Journal of Solid-Phase Biochemistry, Vol. 4, No. 1, 1979

Review

RECENT DEVELOPMENTS IN HETEROGENEOUS ENZYME IMMUNOASSAY

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Accepted January 4, 1979

INTRODUCTION

Radioimmunoassay (1) was the first binding assay utilizing the immunological reaction between an antibody and a labeled antigen. It took, however, more than a decade before an enzyme was used as the necessary label. The use of new marker molecules (2) was accompanied by another important advance, viz., the introduction of the solid phase as carrier of the binding protein or its counterpart. The solid phase made it possible to separate the bound from the unbound fraction and thereby obviated the need of physicochemical precipitating methods. It also facilitated the routine handling of the assay in that it simplified the centrifugation and washing steps. Several solid phases have been used in recent years, such as polyacrylamide, cellulose, glass, Sepharose, and other polysaccharides.

The solid phase in an enzyme immunoassay (EIA) must fill certain basic requirements (Table 1), varying with the type of EIA; e.g., low non-specific adsorption of the solid phase is always desirable, but high flow rate capacity is necessary only in nonequilibrium EIA in continuous flow systems (3,4). In the following sections different types of heterogeneous EIA are discussed.

TABLE 1. **Criteria for a Solid Phase to be Used in EIA**

1. High chemical stability
2. Nonreactive, i.e., low nonspecific adsorption
3. Available at reasonable cost
4. Easy to activate chemically and to which proteins can be easily attached.
5. High buffer flow rate capacity, which is necessary in a continuous flow system

0146-0641/79/0300-0057$03.00/0

TYPES OF EIA

Heterogeneous and homogeneous EIA are the two main categories of EIA and differ from each other in that bound and free label have to be separated in heterogeneous assay. The principles of the existing heterogeneous methods are outlined below.

Competitive EIA

This assay is based on the competition between enzyme-labeled antigen and native, unlabeled, antigen for a limiting number of binding sites offered by the antibodies. The bound antigens are separated from the free ones by a solid phase bound antibody or a second antibody, which precipitates the antigen–antibody complex. The enzyme activity of the bound fraction is then determined (5,6) and related to the amount of native antigen. A disadvantage of competitive EIA is that it may sometimes need a purified antigen when setting up the method. In the sequential saturation approach the addition of labeled antigen is delayed until the binding between antibody and native antigen is complete (7). This delay can increase the sensitivity of the assay. (Fig. 1).

Sandwich EIA

In this technique the native antigen reacts with an excess of solid phase bound antibody and after the reaction products have been washed an enzyme-labeled immunoglobulin is added. The activity of the bound enzyme is directly proportional to the amount of native antigen in the sample (8). An advantage of the sandwich EIA is that it does not require purified antigen, but its application is limited by the fact that the antigen to be assayed has to have at least two antigenic determinants.

If one wishes to use a more universal enzyme conjugate, the sandwich can be extended by a third labeled antibody consisting of an anti-immunoglobulin antibody. This antibody reacts with the unlabeled second antibody already bound to the antigen, after which the enzyme activity can

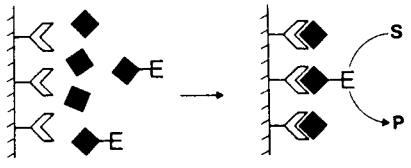

FIG. 1. A schematic presentation of competitive EIA. Symbols used: open chevron, antibody; diamond, antigen; **E**, enzyme; **S**, substrate; **P**, product.

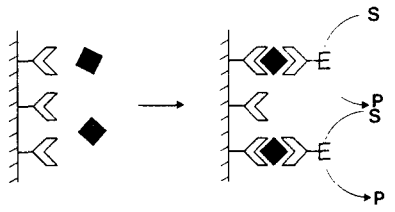

FIG. 2. A schematic presentation of sandwich EIA. Symbols are the same as in Fig. 1.

be monitored and related to the amount of antigen in the sample (Fig. 2). The above methods can also be used for antibody quantification in much the same manner (9).

Immunoenzymometric Assay

In the conventional immunoenzymometric assay (IEMA) the native antigen reacts first with an excess of enzyme-labeled antibodies and, in a subsequent step an excess of solid phase antigen is added to react with the remaining labeled antibodies. After separation from the solid phase the enzyme activity of the soluble immunocomplex is proportional to the antigen concentration (10).

A new approach to the conventional IEMA utilizes an enzyme labeled antiimmunoglobulin antibody in largely the same way as in sandwich EIA. The enzyme-labeled antiimmunoglobulin antibody is added to the sample specific antibody already bound to the solid phase antigen. The enzyme activity measured on the solid phase is related to the antigen concentration. The usefulness of this technique is illustrated by the determination of triiodothyronine, which was detectable down to 0.1 nmol/l (11). An advantage of IEMA is that monovalent drugs/hormones can be assayed with it. Further, with the use of labeled antiimmunoglobulin antibodies the preparation of conjugate is convenient and more practical, since the same conjugate can be used for the assay of several different antigens (Fig. 3). An IEMA for determination of antibodies based on the same principles as those used for antigens has also been described (12).

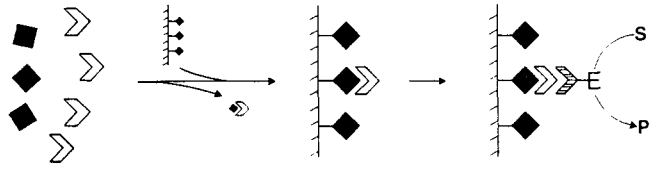

FIG. 3. A schematic presentation of the immunoenzymometric assay. Symbols used: hatched chevron, anti-antibody; for other symbols, see Fig. 1.

Other Types of EIAs

Competitive enzyme-linked immunoassay (CELIA) has recently been used for detecting human chorionic gonadotropin, testosterone, and rubella antibodies (13). This method uses a specific antibody that is competitively bound to free native antigen and to immobilized antigen. In a subsequent step an antiimmunoglobulin antibody is added and, after washing, a further amount of antienzyme antibody-enzyme complex is added. The antibody–enzyme complex binds itself to the second binding site of the anti-immunoglobulin antibody. The amount of bound enzyme can then easily be correlated to the amount of free antigen in the sample (Fig. 4).

In the quantification of specific antibodies in a sample, defined antigen substrate spheres are used with peroxidase-labeled antiimmunoglobulin antibodies as marker (14). Antigen coupled to agarose beads was incubated with serum containing the antibody; the reaction products were then washed and incubated with peroxidase-labeled antiimmunoglobulin antibodies. The result of the enzymic reaction could be readily observed with the naked eye. With this approach anti-*Schistosoma mansoni* antibodies have easily been field-tested in human patients.

NEW ASPECTS OF EIA

Since the principles of radio immunoassay (1) as well as EIA (15,16) were first established, many serious efforts have been made to shorten the time necessary to a single assay, some of which are discussed below.

Magnetic Solid Phases

The use of solid phase antibody in heterogeneous EIA, e.g., competitive and sandwich EIA, simplifies separation. The density of the solid phase facilitates the necessary washing steps using a centrifuge. However, even the many washing/centrifugation steps, which are time consuming and laborious, may prove unnecessary thanks to the introduction of a magnetic

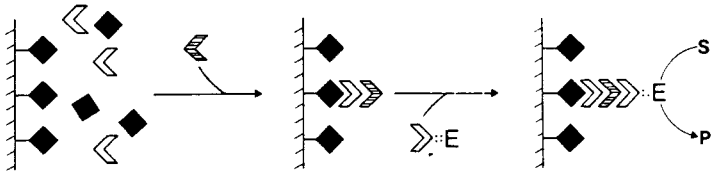

FIG. 4. A schematic presentation of the competitive enzyme-linked immunoassay (CELIA). For symbols used, see Fig. 3.

TABLE 2. **Criteria for use of a Magnetic Solid Phase in EIA**

1. Incorporation by the matrix of large amounts of magnetic materials
2. Easy to activate and to which proteins can be easily attached
3. Relatively high mechanical stability, to prevent the beads from breaking, with consequent interference by the magnetic material
4. Easy to produce in a homogeneous size and amount of incorporated magnetic material

solid phase (17–19). On application of an external magnetic field the gel particles settle and are held in place, while the washing medium, for example, is decanted. Compared with the conventional centrifugation, this procedure is time saving (18) and can easily be introduced as a routine step in automated procedures. To be acceptable under assay conditions the magnetic solid phase must fill certain basic requirements (Table 2).

The magnetic solid phase that has been used most extensively is polyacrylamide-agarose beads with incorporated Fe_3O_4 (18), but other potential solid phases, for examples, might be a copolymerizate of 2-hydroxyethyl metacrylate, acrylamide and Fe_3O_4 (20), or an entrapped ferrofluid in agarose beads (21).

Inhibition EIA

In inhibition EIA the antigen–antibody interaction between a solid phase bound antigen and an enzyme-labeled specific antibody can be inhibited by a sample containing free native antigen. The degree of inhibition can be measured from the enzymic activity detected on the solid phase and related to the amount of native antigen in the sample (Fig. 5).

Since the method is a one-step EIA, using only one incubation, it is one of the most rapid heterogeneous immunoassays available today. In determination of IgG (22) the wells of microtiter plates were coated with pure IgG (5 μg IgG/ml), and a standard or an unknown IgG solution (0.1 ml) was added. This was immediately followed by addition of the enzyme-labeled anti-IgG antibodies. The plates were incubated for 1 h at 35°C by which time 80% of the maximum possible binding had occurred. The enzyme activity in

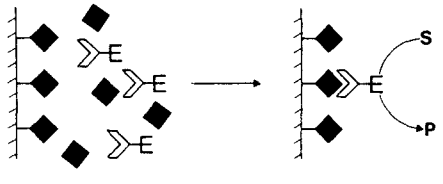

FIG. 5. A schematic presentation of the inhibition EIA. For symbols used, see Fig. 1.

each well was afterwards measured with a photometer and IgG concentrations down to 50 ng/ml could be determined.

Nonequilibrium EIA in Continuous Flow Systems

Continuous flow systems have been used in chemical analysis because they lend themselves well to automation. In recent years this approach has been tried in the field of EIA, and thanks to the isokinetic reaction conditions of a continuous flow system, i.e., well-defined time of contact between antibody and antigen, pH, temperature, etc., it has been possible to use a nonequilibrium approach.

Continuous Flow Tubing Systems. Attempts have been made to design a completely automated continuous flow tubing system. An example is the "Southmead system" (23). However, to be practicable an automated system must fill certain requirements such as the following: (1) it should be easily applicable to a variety of assays, (2) manual steps in an assay should be limited, (3) the assays should be of high precision, quick and inexpensive.

In the Southmead system the antibodies are coupled to agarose beads (diam 40–70 μm, in dry form) and mixed with labeled antigen and free native antigen. After having been mixed and incubated (15 min at 38°C) in an air-segmented continuous buffer stream, the mixture, consisting of an agarose-bound as well as an unbound antigen fraction, is conducted to a separation block. Separation of bound from free antigen takes place continuously with the aid of a membrane (10 μm pore diam) and the stream containing unbound antigen (free as well as labeled antigen) can then be used to quantitate the free antigen in the sample by measuring the enzymatic activity. The percentage of unbound fraction misclassified in the separation block is negligible (<1%). In conclusion, this system operated 40 samples per hour and has been used to detect thyroxine down to 5 nmol/l. It can further be used in different types of EIA or radioimmunoassay (RIA), e.g., sandwich, competitive techniques etc.

Packed Bed Reactors in Continuous Flow. A packed bed reactor placed in a continuous flow yields a system quite different from the continuous flow tubing systems discussed above. A column packed with antibodies bound to Sepharose is connected to a continuous buffer flow. The principle is that of competitive EIA, and after introduction into the continuous buffer flow of the free native antigen as well as enzyme-labeled antigen, a competitive situation is created when the sample passes through the packed bed reactor. The competition for the available antigen binding sites takes place during a time of 0.2–1.0 min which is considered to be under nonequilibrium conditions.

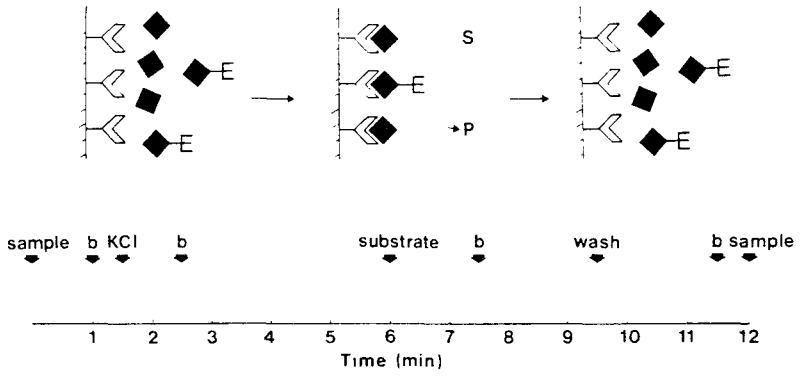

FIG. 6. Diagram of a reaction cycle in the nonequilibrium procedure. The arrows indicate changes in the perfusing medium (flow rate 0.75 ml/min). The cycle starts with a buffer flow (b) and at this time the packed bed reactor contains only immobilized antibodies. The arrow "sample" denotes addition of a mixture of free native antigen and enzyme-labeled antigen. The system is then washed with buffer and 1 M KCl. The antibodies in the reactor are now occupied by native as well as by enzyme-labeled antigen. After measurements of the enzyme activity the system is washed (0.2 M glycine/HCl buffer, pH 2.2) and is ready for another assay.

On application of a pulse of substrate the enzyme reaction occurring in the column is due only to the marker enzyme molecules and is therefore easily related to the amount of free antigen in the sample. After one assay, the antibody–antigen complex is dissociated and the immunosorbent is ready for another assay. A diagram of the assay procedure is given in Fig. 6. This procedure has been used for detecting both haptens and protein antigens, and the advantages over conventional ELISA are, e.g., that one assay takes only 8–12 min, the antibody preparation is reusable, and there are no extensive washing steps. In its present stage of development the method is suited only for manual use, which means that it can analyze only a limited number of samples a day. It may be useful in emergency situations, for example, when close monitoring of a pharmaceutical preparation is desired. However, automation is an obvious possibility, and this would increase the amount of samples that can be handled.

That isokinetic reaction conditions, i.e., time, temperature, sample volume, and antibodies, do not vary from assay to assay, is characteristic of continuous flow packed bed systems, and this also allows nonequilibrium conditions to be used during assays. The solid phase bound antibody can be reused since the column is regenerated, i.e., the antigen–antibody interaction is dissociated. The regeneration step is made possible by the use of a medium capable of breaking antigen–antibody associations (2). An alternative approach to reusable antibodies is the reversible immobilization

technique (24,25). Spectrophotometric analysis (4,23) and recent developments in enzymatic analysis, based on immobilized enzymes, e.g., thermometric analysis (26–29) and specific electrodes (30), have been used as detection devices in continuous flow systems.

MISCELLANEOUS ASPECTS OF EIA

Electrodes, besides the immunoelectrodes in combination with continuous flow (31), can be used as sensors in EIA. Examples of this use are:

1. Indirect measurement of the antigen–antibody interaction using a chemical amplifier (32–35). In this approach the conventional EIA techniques, e.g., sandwich and competitive EIA, are used. If a suitable enzyme, e.g., horseradish peroxidase (HRP), or catalase, is used as antigen label, the amount of enzyme bound to the solid phase can easily be estimated. For example, membrane bound catalase enzymatically generates oxygen, which can be monitored by an oxygen sensitive sensor (36) (Fig. 7). HRP can be used in the same way except that the detection device in this case is a photometer (35).

2. Direct measurement of the antigen–antibody interaction (37–40). In aqueous solutions proteins act as polyelectrolytes having a net electrical charge polarity. When an antigen combines with the specific antibody its electrical charge polarity changes. If an antigen is attached to a membrane that covers the sensor of the electrode, it would be possible to measure the

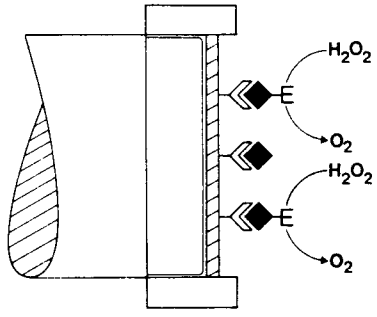

FIG. 7. A schematic presentation of the sensor part of an electrode. The membrane bound antibodies have participated in a competitive binding assay, and the oxygen generated by the marker enzyme is measured.

antigen–antibody association potentiometrically against a reference electrode immersed in the same solution.

There are other ways as well of measuring the antigen–antibody interaction (33,41). For instance, the membrane of an electrode responsive to the hapten trimethylphenyl-ammonium ion ($TMPA^+$) can be coated with $TMPA^+$. Direct antigen–antibody binding can be measured and binding constants can easily be estimated by this method (36), one advantage being that it is much quicker than the conventional dialysis technique.

CONCLUDING REMARKS

Many of the methods discussed in this paper were developed to simplify the assay procedure and to shorten the time necessary for one assay. These goals have been reached, but at the expense of sensitivity. However, increased reproducibility has been gained, and in their present stage of development the methods are quite easy to automate in largely the same way as radioimmunoassay. Furthermore, this development in solid phase immunoassay has been accompanied by the elaboration of new techniques for nonequilibrium assays in a homogeneous system. These methods operate in quite the same concentration range as nonequilibrium solid phase immunoassays. The possibilities of automation of these methods are also good since they can be applied in conventional enzyme assay technology. These new developments, combined with improved methods for producing conjugates, makes the future for quick simplified enzyme immunoassay quite promising.

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