the binding protein are used as solid-phase absorbents (27). With certain exceptions, each of the above procedures is applicable to a large number of different antigen-antibody systems. A variety of factors enter into the selection of a system, including the rapidity and sensitivity required in the immunoassay, unusual properties of the antigen, the affinity and specificity of available antisera, the skill of the technical personnel, and the usual load of samples to be processed. Often the separation method that is chosen is based as much on the preference or the previous experience of the individual investigator as the peculiarities of the particular antigen-antibody system being studied. Nonetheless, an investigator who is setting up an immunoassay for the first

time in his own laboratory should carefully review any published results in the same antigen-antibody system, both for possible useful technical details and any concrete evidence that one separation system is preferable to another. Double antibody immunoprecipitation (3) is probably the most broadly applicable of the separation systems. Double antibody systems have suffered from the disadvantage of the relatively prolonged incubation times needed to maximize the precipitation reaction, but recently it has been shown that the use of polyethylene glycol may permit the precipitation to be completed within 5 to 60 min (28, 29). In general, absorption assays involving charcoal provide rapid measurements but are sensitive to the protein content of the medium, and, depending on the number of samples processed, may be unusually subject to intraassay variation. Salt precipitation with ammonium sulfate is applicable only to radioiodinated antigens that are soluble in 40 to 50% ammonium sulfate. Solid phase immunoassays appear to be particularly sensitive to the exact nature of the complexes formed between antigen and antibody. Although a number of solid phase immunoassay systems have been shown to give highly satisfactory results, others are less practical because of delayed equilibration times, problems with reproducibility, or high nonspecific binding blanks.

Once the separation of bound and free antigen has been initiated, nonequilibrium conditions are established, and depending on the conditions of washing and the particular antigen-antibody system, significant amounts of previously complexed antigen may dissociate. This is not necessarily a major disadvantage since relatively low affinity interactions involving cross-reacting antigens may be particularly subject to reversal. Some investigators have proposed the use of nonequilibrium assays in which the ability of unknown antigen to displace radiolabeled antigen from preformed antigen-antibody complexes is measured (30). While such nonequilibrium assays are potentially useful because of their simplicity and decreased dependence on sample volume, they also may be subject to seemingly minor variations in assay time and temperature, and special care may be needed to minimize intraassay variation. Regardless of the procedure used, separation of free and bound antigens should be performed as rapidly and reproducibly as possible, and for this reason it may be unwise to process too many samples simultaneously.

In general, complete automation is more easily achieved with fluorometric enzymatic immunoassay techniques than with radioimmunoassay (31). Although the complete automation of radioimmunoassays has recently been described (32), at this time the procedure is relatively complex and expensive, making it practical only in laboratories performing large numbers of assays.

VALIDATION OF THE IMMUNOASSAY

Regardless of the immunoassay system used, the specificity and sensitivity of the system and adequacy of recovery of the unknown substance from biologic samples must be fully evaluated, and rigorous controls are needed. It cannot be assumed that immunoassays that give sensitive and reproducible results in buffer will necessarily give valid results in biological samples (1). Tissue and blood samples, for example, may contain interfering molecules that either degrade the antigen, decrease antigen binding nonspecifically, exhibit unexpected immunologic cross reactivity, quench β radiation (in pigment-containing samples), or interfere with the separation of bound and free Ag*. Antigenic reactivity may be lost either before or during the immunoassay. In some systems insoluble debris in the tissue sample may be detrimental. There may be effects on the assay due to alterations in pH or the presence of salts that affect antigen-antibody binding. Susceptibility to these influences may vary with the antigen-antibody system or even the individual antiserum. For example, in the cyclic AMP immunoassay, Ag* binding is highly sensitive to pH (in the range of 6.0 to 8.0), ionic strength (decreased binding at NaCl concentrations above 0.2 M), and the concentration of Mg^{2+} and Ca^{2+}. The antigen itself may be heterogenous. Protein and polypeptide hormones and enzymes may be secreted in multiple molecular forms, exhibit partial structural homology with other hormones and enzymes, undergo partial proteolysis or become attached to naturally occurring inhibitors or carriers that may or may not affect immunologic reactivity. Drugs may be metabolized to products that cross react to a variable extent with antibody to the original drug or be bound by macromolecules in plasma or tissue that affect their accessibility to antibody.

Procedures of established value in the validation of immunoassay results include the following: (*a*) Parallel studies in another assay system using a different principle of measurement (such as, for example, enzymatic or mass spectroscopic studies). (*b*) The use of internal standards (the addition of known amounts of purified antigen to tissue samples) to see whether the expected increase in Ag concentration is demonstrated. (*c*) The use of enzymes that selectively destroy the antigen (for example, the treatment of samples containing cyclic AMP or cyclic GMP with the appropriate cyclic nucleotide phosphodiesterase for various time periods) (9). (*d*) Comparison of the slopes of the Ag dose inhibition curves in the unknown sample and the standard. (*e*) Screening studies with substances known to be present in biologic samples that might theoretically cross react and therefore inhibit in the immunoassay (for example, the evaluation of anticyclic AMP antisera with ATP, ADP, 5'-AMP, and cyclic GMP) (Figure 2). (*f*) Demonstration that the immunoreactivity in the tissue samples comigrates with the antigen

in question through a series of chromatographic purification steps or is removed under conditions in which the antigen is selectively adsorbed. Adding known amounts of radioactive antigen may be helpful in monitoring for recovery during the purification provided the antigen has a sufficiently high specific activity that it does not unduly increase the total antigen content of the sample and does not interfere in measurements of the radioindicator molecule used in the immunoassay itself. (*g*) Studies under conditions in which the results of the assay are expected to be markedly positive or negative (for example, in an immunoassay for a drug, screening individuals taking various doses of the drug or in whom no drug or an unrelated drug is being taken; in an immunoassay for a hormone, screening individuals in whom the hormone should presumably be greatly reduced or absent or present in markedly increased amounts). An example of an unexpected cross reaction picked up by routine screening is the occurrence of a positive urine immunoassay for morphine in individuals ingesting bread or coffee cake that contains poppy seeds (33). (*h*) Analysis during pregnancy or pathologic states such as hyperthyroidism in which binding proteins in plasma that might affect the assay (by competing with the antibody) are altered. Similar screening studies are desirable in individuals with conditions such as hypoalbuminemia, uremia, and paraproteinemia where nonspecific effects in an assay might be anticipated (34). (*i*) Exchange of samples between different laboratories. (*j*) Serial analyses of stored samples to determine how much decomposition may normally be occurring prior to the assay.

Replicate samples should always be analyzed, ideally using at least two different dilutions of the unknown sample. In so far as possible each sample should be treated identically. The assay must be rigidly standardized in terms of total reaction volume, buffer content, quantity of radioactive antigen, and duration and temperature of incubation. Every assay should include a full antigen standard curve. In an immunoassay of any size binding controls and antigen standards should be interspersed at the beginning, middle, and end of the assay to detect any systematic variations in the assay related to the number of samples involved.

Interassay variation can be a major problem. Large-scale collaborative studies in which immunoassay results have been compared in different laboratories indicate that the major source of immunoassay variation is interassay variation (35), which may be due to a variety of factors such as fluctuations in the quality of the radioiodinated antigen marker or unstable or improperly prepared antigen standards.

Recently, a large number of immunoassay kits or individual immunoassay components have become available, permitting the reagents needed to perform an immunoassay to be purchased. Recent reviews in the literature

may be helpful in selecting a commercial supplier [see for example, the reviews in references (36, 37)], but it is nonetheless incumbent upon the individual investigator to verify the sensitivity and specificity of the assay in his own laboratory.

Many different ways have been suggested for treating radioimmunoassay data statistically. Frequently laboratories use a logit/log transformation of the data (38) to linearize the standard binding curve and permit easy comparisons among assays performed on different days. This transformation has been shown to be applicable to a large number of antigen-antibody systems, and in general there appears to be no real advantage to the use of more complex mathematical analyses. It should be recognized, however, that results are not as precise at the extreme ends of the binding curve.

RADIORECEPTOR ASSAY

In some circumstances naturally occurring receptor proteins present in tissue or plasma may be used instead of antibodies for sensitive competitive binding measurements (reviewed in 1). Radioreceptor assays are theoretically advantageous in that they are less likely to detect partially degraded or otherwise altered ligands, and immunization is not required to generate the receptor protein. However, binding is much more susceptible to seemingly minor alterations in the ligand associated with labeling particularly when ^{125}I is used, and the receptor proteins themselves may be unstable and difficult to purify. Now that monoclonal antibodies can be produced more or less at will, it seems likely that antibodies will continue to be the major binding proteins used in competitive binding assays.

PROCEDURES TO IMPROVE ASSAY SPECIFICITY

One of the continuing problems in immunoassay development is that of partial antigenic cross reactivity between structurally related molecules. A good example is the immunologic cross reactivity between the polypeptide hormones LH, TSH, HCG, and FSH which share a common polypeptide chain (the α chain) but also have a second polypeptide chain that is highly individualized (39). Another example is the creatine phosphokinase isozyme immunoassay referred to earlier (20). Cross reacting antigens may produce parallel or nonparallel dose inhibition curves in the immunoassay. When the curves are parallel the cross reactivity may be expressed quantitatively by comparing the concentrations required for 50% inhibition. Possible approaches to the problem of antigenic cross reactions either at the level of the tissue sample itself, the antiserum, or the radioactive antigen are discussed below.

Fractionation of the Tissue Sample

The unknown antigen in the tissue sample may be partially purified before being assayed. Many examples of the use of this approach are available. For example, this strategy was used by our laboratory to help discriminate among the various prostaglandin classes (11). Although antisera had been prepared with relatively high specificities for the E and F prostaglandins, a certain amount of cross reactivity still remained, and therefore a simple preliminary column fractionation was carried out immediately before the immunoassay to separate the major prostaglandin classes from one another. This virtually eliminated the problem. Although such separations increase the time involved in processing samples, if the chromatograph separation procedure is sufficiently simple and rapid, substantial numbers of samples may be processed simultaneously. Indeed, with the advent of rapid, very high resolution systems such as high pressure liquid chromatography, even extremely high degrees of specificity are obtainable without unacceptable increases in sample processing times.

Simultaneous Immunoassays

Simultaneous immunoassays may be carried out using a specific antiserum and Ag* from each of the cross reacting systems present. Alternatively, if the circumstances are favorable the assay may be carried out in the presence of excess cross reacting antigen.

Use of an Antiserum with Minimal Cross Reactivity

When a large number of animals are immunized with a given antigen the antisera may differ substantially in the areas on the antigen that they recognize. The production of antibodies to different antigenic determinants on the same protein is in part genetically controlled and is linked to proteins in the major histocompatibility antigen complex (40). Antiserum with minimal cross reactivity may be obtained by (*a*) screening large numbers of antisera from different animals of the same species (1), (*b*) empirical immunization of a variety of species, (*c*) immunization with antigen from some other animal source in which the cross reacting antigenic determinant may be less prominent or otherwise altered, (*d*) immunization with an antigen that has been partially altered enzymatically or chemically to remove or mask possible cross reacting antigenic determinants; immunization with a hapten that has been conjugated to protein so that the portion of hapten most important for immunologic discrimination is maximally exposed, (*e*) absorbing the antiserum with cross reacting antigen, or (*f*) selecting for a monoclonal antibody directed toward a non-cross reacting antigenic determinant (1).

Use of a Non-Cross Reacting Radioactive Antigen Marker

At the time of the immunoassay partially degraded or otherwise altered antigen preparations in which cross reactivity is minimized may be used as the radioindicator molecule.

In addition to cross reacting antigens, considerable difficulty may be experienced with contaminating antigen-antigen systems. Contaminating antigens, if present in large quantities in the antigen preparation used for immunization, may interfere with the desired antibody response. Even in trace amounts a contaminant may prove to be an unexpectedly potent immunogen and cause major problems in interpretation. Many of the above approaches for cross reacting reagents may be used to minimize problems due to antigen-antibody systems. However, all that is really needed is an uncontaminated radiolabeled antigen preparation (1). Since only limited quantities of radioactive antigens are needed in immunoassays, it should generally be possible to obtain the amounts of pure labeled antigen that will be needed.

MONOCLONAL ANTIBODIES

It is apparent that there are a variety of possible ways of dealing with the problem of antigenic cross reactivity and contamination. However, the solutions are frequently not as easy as they might appear to be on paper. This is particularly true when the antigen is difficult to purify and is available only in limited quantities. Under these circumstances systematic exploration of different immunization and antigen purification and labeling procedures may be difficult. Recently, an important new approach in immunoassay development has been described by Milstein and his colleagues (41, 42) involving the production of monoclonal antibodies. Although the experience with this technique is still limited, it has already been shown to be potentially very useful when ordinary immunologic approaches appear to have only a limited chance for success.

In 1973 Cotton & Milstein (41) reported adaptation of the technique of mammalian cell fusion to obtain hybrid cell lines producing two different types of antibodies. This suggested that the fusion of antibody secreting cells with permanent myeloma cell lines might provide a general approach to the production of homogenous antibodies. As later refined in Milstein's and other laboratories (42–46), animals (usually mice) are immunized and their spleen or lymph node cells are prepared as a suspension and incubated with a permanent nonsecreting mouse plasma cell line in the presence of a fusion-promoting reagent such as polyethylene glycol, Sendai virus, or dimethyl sulfoxide. Fusomas secreting the desired antibody are obtained

which can be cloned and maintained more or less indefinitely either in tissue culture or as ascites tumors in mice. The antibodies are apparently homogenous with respect to their electrophoretic mobility (both before and after separation of their individual heavy and light chains), amino acid composition, and antigen binding properties, confirming their monoclonal origin. Because even under favorable circumstances the frequency of hybridization does not usually exceed one hybrid for 2×10^5 input spleen cells (45) and cells making other antibodies also undergo fusion, it is necessary to select for the desired antibody-producing cells. Unfused cells from the original plasma cell line are eliminated by utilizing a mutant line that does not survive unless it undergoes fusion with a normal cell or is supplied with specific growth factors. As a rule mutant cells lacking the enzyme hypoxanthine guanine phosphoribosyl transferase are used making them unable to grow in medium containing hypoxanthine, aminopterin, and thymidine (HAT medium) (42). When the cells from the fusion mixture are grown in the selective medium, the cells in the original mouse plasma cell line and the normal lymphoid cells largely die, and the culture is enriched with cells that have undergone fusion. It is then necessary to separate clones producing the desired antibody from those making other antibodies. This is done by culturing cells in multiple small tissue culture wells and screening the individual wells. Once clones of antibody-producing cells have been isolated, the antibodies are characterized in detail in regard to their binding properties. The most favorable clones are retained and the antibodies are then generated on a larger scale. Many laboratories have now successfully utilized this approach. Homogenous antibodies to a variety of cell-associated and soluble antigens have been obtained, indicating that the technique is probably applicable to any reasonably effective immunogen. In general, the cell lines have been sufficiently stable to permit the generation of considerable quantities of antibodies.

The advantages of monoclonal antibodies are multiple: 1. Assuming that the original cloning procedure was adequate and antibodies to a non-cross reacting antigenic determinant have been selected, the antibody will be specific for a single antigenic determinant, and antibodies to contaminating proteins and cross reacting antigenic determinants will have been eliminated. Thus there is no necessity for undertaking the extensive losses of antibody that may be associated with selective adsorption techniques used in an effort to improve antibody specificity. Moreover, highly purified antigen is not needed for immunization since the appropriate antibody-producing cell line can be selected after the immunization is completed. 2. The antibody that is obtained is a defined chemical reagent easily separated from most of the contaminating proteins in the tissue culture medium and is

potentially available in large quantities. The antibodies or antibody-producing cell lines can be widely disseminated to other laboratories providing investigators in an entire field of biologic research with the same analytical reagent. Moreover, the antibody is homogenous with respect to its binding affinity for antigen, helping to normalize the antigen binding curve.

The success of this approach is already evidenced by the number of research applications involving monoclonal antibodies in the limited number of years since the technique was first described. Nonetheless, the generation of monoclonal antibodies is easier for some antigens than others and in any case is a formidable undertaking: 1. Obviously, if the antigen has stimulated only a very feeble response, the statistical probability of obtaining the desired fusion product may be unacceptably low. This may be a particular problem if antigen is highly contaminated. 2. Even though an antibody has been selected that is unquestionably directed toward the appropriate antigen, there is no assurance that this will be the most appropriate antibody for immunoassay purposes since the binding affinity or specificity may not be optimal. While the antibody is homogenous, nonspecific cross reactivity is not necessarily eliminated because this may be an intrinsic property of the antibody-combining site itself (47). For example, monoclonal myeloma proteins with binding affinity for the 2,4-dinitrophenyl (DNP) hapten may also bind to DNA with a relatively high affinity (48). This dual reactivity for DNP and DNA would not be predicted on purely immunochemical grounds. Thus, regardless of the apparent specificity of an antibody, it will need to be screened empirically for unexpected cross reactions. Ideally this should be done under the conditions of eventual use in the immunoassay. Crude qualitative assays of the types often used for rapid screening may lack the necessary level of discrimination to determine which antibody is most suitable for later applications. 3. A considerable amount of work is involved. After immunization, fusion, identification, and isolation of the hybridized antibody-producing cells, the cells must still be maintained until the analysis of the antibodies is completed. Apparently desirable cell lines may be lost during this period, as a result of technical problems or reversion. 4. Up to now there have been difficulties in fusing cells from widely differing animal species, perhaps because of chromosomal segregation (43), and almost all of the work has been with mouse plasma cell lines fused either with mouse or rat lymphoid cells. Although other combinations may be attempted and successful fusion of human to mouse cells has been reported (49), the likelihood of success appears to be very considerably diminished. Therefore for most purposes the use of mice or rats as the source of antibody-producing cells for fusion appears highly desirable. In general, this is not a serious drawback, but in some situations it may be preferable to have species other than mice or rats as the antibody-

producing species. This problem may eventually be solved by increases in the availability of nonsecreting plasma cell lines from many different animal species or by improvements in fusion or culture methodology. 5. Although there are theoretical and practical advantages to the use of a highly purified antibody preparation which is homogenous in its binding affinity for antigen, in many situations these advantages may be more apparent than real. Frequently it may be *advantageous* to have a mixture of different antibodies to the various antigenic determinants on a protein as occurs normally in the serum of immunized animals rather than an antibody to a single determinant. As discussed above, the greater stabilization possible when multiple rather than single antigenic determinants are interacting with antibody may create a higher operational immunologic sensitivity than would be possible with an antibody directed toward a single determinant. In some cases antigens will have only one copy of a determinant per molecule, making lattice formation impossible with monoclonal antibodies. Depending on the assay system used such antibodies may not even be detected. Of course, there is no a priori reason why a number of monoclonal antibodies could not be generated against different determinants on the same antigen and used as a mixture to ensure that the usual types of stable aggregates would be formed. On the other hand, because multiple animals are ordinarily utilized to generate standard antisera and screening assays for antibody binding and specificity are normally not difficult to perform, one is in a position to rapidly select for the antiserum with the most favorable binding properties. While even carefully selected antisera will contain a mixture of antibodies with differing binding specificities and affinities for the antigen in question, under the usual conditions of a sensitive immunoassay, only the antibodies of highest affinity contribute significantly to antigen binding. Moreover, even though serum contains a large number of unrelated antibodies that might theoretically be undesirable, if the radioactive antigen marker is pure there is normally no interference in the assay. And while many enzymes are present in serum, at the high serum dilutions used in sensitive immunoassays substantial effects of these enzymes on antigen binding are unusual. If necessary, a purified γ globulin preparation can be used. 6. The immunization procedures classically used by immunologists and other investigators to maximize antibody affinity involves the use of injections of small amounts of antigen in complete Freund's adjuvant in an effort to maximize antibody affinity. The use of limited amounts of antigen is based on the assumption, well supported by the existing experimental data, that the generation of antibody-producing cells in vivo is a thermodynamically driven process (50). When only small amounts of antigen are available, the cells best able to bind the available antigen are selectively stimulated. It is not yet entirely clear how best to manipulate the immuniza-

tion procedure so as to obtain substantial numbers of high affinity antibody-producing cells for fusion. If the frequency of such cells is too low, the development of a suitable hybridoma may present real difficulties, and the normal in vivo selection process used in the production of high affinity antisera, although less elegant, may actually be preferable. Presumably the same immunization procedures that produce high affinity serum antibodies will also be optimal for obtaining high affinity cell fusion products, but this has not yet been carefully studied. For these and other reasons, despite the undoubted value of using monoclonal antibodies for immunoassay development, there are many situations in which serum antibodies obtained in the usual way will continue to be highly satisfactory.

SPECIAL FEATURES OF IMMUNOASSAYS TO DRUGS

It is now apparent that virtually any drug of reasonable size and structural complexity can be conjugated to a protein and produce antibodies suitable for the development of a sensitive immunoassay. While immunoassays lack the ability to screen for many different drugs simultaneously, there is evidence that when an appropriate combination of markers is used several unrelated drugs may be analyzed in the same assay tube (51, 52) which may be of value in drug abuse detection programs. Obviously, even under optimal circumstances, immunoassays suffer from the same limitation as other sensitive assays in that when a drug is concentrated to a major extent in tissue, serum or plasma measurements may not be particularly useful because they may not accurately reflect the total quantity of drug present in the body. As in other assays, if the drug is unstable or difficult to extract from blood or tissue and adequate precautions are not taken, inaccurate results may be obtained. There is the additional problem that active metabolites with altered pharmacologic reactivity may cross react in the immunoassay, creating possible problems in interpretation. Depending on the drug, its mode of conjugation to protein, the antiserum, and the radioactive marker, drugs or drug metabolites that are closely related structurally may either react almost indistinguishably or quite differently with an antibody. If several antisera are available with differing specificities and if simultaneous immunoassays are performed, adequate discrimination, even in mixtures of several different metabolites, is possible. On the other hand, the use of an immunoassay in conjunction with a high resolution technique such as high pressure liquid chromatography may more readily solve the problem, providing both high sensitivity and specificity.

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