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Alternative Immunossays

Barnard. G.J.R.,* J.B. Kim and W.P. Collins***

* King's College Hospital Medical School,Dept. of Obstetrics & Gynaecology
University of London, London, SE5, 8RX, England

** Dept. of Animal Products Science,College of Animal
Husbandry,Kon-Kuk University

Introduction

An immunoassay may be defined as an analytical procedure involving the competitive reaction between a limiting concentration of specific antibody and two populations of antigen, one of which is labelled or immobilized. The advent of immunoassay has revolutionised our knowledge of reproductive physiology and the practice of veterinary and clinical medicine. Radioimmunoassay (RIA) was the first of these methods to be developed, which measured the analyte with good sensitivity, accuracy and precision (1,2). The essential components of RIA are:– (i) a limited concentration of antibodies, (ii) a reference preparation, and (iii) an antigen labelled with a radioisotope (usually tritium or iodine-125). Most procedures involve isolating the antibody-bound fraction and measuring the amount of labelled antigen. Good facilities are available for scintillation counting, data reduction and statistical analysis. RIA is undergoing refinement through:- (i) the introduction of new techniques to separate the antibody-bound and free fractions which minimize the misclassification of labelled antigen into these compartments, and the amount of non-specific binding (3), (ii) the development of non-extraction for the measurement of haptens (4), (iii) the determination of apparent free (i.e. non-protein bound) analytes (5), and (iv) the use of monoclonal antibodies (6).

In 1968, Miles and Hales introduced an important new type of immunoassay which they termed immunoradiometric assay (IRMA) based on the use of isotopically labelled specific antibodies (7) in a move from limited to excess reagent systems. The concept of two-site IRMAs (with a capture antibody on a solid-phase, and a second labelled antibody to a different antigenic determinant of the analyte) has enabled the development of more sensitive and less-time consuming methods for the measurement of protein hormones over a wide concentration of analyte (8). The increasing use of isotopic methods for diverse applications has exposed several problems. For example, the radioactive half-life and radiolysis of the labelled reagent limits assay sensitivity and imposes a time limit on the usefulness of a kit. In addition, the potential health hazards associated with the use and disposal of radioactive compounds and the solvents and photofluors necessary for liquid scientillation counting are incompatable with the development of extra-laboratory tests. To date, the most practical alternative labels to radioisotopes, for the measurement of analytes in a concentration > 1 ng/ml, are erythrocytes, polystyrene particles, gold sols, dyes and enzymes or or cofactors with a visual or colorimetric end-point (9). Increased sensitivity to < 1 pg/ml may be obtained with fluorescent and chemiluminescent labels, or enzymes with a fluorometric, chemiluminometric or bioluminometric end-point. The sensitivity of any immunoassay or immunometric assay depends on the affinity of the antibody-antigen reaction, the specific activity of the label, the precision with which the reagents are manipulated and the nonspecific background signal (10). The sensitivity of a limited reagent system for the measurement of haptens or proteins is mainly dependent upon the affinity of the antibodies and the smallest amount of reagent that may be manipulated.

Consequently, it is difficult in practice to improve on the sensitivity obtained with iodine-125 as the label. Conversely, with excess reagent systems for the measurement of proteins it is theoretically possible to increase assay sensitivity at least 1000 fold with alternative luminescent labels. To date, a 10-fold improvement has been achieved, and attempts are being made to reduce the influence of other variables on the specific signal from the immunoreaction.

Particle Immunoassays

Relatively simple tests collectively called particle immunoassays, of medium sensitivity (i.e. analyte concentrations > 1 ng/ml) have been developed for extra laboratory use. Erythrocytes or polystyrene (latex) spheres are linked to the antigen or antibody; the separation system does not require the intervention of the assayist, and the end-point may be semi-quantitative (by serial dilution of the sample) or quantitative. A threshold value is used to divide positive from negative results (e.g. in a pregnancy test). Semi-quantitative methods are performed in tubes with hemi-spherical bases and the quantitative procedure in open-ended, horizontal glass capillaries (11). In the latter system the distance between the parallel lines of precipated reagent (read through eyepiece with a graticule) is proportional to the concentration of analyte. The tests may be used to measure haptens or proteins and take approximately [illegible] to complete without the use of equipment. Alternatively latex particles are being used in methods which involve angular anisotropic measurement (12) or particle size analysis (13) to distinguish between the antibody-bound and free fractions. Gold sols (14) or disperse dye sols (15) have also been used as labels in tests for the semi-quantitative or quantitative measurement of haptens and proteins.

Luminescence Immunoassays

Various forms of luminescence (i.e. the dissipation of energy in the form of light from a substance in an electronically excited state) have been used to monitor immunological reactions. There are several forms of luminescence which only differ in the source of energy involved in exciting the electrons to a higher energy level. These include: – (i) [illegible]luminescence, where excitation is effected by beta or gamma rays, (ii) fluorescence or phosphorescence where excitation is produced by photons of infra red, visible or ultraviolet light, (iii) chemiluminescence where activation is affected by a chemical reaction, and (iv) bioluminescence, where the chemical reaction is mediated by enzymes. Recent developments in immunoassay procedures with fluorescent labels have been reviewed (16). To date, the most commonly used fluorophores have been derivatives of fluorescein, rhodamine or umbelliferone. Recently erythrosin has been used as a label for the measurement of haptens by phosphorescence immunoassay. The most robust fluorescence immunoassays (FIAs) and phosphorescence immunoassays have been applied to the measurement of analytes that are present in amounts > 1 ng/ml of biological fluid (16). Attempts to increase sensitivity have involved the attachment of fluoreophore to polymers which are subsequently linked to the analyte to form a labelled antigen, and by the use of immunofluorometric assay for the measurement of proteins. Non-separation assays have been described which either involve fluorescence polarisation, energy transfer from the labelled antigen to a labelled antibody or the use of antibodies against the label (16). Various factors present in biological fluids can cause apparent enhancement or quenching of the fluorescent signal which include: – (i) light scattering, (ii) endogenous fluorescence; and (iii) inner filter effects. Recently, excess reagent methods involving the use of time-resolved fluorometry have been introduced for the measurement of protein hormones (17). Europium chelates covalently linked to mono-

clonal antibodies constitute the label and become fluorogenic when attached to an organic moiety surrounded by detergent in an aqueous solution. Assays for the measurement of hCG and TSH have good sensitivity, accuracy and precision. Limited reagent methods with a second antibody labelled with europium are being developed for the measurement of haptens.

Chemiluminescent compounds (particularly cyclic hydrazides and acridinium esters) have been used to monitor immunoassays (18). Bioluminescence is a special form of chemiluminescence, which is found in living organisms, and has also been used as a method of detecting immunological reactions (19). The reactions are catalysed by an enzyme (luciferase), which oxidises a specific substrate (luciferin). The structure of both reactants vary with the species. Solid-phase chemiluminescence immunoassays (CIAs) have been described and evaluated for the meausrment of plasma steroids and urinary steroid glucuronides (20), hCG (21), thyroxine (22) and thromboxane B_2 (23) using derivatives of isoluminol to form the labelled antigens. Recently substances which dissociate the antibody-antigen complex and enhance the signal (approximately 100 fold) have been identified, and may replace the use of concentrated sodium hydroxide. Immunochemiluminometric assays (ICMAs) using monoclonal antibodies labelled with acridinium ester are preferred for the measurement of protein hormones. Methods for the measurement of hCG, α-fetoprotein (24) and TSH (25) have improved sensitivity, and equivalent accuracy and precision to a corresponding RIA or IRMA. Nonseparation CIAs for haptens using chemical energy transfer (26) or differential activation of the antibody-bound or free fractions of labelled antigen (27) have been developed. The end-point of the assay can be extremely rapid < 10 secs as in chemiluminescence, or continuous as in bacterial or firefly bioluminescence. Aspects of CIA or ICMA under active development are:– (i) the synthesis of alternative chemiluminescent labels with a higher quantum yield, (ii) the identification of substances which enhance or inhibit the chemiluminescence of labelled antigens and antibodies; (iii) the development of improved chemiluminometers with online data reduction systems; and (iv) the purification of reagents (cofactors, enzymes and substrates) for use in bioluminescence immunoassays and immunobioluminometric assays.

Enzymeimmunoassays

Enzymes are versatile and sensitive labels of immunoassay reactions (28). Enzyme immunoassay (EIA) may be subdivided into separation systems (e.g. enzyme linked immunosorbent assay-ELISA) for the measurement of haptens (29) and proteins (30). Non-separation systems (e.g. enzyme multiplied immunoassay -EMIT) have also been reported for the measurement of haptens (31) and proteins (32). The latter approach capitalizes on the finding that relatively small enzymes are inactivated when the labelled antigen is bound to antibody-leaving the free fraction available for measurement. Factors affecting the choice of enzyme include:– (i) the turnover number, (ii) the purity of the preparation, (iii) the sensitivity, ease and speed of product detection, (iv) the absence of interfering factors in the fluid for analysis, (v) the presence of reactive groups for coupling, (vi) the stability of the labelled reagent, (vii) the availability and cost, and (viii) the suitability for nonseparation assays. The enzymes that have been used most extensively in separation assays are horseradish peroxidase, alkaline phosphatase from calf intestinal mucosa, β-D-galactosidase from Escherichia coli, glucose oxidase from Aspergillus niger, penicillinase and luciferase. Lysozyme, malate dehydrogenase, glucose-6-phosphate dehydrogenase and β-D-galactosidase have been used in non-separation assays. A variety of alternative end-points to colorimetry have been used and include fluorometry (33), chemiluminometry (34) and potentiometry (35). The sensitivity of detection is good ($> 10^{18}$ moles), but is limited in practice by the characteristics of the antibodies and background interference. Recently, the production of purified enzymes by affinity chromato-

graphy, together with the use of monoclonal antibodies and solidphase systems has enabled the development of two-site immunoenzymometric assays for the measurement of protein hormones (36). To date, EIA has not been widely applied to the measurement of haptens for the following reasons: (i) the large molecular weight of the label may interfere with the antibody-binding reaction; (ii) the synthesis of the labelled antigen is difficult to control and the product needs to be characterised for enzyme and antibody-binding activities, (iii) the non-separation systems are relatively insensitive (> 1 ng/ml) due to non-specific interference from the biological sample; and (iv) the end-point determination is more complex and time-consuming than for RIA. Nevertheless, the availability of automatic equipment for taking spectrophotometric, fluorescent, or chemiluminescent measurements from microtitre plates, together with the appropriate software for data reduction, makes this approach attractive for the routine analysis of a large number of samples. Recently, an immunometric type assay has been described for the measurement of haptens (37). In addition, antibodies attached to dipsticks have been produced for the measurement of hCG and LH and for the measurement of haptens. An enzyme or an apo-enzyme has been used as the label to produce a visual end-point.

Future Developments

It is anticipated that the trend towards the development of excess reagent assays for the measurement of all analytes will continue, and the pressure to find alternative biochemical labels may become more intensive. The tendency towards the use of non-invasive immunochemical tests e.g. on saliva (38), defined collection of urine (39), milk (40) and constituents of fluid from the reproductive tract (41) in extra-laboratory locations, together with the requirement for more sensitive assays e.g. for the detection of viral antigens, will facilitate the acceptance of nonisotopic procedures. Multiple immunoassays for the simultaneous measurement of more than one analyte (42) and automated systems (43) are being developed. There is also a concerted effort to product more appropriate reference preparations for the immunoassay of protein hormones (44). An interesting new approach to immunoassay involving the use of microscope slides or fibre optics and the production of evanescent waves may:– (i) avoid the use of lables, (ii) lead to a universal excess reagent system for the measurement of haptens and proteins, and (iii) involve a rapid-end-point determination, which is based on the antibody-antigen binding rate (45). In addition, it is anticipated that the continuous refinement of solid state biochemical sensors will enable signals from immunochemical reactions to be recorded and processed (46).

REFERENCES

1. Hunter W.M., 1978. Radioimmunoassay. In: Handbook of experimental immunology, Vol. 1, 3rd edn. Weir D.M. (ed): ch 14, p. 14.1-14.4, Blackwell, Oxford.
2. Van Vunakis H., 1980. Radioimmunoassays: an overview. In: Methods in Enzymology Vol. 70 Immunochemical Techniques Part A Van Vunakis H, Langone J.J. (eds): 201-209, Academic Press, New York.
3. Ratcliffe J.G., 1983. Requirements for separation methods in immunoassay. In: Immunoassays for clinical chemistry, 2nd edn. Hunter W.M. and Corrie J.E.T. (eds): 135-138, Churchill Livingstone, Edinburgh.
4. Ratcliffe W.A., 1983. Direct (non-extraction) serum assays for steroids. In: Immunoassays for clinical chemistry, 2nd edn. Hunter W.M. and Corrie J.E.T. (eds): 401-409, Churchill Livingstone, Edinburgh.
5. Ekins R.P., 1983. The direct immunoassay of free (non-protein bound) homones in body fluids. In: Immunoassays for clinical chemistry, 2nd edn.

Hunter W.M. and Corrie J.E.T. (eds): 319-337, Churchill Livingstone, Edinburgh.

6. Galfre G. and Milstein C., 1981. Preparation of monoclonal antibodies: strategies and procedures. In: Methods in Enzymology Vol. 73 Immunochemical Techniques Part B. Langone J.J., Van Vunakis H. (eds): 1-46, Academic Press, New York.
7. Hales C.N., Woodhead J.S., 1981. Labelled antibodies and their use in immunoradiometric assay. In: Methods in Enzymology Vol. 70 Immunochemical Techniques Part A. Van Vunakis H., Langone J.J. (eds): 334-355 Academic Press, New York.
8. Hunter W.M., Budd P.S., 1981. Immunometric versus radioimmunossay: a comparison using alphafeto protein as the model analyte. J. Immunol. Meth. 45: 255-273.
9. Schall R.F., Tenoso H.J., 1981. Alternatives to radioimmunoassay: labels and methods Clin. Chem. 27: 1157-1164.
10. Ekins R.P., 1980. More sensitive immunoassays. Nature 284: 14-15.
11. Ittrich G., 1980. A simple technique for quantitative measurement of erythrocyte settling patterns in haemagglutination inhibition tests for immunologic hormone assay. Endokrinologie 75: 13-19.
12. Von Schulthess K.G., Giglio M., Cannel D.S., Benedek G.B., 1980. Detection of agglutination reactions using anisotropic light scattering. An immunoassay of high sensitivity. Mol. Immunol. 17: 81-92.
13. Collect-Cassart D., Mareschal J.C., Sindic C.J.M., Tomassi T.P., Masson P.L., 1983. Automated particle counting immunoassay of C-reactive protein. Application to serum, cord serum and cerebrospinal fluid. Clin. Chem. 29: 1127-1131.
14. Leuvering J.H.W., That P.J.H.M., Van Der Waart M. Schuurs A.H.W.H., 1980. Sol particle immunoassay (SPIA) J. Immunoassay 1: 77-91.
15. Gribnau T., Roeles R., Biezen J.U.D., Leuvering J., Schuurs A.H.W.M., 1982. The application of colloidal dye particles as label in immunoassay: Disperse (d) dye immunoassay (DIA). In: Affinity chromatography and related techniques - Analytical chemistry symposia series. Vol. 9. Gribnau T.C.J., Visser J, Nivard R.J.F., 8eds): 411-424, Elsevier, Amsterdam.
16. Smith D.S., Al-Hakiem H.H., Landon J., 1981. A review of fluoroimmunoassay and immunofluorometric assay Ann. Clin. Biochem. 18: 253-274.
17. Soini, E., Kojola H., 1983. Time-resolved fluorimeter for lanthanide chelates - a new generation of nonisotopic immunoassays. Clin. Chem. 29: 65-68.
28. Simpson J.S.A., Campbell A.M., Ryall M.E.T., Woodhead J.S., 1979. A stable chemiluminescence-labelled antibody for immunological assays. Nature 274: 646-647.
19. Fricker H., Strasburger C.J., Wood W.G., 1982. Enzyme enhanced luminescence immunoassay for the determination of transferrin concentrations in serum. J. Clinic. Chem. Clin. Biochem. 20: 91-94.
20. Collins W.P., Barnard G.J., Weerasekera D.A., Kohen F., Eshhar Z., Lindner H.R., 1983. Chemiluminescence immunoassays for plasma steroids and urinary steroid metabolites. In: Immunoassays for clinical chemistry, 2nd edn. Hunter W.M. and Corrie J.E.T. (eds): 373-397, Churchill Livingstone, Edinburgh.
21. Barnard G., Kim J.B., Brockelbank J.L., Collins W.P., Kohen F., Gaier B., 1984. The measurement of choriogonadotropin by chemiluminescence immunoassay and immunochemiluminometric assay. 1. Use of isoluminol derivatives. Clin. Chem. 30: 538-541.
22. Weerasekera D.A., Kim J.B., Barnard G.J., Collins W.P., 1983. The measurement of serum thyroxine by solid-phase chemiluminescence immunoassay. Ann. Clin. Biochem. 20: 100-104.
23. Weerasekera D.A., Koullapis E.N., Kim J.B., Barnard G.J., Collins W.P., Kohen F., Lindner H.R., 1983. Chemiluminescence immunoassay of thromboxane B_2. In: Advances in prostaglandin, thromboxane and leucotriene research. Vol. II. Samuelsson B, Paoletti R, Ramwell P. (eds):

185-190. Raven Press, New York.

24. Weeks I., Campbell A.K., Woodhead J.S., 1983. Two-site immunochemiluminometric assay (ICMA) for human α-fetoprotein. Clin. Chem. 29: 1480-1483.

25. Weeks I., Sturgess M., Siddle K., Jones M.K., Woodhead J.S., 1984. A high sensitivity immunochemiluminometric assay for human thyrotrophin. Clin. Endocrinol. 20: 489-495.

26. Patez A, Campbell A.K., 1983. Homogeneous immunoassay based on chemiluminescence energy transfer. Clin. Chem. 29: 1604-1608.

27. Kohen F., Kim J.B., Barnard G., Lindner H.R., 1980. An assay for urinary estriol-16α-glucuronide based on antibody-enhanced chemiluminescence. Steroids 36: 405-419.

28. Oellerich M., 1980. Enzyme immunoassays in clinical chemistry: Present status and trends. J. Clin. Chem. Clin. Biochem. 18: 197-208.

29. Van Weeman B.K., Bosch A.M.G., Dawson E.C., Schuurs A.H.W.N., 1979. Enzyme-immunoassay of steroids: possibilities and pitfalls. J. Steroid Biochem 77: 147-151.

30. Brock D.J.H., Barron L., Van Heyningen V., 1982. Enzyme-linked immunospecific assays for human AFP using monoclonal antibodies. Clinica Chim. Acta 122: 353-358.

31. Ghosh M., Dhar T.K., Ali E., Bachhawat B.K., 1983. Homogeneous enzyme immunoassay of estriol in picogram amounts. Clin. Chim. Acta 128: 223-232.

32. Gibbons I., Skold C., Rowley G.L., Ullman E.F., 1980. Homogeneous enzyme immunoassay for proteins employing β-galactosidase. Anal. Biochem. 102: 167-170.

33. Turkes A.O., Turkes A., Joyce B.G., Riad-Fahmy D., 1980. A sensitive enzymeimmunoassay with a fluorimetric end-point for the determination of testosterone in female plasma and saliva. Steroids 35: 89-101.

34. Whitehead T.P., Thorpe G.H.G., Carter T.J.N., Groucutt C., Kricka L.J., 1983. Enhanced luminescence procedure for sensitive determination of peroxidase-labelled conjugates in immunoassay. Nature 305: 158-159.

35. Boitieux J.L., Lemay C., Desmet G., Thomas D., 1981. Use of solid phase biochemistry for potentiometric enzyme immunoassay of oestradiol-17β-preliminary report. Clinica Chim. Acta 113: 175-182.

36. Wada H.G., Danisch R.J., Baxter S.R., Federici M.M., Fraser R.C., Brownmiller L.J., Lankford J.C., 1982. Enzyme immunoassay of the glycoprotein tropic hormones - CGH, lutropin, thyrotropin - with solid-phase monoclonal antibody for the α-subunit and enzyme coupled monoclonal antibody specific for the β-subunit. Clin. Chem. 28: 1862-1866.

37. Ferytag J.W., Dickinson J.C., Tseng S.Y., 1984. A highly sensitive affinity-column-mediated immunometric assay, as exemplified by digoxin. Clin. Chem. 30: 417-420.

38. Riad-Fahmy D., Read G.F., Walker R.F., 1983. Salivary steroid assays for assessing variation in endocrine activity. J. Steroid Biochem. 19: 165-272.

39. Branch, C.M., Collins P.O., Collins W.P., 1982. Ovulation prediction: changes in the concentrations of estrone-3-glucuronide, estradiol-17β-glucuronide and estriol-16α-glucuronide during conceptional cycles. J. Steroid Biochem. 16: 345-347.

40. Arnstadt K.I., 1983. Steroid determination in milk by enzyme immunoassay. J. Steroid Biochem. 19: 423-424.

41. Zanchetta R., Busolo F., Mastrogiacomo I., 1982. The enzyme-linked immunosorbent assay for detection of the antispermatozoal antibodies. Fert. Steril. 38: 730-734.

42. Weerasekera D.A., Kim J.B., Barnard G.J., Collins W.P., 1983. Multiple immunoassay: the simultaneous measurement of two urinary steroid glucuronides as an index of ovarian function. J. Steroid Biochem. 18: 465-470.

43. Forrest G.C., 1983. A general review of automated Ria. In: Immunoassays for clinical chemistry, 2nd

edn. Hunter W.M. and Corrie J.E.T., (eds): 211 220. Churchill, Livingstone, Edinburgh.

44. Storring P.L., Gaines-Das R.E., Bangham D.R., 1980. International reference preparation of HCG for immunoassay: potency estimates in various bioassay and protein binding assay systems; and international reference preparations of the α and β subunits of HCG for immunoassay. J. Endocr. 84: 295-310.

45. Sutherland R.M., Dahne C., Place J.F., Ringrose A.S., 1984. Optical detection of antibody-antigen reactions at a glass-liquid interface. Clin. Chem. in print.

46. Janata J., Blackburn G.F., 1984. Immunochemical potentiometric sensors. Ann. N.Y., Acad. Sci., in print.