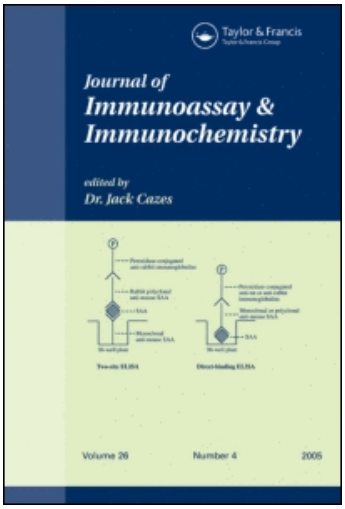

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Enzyme-Immunoassay: A Powerful Analytical Tool

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ENZYME-IMMUNOASSAY: A POWERFUL ANALYTICAL TOOL

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ABSTRACT

This paper reviews various aspects of enzyme-immunoassay (EIA). Firstly it summarizes the principles of tests in which use is made of labelled antigen or antibody. Since these constitute essential reagents in EIA they are discussed next. Many assay principles call for a bound/free separation. The various methods to accomplish this are therefore briefly discussed. Very important are the characteristics of EIA: specificity, sensitivity, precision and practicability, the latter including reagent stability, performance requirements, assay times and automation potential. Finally, fields of application are listed and some more recent developments are mentioned.

INTRODUCTION

The glorious story of radio-immunoassay (RIA) has been aptly described by Yalow (1) in her Nobel Prize lecture. It shows how this new analytical tool has caused a revolution in endocrinology.

The story of enzyme-immunoassay (EIA) is a different one in many respects. Both groups (2, 3) who pioneered first in this field had in mind to develop a new type of test maintaining the great

advantages of RIA but without some of the limitations set by the use of radio-isotopes. In its development RIA differs from EIA in that in the first ten to fifteen years of its existence RIA was mainly applied for scientific purposes while EIA from its early stages was primarily aimed at practical diagnosis by simple means. Another difference is that early applications of EIA were not limited to one field but covered very different substances such as hormones (3, 4, 5), serum proteins (2, 5, 6), drugs (7, 8) and antibodies against microorganisms (9). From the very beginning of EIA work various assay principles previously used with radioactive labels, such as the competitive, the immunometric and the sandwich type, were more or less simultaneously explored with enzyme as labels. A final difference is that the use of certain enzymes as label appeared to permit an assay whereby the separation of free label from the label bound to its counterpart in the immune reaction was no longer needed. Where radioactivity is unaffected whether the label is free or bound, enzyme activities sometimes differ in free and bound labels (7, 8). This type of assay has been coined homogeneous EIA (7) in contrast to the heterogeneous EIA where a bound/free (B/F) separation is needed as in RIA.

All these aspects have to be considered when one wants to compare EIA with RIA as if they were competitors. However, in the field of microbiological tests EIA has certainly to be compared with the usual methods in that field, particularly with immunofluorescence (IF).

Quite a few test characteristics have to be reviewed in order that a proper evaluation of EIA be possible. The following points will be discussed: preparation and properties of enzyme-labelled reagents, B/F separation (where applicable), specificity, precision, sensitivity and practicability. However, we shall first briefly review various

test systems in which labelled immune reactants are used. At the end, the various fields of applications will be summarised.

TEST PRINCIPLES

The competitive antibody binding technique is the one most frequently used in RIA (1). It requires labelled antigen, which competes with unlabelled antigen in the sample for a limited amount of antibody, and a method for separating antibody-bound and free labelled antigen.

Immunoradiometric techniques are based on the reaction of antigen in the sample with labelled antibody and the "back-titration" of non-reacted labelled antibody with antigen bound to an insoluble material (10).

Sandwich techniques have been described in a number of versions, some of which can also be coined as of the (pseudo) competitive type.

A summary of various test principles is presented in figure 1.

ENZYME-LABELLED IMMUNE REAGENTS

The primary requirement for an enzyme to be used as label is the possibility of chemically binding the enzyme to the immune reactant in an acceptable yield. Furthermore, an easy way of measuring enzyme activity, a low molar detection level, and a low cost of highly purified enzyme are desirable. In systems in which B/F separation is needed the enzymes most frequently used are horseradish peroxidase (HRP), alkaline phosphatase and β-galactosidase. In the homogeneous EIA's lysozyme, malate dehydrogenase and glucose-6-phosphate dehydrogenase are, at present, the enzymes of choice.

Competitive

homogeneous EIA: no B/F separation needed; heterogeneous EIA: B/F separation needed

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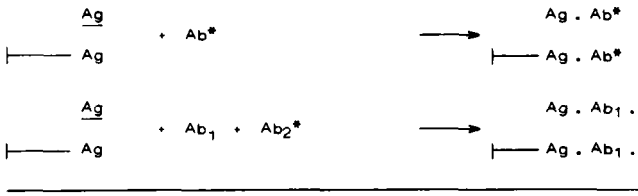
Immuno $\frac{\text{radio}}{\text{enzymo}}$ metric



Sandwich

Ag + Ab1 + Ab2*	→ Ag . Ab1 . Ab2*	1)
Ag + Ab + protein A*	→ Ag . Ab . protein A*	
Ag + Ab + anti-μ*	→ Ag . Ab . anti-μ*	1,2,3)
Ag + Ab + Ag*	→ Ag . Ab . Ag*	
Ab + Ag + Ab*	→ Ab . Ag . Ab*	1)
Ab + Ag • Ab1 + Ab2*	→ Ab . Ag . Ab1 . Ab2*	1,4)
Ab + Ag + Ab1 + anti-μ*	→ Ab . Ag . Ab1 . anti-μ*	1,2,3)
Ab2 + Ab1 + Ag*	→ Ab2 . Ab1 . Ag*	
anti-μ + $\frac{\text{Ab1}}{\text{IgM}}$ + Ag*	→ anti-μ . $\frac{\text{Ab1}}{\text{IgM}}$. Ag*	3)
Ab2 + Ab1 + Ag + Ab*	→ Ab2 . Ab1 . Ag . Ab*	1,5)
anti-μ • $\frac{\text{Ab1}}{\text{IgM}}$ • Ag • $\frac{\text{Ab*}}{\text{IgG}}$	→ anti-μ . $\frac{\text{Ab1}}{\text{IgM}}$. Ag . $\frac{\text{Ab*}}{\text{IgG}}$	3)

Sandwich/Competitive

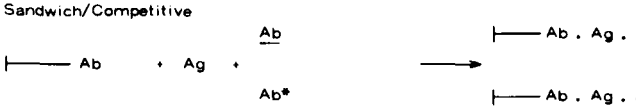


Figure 1: SURVEY OF TEST PRINCIPLES

F	- free
B	- bound
Ab IgG	- IgG class Ab
Ab IgM	- IgM class Ab
Ab_2	- antibody against Ig of the species in which Ab_1 was raised
anti-μ	- Ab_2 specifically directed against IgM
⊢———	- solid phase (e.g. cellulose particles, tube wall, wall of well in microtitration plate) to which an immune reactant is bound
*	- (enzyme or radioactive) label
———	- underlining designates immune reactant under test

Notes:

1. Antibody against Ag or against (specific) Ig(-classes) of other species and labelled with enzyme (Ab^*, Ab_2^*, anti-μ^*) can be replaced by $F(ab')_2$ or F(ab) fragments of these antibodies.
2. Ab can be of all Ig-classes; a disadvantage of this system is that Ab of a specific class can be underestimated, if Ab of other classes are also present.
3. This test system is also possible for other Ig-classes by replacement of anti-μ^* by labelled specific antibodies against other Ig-classes.
4. Ab_2^* or anti-μ^* should be unable to react with Ab, the latter being of a different species than Ab_1.
5. Ab_2 should be unable to react with Ab^*, the latter being of a different species than Ab_1.

The method of coupling depends on the chemical properties of the immune reactant. Proteins (both antigens and antibodies) are usually coupled by means of glutaraldehyde (11, 12), or periodate (13). Other methods are, for instance, based on the use of maleimides (15, 17). Small organic molecules (haptens) are provided with a carboxyl group which is then linked to an amino group of the enzyme by mixed anhydride or carbodiimide reactions. These latter methods have already been known from the preparation of immunogens such as steroid-protein conjugates, see e.g. ref. 18. Maleimides can also be used for coupling haptens to proteins (19-21).

Purification of enzyme-labelled reagent is performed with usual biochemical techniques including affinity chromatography (22). For (partial) purification of enzyme-hapten conjugates simple dialysis is more convenient. For haptens such as steroids which easily adsorb to charcoal the dialysis can be made more efficient by adding charcoal to the dialysis fluid (23). Removal of enzyme which has not become bound to the immune reagent is not essential for those assays in which the activity is measured in the bound (insoluble) phase.

The characterisation of the enzyme conjugates includes the following aspects (22):

(1) activity of the enzyme (specific activity, Michaelis constant, maximal velocity, pH optimum);
(2) immunological activity (affinity);
(3) amount of non-coupled enzyme;
(4) amount of non-coupled immune reactant;
(5) molar ratio immune reactant/enzyme and its relation to the eventual assay characteristics;

(6) biochemical or biophysical properties (homogeneity, molecular weight, stability);

(7) performance in EIA.

The latter aspect has always been used as the final criterion. (The proof of the pudding is in the eating). However, the last year or two increasing attention is being paid to the other, often more fundamental properties of the enzyme-labelled immune reactants. An example is the study of steroid-enzyme conjugates as described recently (23,24).

BOUND/FREE (B/F) SEPARATION

In all assay types depicted in figure 1 except the homogeneous EIA, the free enzyme-labelled immune reactant has to be separated from the labelled immune reactant which is bound to its reaction counterpart. In the immunometric and sandwich type tests one of the reactants participates in the reaction while bound to an insoluble phase. In these tests B/F separation is realised by the simple removal of the fluid from the solid phase and washing of the latter. This situation is also found in the competitive assay with a solid phase antibody: e.g. solid phase radio-immunoassay or SPRIA. However, competitive RIA or EIA proper can also be performed in the fluid phase, whereby the B/F separation is performed in a subsequent step. The classical example is the B/F separation in the insulin RIA by means of electrophoresis alone or combined with hydrodynamic flow chromatography as developed by Berson and Yalow (see ref. 1). Other methods are dextran-coated charcoal (for steroids), precipitation of the immune complex with ammonium sulphate or an organic solvent, the double antibody procedure or the double antibody solid phase method. In EIA the latter two methods are used next to the solid phase procedure.

An important facet is in which fraction, B or F, the enzyme activity is measured. It has become clear that the B fraction is to be preferred for several reasons:

(i) contaminants present in the test fluid and interfering with the enzyme reaction are easily removed;

(ii) the enzyme reaction can be allowed to proceed under optimal conditions, e.g. pH, and

(iii) the conjugate need not be completely free from unconjugated enzyme.

SPECIFICITY

Immunological specificity is one of the basic assets of a good immune test and EIA is no exception to this rule. General aspects of specificity of EIA's have been discussed earlier (22). It suffices to give a few capita selecta from recent experimental data.

In our laboratory we made a careful comparison between RIA and EIA for human chorionic gonadotrophin (HCG). Two highly purified HCG preparations were labelled with ^{125}I and with HRP, and combined with two antisera obtained from different rabbits immunised with different HCG preparations. It appeared that the results of EIA and RIA did not differ significantly and that the antiserum effect which was found was equally prevalent in both EIA and RIA (25,26).

Cross-reactions are usually established by determining the ratio between the amounts of cross-reacting and parent compounds needed to give the same response. However, in the case of oestrogens it has been shown that this simple approach may lead to a considerable overestimation of the true amount of one oestrogen assayed in the presence of other oestrogens (25). This was found for both RIA and

EIA. Such discrepancies may also occur in the assays of other haptens, so that "simple" cross-reaction data may give an over-optimistic picture of specificity.

SENSITIVITY

Too many variables are involved to allow general statements as to the sensitivity of EIA in comparison with that of other immunoassay techniques (22). The importance of some of such variables was recently illustrated for EIA's of steroids (27). Yet, statements have, for instance, been made that RIA is more sensitive than EIA (28, 29). That this generalization is not justified has already been shown by experimental data reviewed in 1977 (22). Newer data confirm this, for instance: RIA's and EIA's of hepatitis B surface antigen are of the same order of sensitivity (30, 31, 33-35); the same was reported for tetanus antitoxin (36); two groups of investigators have found EIA's of steroids to be more sensitive than the corresponding RIA (27, 37, 38); EIA's of triiodothyronine were reported which were less than a factor of two less sensitive than RIA (39), or equally sensitive (40). Of course, many EIA's described in the literature are less sensitive than corresponding RIA's but it does not seem likely that this difference is caused by an inherent difference between both types of assay. A more thorough discussion of the sensitivity of heterogeneous EIA can be found elsewhere (41).

In the field of microbiology EIA's are mostly compared with immuno-fluorescence, agglutination, complement fraction and several other methods. In these comparisons EIA scores very high indeed (22, 42-47).

The homogeneous EIA is less sensitive than the heterogeneous type assays and can until now not compete with RIA with respect to sensitivity.

PRECISION

Although data related to precision are often somewhat difficult to interpret it looks as if RIA and EIA do not differ very much in their attainable precision (22). Some data from our laboratory are collected in table 1. Recent papers in the literature confirm that the precision of both heterogeneous and homogeneous EIA's compare favourably with that of RIA (16, 32, 38).

PRACTICABILITY

Under this heading a number of points can be discussed.

Stability of the enzyme-labelled reagents has been shown to be high by many laboratories. This carries quite important advantages over radio-active labelled reagents:

(i) Standardisation of the test system needed because of change of label becomes less frequent, and continuity of results is better guaranteed.

(ii) Inter- and intra-laboratory comparisons become more meaningful since identical reagents can be used.

(iii) Loss of label because of infrequent or irregular use no longer occurs.

Apart from their limited shelf life radio-active labelled reagents have other limitations:

(i) Many countries are imposing rather strict rules for the transport, use and disposal of radio-active isotopes; requirements are defined for laboratory facilities (separate rooms, separate air conditioning,

TABLE 1

Coefficients of variation (CV) for RIA's and EIA's of human chorionic gonadotrophin (HCG), human placental lactogen and total oestrogens (TE) or oestriol (E_3)

Method	Reference	CV	
		within-assay	total
HCG			
EIA[1)]	2)	4,5	3)
RIA[1)]	2)	4,8	3)
RIA[1)]	60,61	6	7-11
HPL			
EIA[1)]	25	6,0	9,6
RIA[1)]	25	5,1	5,1
RIA	62,63	5,5-6,5	8
TE			
EIA[1)]	25,37	6,4	11,8
RIA[1)]	25,37	12,4	19,1
E_3			
RIA	64	7	9

1) The same antisera were used for RIA and EIA of each antigen

2) A.M.G. Bosch & H. van Hell, unpublished data

3) Data not available

seamless floors and tables, etc.), special training for personnel, special clothing, book-keeping of incoming and outgoing radio-active material. Work with radio-active isotopes is often subject to government licences. All such rules lead to higher capital investments and higher personnel costs. Health hazards of radio-active material are repeatedly discussed in scientific and political circles and may lead to further constraints.

(ii) Capital investments for radio-active equipment, particularly of automatic equipment allowing overnight counting of a large number of samples, are quite high. The use of ^{3}H requires scintillation fluid and special liquid scintillation spectrometers.

(iii) EIA's, in particular those done in the microbiological field, are often performed in microtitration plates. Use of such plates in connection with radio-isotopes, is impractical, since each well has to be cut apart and transferred into a counting tube prior to counting.

Assay times of heterogeneous EIA's and of RIA's are comparable with respect to both working and waiting periods. In principle, EIA's require extra time and handling for enzyme assay. On the other hand, colorimetric or fluorimetric determination requires less time than radio-active counting, when the degrees of automation of both are comparable. The assay time of homogeneous EIA's can usually be counted in minutes rather than in hours. In all assay types incubation times are in principle inversely related to the desired detection levels, so that assay times are often more related to the desired sensitivity than to the type of assay.

Automation potential is high for homogeneous EIA's. Heterogeneous EIA's and RIA's can be automated, but with some more difficulty because of the B/F separation. Automation of colori-

metric tests is easier than of radio-active counting in one respect: absorbances are measured instantaneously while counting requires minutes; the latter can therefore become the limiting factor in the whole assay process, particularly when high sensitivities are required.

Automation of tests on microtitration plates - so important for purposes of screening - is only possible for EIA's (see above). Some more data on automation of various test types can be found elsewhere (48, 49).

A comparison of EIA's with IF is in order for microbiological tests. IF has the advantage that fluorescent structures can be recognised under the microscope and used for the assessment of specificity. In EIA specificity has to be ascertained differently, e.g. by confirmation tests. On the other hand, recognition of histological structures requires know-how, cannot be expressed objectively, and is not suitable for quantitative interpretation. IF requires rather expensive fluorescence microscopes. The automation potential of IF is very low, so that IF is not suitable for large scale screening.

APPLICATIONS

Everything can be tested provided antibodies, the essential reagents, can be prepared. All antibodies can be tested provided one knows against what antigen or hapten they are directed. These statements are, in principle, valid for all immune tests. They are also valid for EIA's. Of course, test characteristics as discussed above play an important role in the choice of a test.

Various recent review articles (22, 42, 50, 51) or books (14, 16, 32, 52) summarise the fields of application:

microbiology including virology and parasitology

- antibodies against microorganisms
- antigens from microorganisms

endocrinology

- peptide hormones
- steroids
- others

serum proteins (normal and pathologic)

- albumin
- immunoglobulins
- ferritin
- lipoproteins
- blood clotting factor
- complement components
- pregnancy-specific proteins
- rheumatoid factor
- immune complexes
- auto-antibodies (see also ref. 53, 54)

cancer antigens

drugs

allergens

NEW DEVELOPMENTS

Most microbiological tests are aimed at the diagnosis of human or animal disease. However, EIA's for detecting plant viruses have also been described (e.g. ref. 55). Untreated plant extracts can be tested in microtitration plates, such tests being suited to large scale screening.

Detection and estimation of defective phages is difficult and time-consuming: couting can be done either by the use of prelabelled

phages or by electron microscopy. A sandwich type EIA was developed which was able to detect virus at a lower detection level than was hitherto possible (56).

An EIA for the detection of immunoconglutinin was recently reported (57). The test was based on the following principle (for explanation see caption to figure 1):

$$\vdash\!\!\!-C3 + Ab_1 + Ab_2^{*} \longrightarrow \vdash\!\!\!-C3 \,.\, Ab_1 \,.\, Ab_2^{*}.$$

Until recently, homogeneous EIA had only been reported for hapten. A recent paper describes the application of this test type on a large molecule, staphylococcal enterotoxin (58).

Another type of homogeneous EIA is based on what is called coenzymic cycling reactions. This test principle is different from the other discussed in this review in that it is a coenzyme rather than an enzyme which is coupled to an immune reactant, viz. a hapten. The coenzyme in the conjugate is inhibited by antibody against the hapten. This inhibition is lifted by reaction of unconjugated hapten with the available antibody. In the latest publication on an oestriol assay a sensitivity of 2 pmol/ml is claimed. The system has not yet been applied on serum, and precision and accuracy data are not yet available (59).

CONCLUSION

The term EIA covers a whole series of test systems which are being developed or already applied for a large variety of purposes. Looking at the flood of publications one can but conclude that EIA is becoming an increasingly important analytical tool in many a scientific field.

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