CHROMBIO. 2944

PARTICLE-LABELLED IMMUNOASSAYS: A REVIEW

T.C.J. GRIBNAU*, J.H.W. LEUVERING and H. van HELL

Organon International B.V., P.O. Box 20, 5340 BH Oss (The Netherlands)

SUMMARY

Up to now, various types of particles have been used as labels in immunoassay. Well known examples are erythrocytes and latex particles. More recently, colloidal gold and dye particles have been introduced as a label. Each type of particle offers one (or more) method(s) of detection which depend(s) on the physical properties of the particles. In this paper, the present state-of-the-art regarding particle-labelled immunoassays will be reviewed.

INTRODUCTION

Immunoassays are based on the reaction of an antibody with the corresponding antigen. If both reactants are present at a sufficiently high concentration, and in the right proportion, this reaction leads to a visible precipitate. The concentrations at which antigens and antibodies, relevant for diagnostic purposes, occur in body fluids are usually too low, however, to produce a visible effect. Immunoassay development has concentrated, therefore, on ways to detect the primary antigen—antibody reaction by means of secondary phenomena. Almost invariably, this objective has been achieved by labelling the antigen or antibody with compounds or particles which allow easy or extremely sensitive detection.

Much attention has been focused on sophisticated methods like radio-immunoassay (RIA), enzyme immunoassay (EIA), fluoroimmunoassay and chemi-, thermo- and bioluminescence immunoassay. These methods enable the measurement of low levels of analyte, but are often relatively complicated and require rather expensive equipment. Less attention has been given to earlier methods in which particles (such as erythrocytes or polystyrene latex spheres) were used as label for the antigen or antibody. These methods, however, have the advantage of simplicity of performance combined with a sensitivity which is sufficient for many diagnostic applications. Such characteristics account for the widespread use of these methods (e.g. for blood group typing, pregnancy

and rheumatoid factor tests), and for further attempts to improve the assay performance.

During the last decade, the concepts of (latex) particle counting immuno-assay, PACIA® (recently renamed IMPACT®), sol particle immunoassay (SPIA) and disperse dye immunoassay (DIA) were described. These techniques have the potential to increase the number of analytes that can be detected or quantitated by means of particle-labelled immunoassays. The following sections contain more detailed information concerning the present state-of-the-art regarding particle-labelled immunoassays.

IMMUNOASSAY SYSTEMS USING ERYTHROCYTES

The ability of erythrocytes to agglutinate was first used for blood group typing [1]. Antibodies in serum of persons with blood group A react with antigens present on the erythrocytes of individuals with blood group B. The agglutination of the cells can be visualized by performing the reaction on a glass slide. The reaction, in which the reacting antigen is a natural part of the cell surface, was called haemagglutination (H). When an antigen, artificially attached to the cell, acts as the reactant the reaction is called passive haemagglutination (PH). Reverse passive haemagglutination (RPH) is the reaction of an antibody bound to the cell surface with its antigen. Better use of the sensitivity of the agglutination reaction of erythrocytes can be made by letting the cells sediment in test tubes or wells of microtitration plates. In the case of agglutination, a diffuse pattern is formed on the bottom of the tube or well, in the absence of a reaction a ring or button pattern is observed (Fig. 1). In tests depending on H, PH or RPH, a ring or button pattern therefore indicates a negative result; a diffuse pattern indicates a positive result.
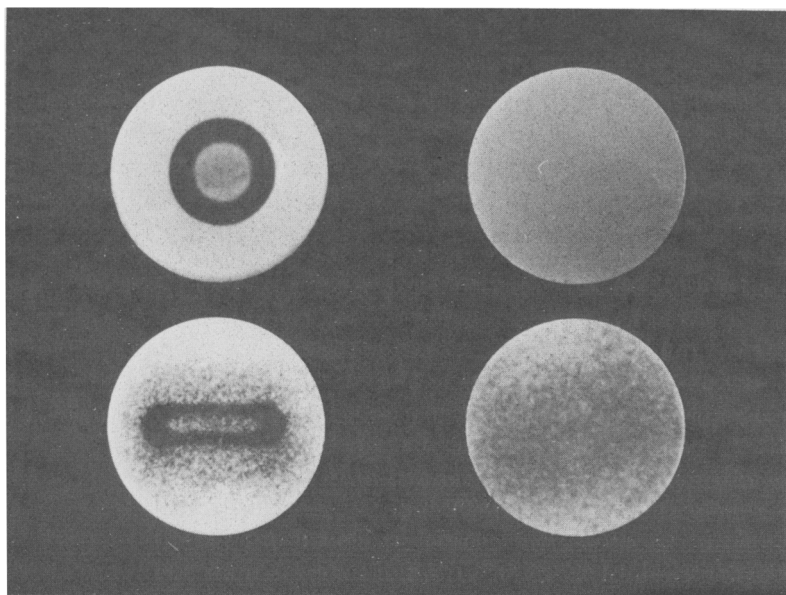

Fig. 1. Sedimentation patterns of tube tests using erythrocytes.

Tests with red blood cells can also be performed in the inhibition mode: antigen in the sample inhibits the reaction of antibody in solution with antigen attached to the erythrocytes. These types of tests are called passive haemagglutination inhibition (PHI) tests. A ring pattern (i.e. the absence of agglutination) in this case signals the presence of antigen, i.e. a positive result. To avoid confusion in the interpretation of results with HI and H tests, we recently introduced a specially designed tube in an RPH test that produces a minus-like sedimentation pattern in the absence of agglutination, signifying a negative result (Fig. 1).

Fresh or stabilized erythrocytes can be used to bind antigens or antibodies. Stabilized erythrocytes are usually preferred since they can be stored for longer periods. Stabilization can be performed by treatment with aldehydes or sulphosalicylic acid, and numerous procedures and modifications have been described.

The cell surface of erythrocytes appears to allow the attachment of many different antigens. Since the sensitivity of assays using erythrocytes depends on the amount of antigen or antibody bound per cell, the method used to adsorb or bind the protein to the cell surface is of great importance [2]. There are three types of coating or sensitization of red blood cells for immunoassay: (i) the direct adsorption of antigen by the cells, (ii) the tanned cell technique and (iii) covalent binding. The first method can be used for most proteins, but the sensitivity of the resultant cells can be much improved by previous tanning. The tanned cell technique [3], in which fresh cells are treated with tannic acid, greatly enhances the amount of antigen that can be adsorbed, but also increases the tendency of the cells to agglutinate non-specifically. This tendency to self-agglutination can be reduced by the addition of normal serum. Various methods have been developed to bind antigen or antibody covalently to the cells, such as those using bis-diazotized benzidine [4], difluorodinitrobenzene [5], toluene 2,4-diisocyanate [6], carbodiimide [7], bis-diazotized o-dianisidine [8] or, indirectly, via lectins [9].

Although many alternative methods exist, a common method of choice is still lacking. In the viral haemagglutination inhibition tests, use is made of viral receptors, which occur naturally on the surface of erythrocytes from many species. Antibodies against such viruses can be detected by their ability to inhibit the agglutination of such erythrocytes by the virus. Care should be taken to avoid interference of, for example, lipoproteins. Tests, like these, are widely used in the serology of rubella [10]. Other examples of haemagglutination tests are: (i) hepatitis B surface antigen (HBsAg) and antibody [11—13], used in particular for the large-scale screening of blood donors and (ii) haemagglutination inhibition tests for human chorionic gonadotrophin (hCG) in urine [14—16], to detect pregnancy (sensitivity: 1000 I.U. hCG per l urine). The latter test, and one for human lutropin (hLH) [17], was also used for the quantitative estimation of these hormones in urine.

By using RPH and two different monoclonal antibodies against hCG, one specific for the intact molecule and the other for the β-subunit, the sensitivity could be improved to 75 I.U. hCG per l urine ($\approx$ 10 ng/ml) [18].

These so-called tube tests are very popular because they are sensitive, easy to perform and need no equipment other than a few pipettes and a settling rack.

However, they are sensitive to vibrations that can disturb sedimentation patterns. A drawback of all assay systems using erythrocytes lies in their production. The red blood cell is a natural product and exhibits variable characteristics. Consequently, it is still difficult to manufacture tests with defined properties over long periods of time.

Various other types of immunoassay (e.g. mixed agglutination, rosetting reactions, solid-phase aggregation of coupled erythrocytes) in which erythrocytes are used as a label have been reviewed by Coombs [19].

IMMUNOASSAY SYSTEMS USING LATEX PARTICLES

These assays make use of the agglutination of antigen- or antibody-coated polystyrene (latex) spheres caused by analyte added to the aqueous suspension of particles. The first application [20] was for the detection of rheumatoid factor (RF). The reaction was carried out in a test tube and the agglutination of the particles could be observed with the naked eye.

Further development proceeded in two directions: the first led to the further perfection of tests that could be read by the naked eye, while the second aimed at the development of equipment to measure the agglutination in the initial phase of the process. For the visual detection, a number of tube tests were developed for RF [20], anti-DNA antibodies [21], hCG [22] and Brucella antigen and antiserum [23]. The real advantage of this new particle, however, only became apparent when the agglutination was allowed to develop on a slide. A drop of reagent, containing antigen- or antibody-coated latex in suspension, was mixed on a flat surface (a slide) with a drop of sample (urine, serum or plasma). After gentle rocking of the slide for 2 or 3 min, the agglutination pattern could be observed visually.

These so-called latex tests became very popular in serology, and as pregnancy tests, although they were generally less sensitive than the tube tests using erythrocytes.

Recently, it has been shown that by using direct agglutination, with smaller particles and monoclonal antibodies, the sensitivity of latex slide tests for hCG can be increased to ca. 250—500 I.U. ($\approx$ 25—50 μg) hCG per l urine. This example illustrates that improvements are possible. However, the formation of a visible agglutination in such a short time still requires a relatively high concentration of the substance to be measured, which explains the lower sensitivity of slide tests as compared with RIA and EIA. Obviously, the sensitivity of latex tests could be greatly improved if an earlier phase of the agglutination, invisible to the naked eye, could be detected. Quasi-elastic light-scattering spectroscopy [24] measures the average diffusion constant of the particles, which is a function of their size. A sensitivity of 10—20 μg antibody/l could be realized with this technique [25]. Since a visible agglutinate with the same reagents required a concentration of 2—5 mg/l, this technique yielded a 100-fold increase in sensitivity. A minimum concentration in urine of 16 I.U. hCG/l could be detected [26]. By using angular anisotropy measurement, the sensitivity could be further improved to 2 I.U. hCG per l urine [27]. However, when this technique was used for measurements in serum, non-specific agglutination and inhibitory effects were observed. New developments in this

area became possible by the introduction of immunoassay by particle counting (IMPACT) [28]. This technique measures the number of residual non-agglutinated particles by means of equipment designed to count blood cells. Using the immunoglobulin G (IgG) fraction of goat antisera against human placental lactogen (hPL) and α-fetoprotein (AFP) to coat latex particles, threshold sensitivities of 10 $\mu g/l$ of buffer were attainable for these antigens. However, to suppress serum interference, the samples had to be treated with dithiothreitol and subsequently diluted, which reduced the sensitivity to 100 $\mu g/l$. The use of F(ab')$_2$ fragments to coat latex particles and a high ionic strength in the reaction medium considerably reduced the interference from serum. In this way the dose range for AFP could be improved to 10– 500 $\mu g/l$ of serum [29].

The combined use of F(ab')$_2$ fragments of a chaotropic agent such as ammonium thiocyanate and of EDTA enabled ferritin to be detected at a lower limit of 13 $\mu g/l$ of serum [30]. The assay of C-reactive protein in serum, cord serum and cerebrospinal fluid was reported recently [31]. Sera were diluted ten times and cerebrospinal fluid twice with an assay having a range extending from 1 to 100 $\mu g/l$. IMPACT obviously has several advantages, such as: (i) no separation steps; (ii) short incubation times; (iii) fully automated process. A disadvantage is the need to use large and expensive equipment and the sensitivity obtained does not completely match that of RIA and EIA.

IMMUNOASSAYS USING INORGANIC COLLOIDAL PARTICLES

The use of colloidal particles, consisting of a metal (or an insoluble metal compound) as a label in immunoassay has been introduced by Leuvering et al. [32]. Since a dispersion of colloidal particles in a liquid is called a "sol", this technique was called "sol particle immunoassay" (SPIA).

The literature provides many examples of colloidal particles which, in principle, can be used as a label in immunoassay, e.g. gold, silver, silver iodide, barium sulphate, etc. [33]. Because of its special optical properties, absence in body fluids, and a suitable method for preparation, gold was eventually chosen by our research group. Immunochemically reactive gold particles are obtained by adsorption, under specific conditions, of antibody molecules (Ab) onto the surface of 50-nm gold particles (Au$_{50}$—Ab). This type of labelled antibody can be used in both heterogeneous and homogeneous immunoassays. The main advantages of SPIA with respect to other types of labels are: the possibility to use various methods of detection, including visual inspection; the use of a non-isotopic label; a better stability than, for example, enzymes; and, in contrast to enzymes, no need for an extra chemical reaction.

Heterogeneous SPIA

In heterogeneous immunoassays, a separation of the free and bound fractions of the labelled immunocomponent is required. The "sandwich" and "sandwich inhibition" SPIA belong to this group (Fig. 2). In the sandwich SPIA, the magenta colour of Au$_{50}$—Ab dispersions is used to detect the presence of an antigen by visual inspection or to measure its concentration using a colorimeter to measure the absorbance of the bound [32] or free [34]
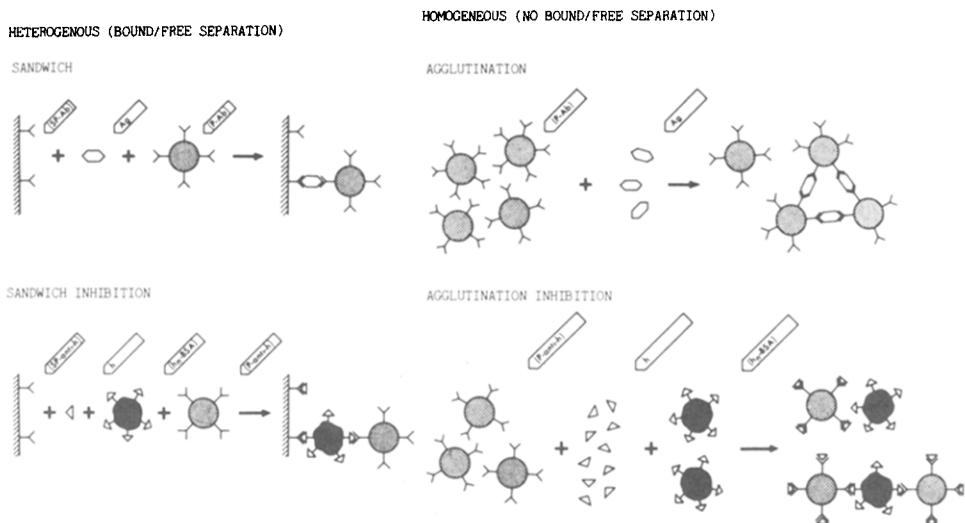
Fig. 2. Schematic representation of the various SPIA models. SP = solid phase; h = hapten; Ab = antibody; anti-h = antibody to the hapten; Ag = antigen; BSA = bovine serum albumin; P = particle. (Reprinted with permission from ref. 33.)
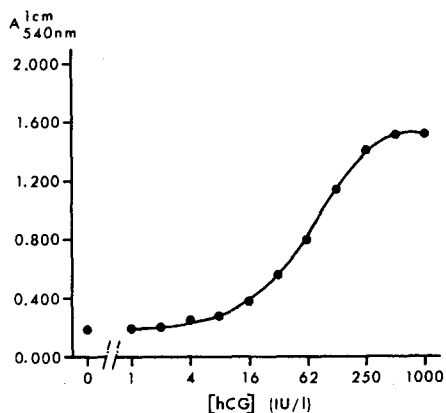

Fig. 3. A typical dose—response curve for a colorimetric sandwich SPIA. The absorbance of the bound fraction of Au_{50}—(anti-hCG) was measured after dissociation of the immune complex.

fraction of the added amount of Au_{50}—Ab. Since the particles consist of a metal (gold), atomic absorption spectrophotometry (AAS) could also be used for detection [32, 33]. With this method, it is possible to develop simultaneous sandwich immunoassays for two non-cross-reacting antigens using gold and silver particles as labels [32]. A typical dose—response curve of a colorimetric sandwich SPIA for hCG is shown in Fig. 3. The limit of detection of the antigen dissolved in buffer depends on the method by which the bound fraction of Au_{50}—Ab is measured: visual inspection 170 pmol/l [32], colorimetry 5 pmol/l [34] and AAS 0.02 pmol/l [33]. These detection limits were obtained, using hCG and hPL as models, with a sandwich SPIA requiring an overnight incubation with the Au_{50}—Ab dispersion. Shorter incubation periods are possible but result in less sensitive assays.

In a preliminary study [33], it was demonstrated that it is possible to develop a colorimetric sandwich inhibition SPIA for haptens such as testosterone.

Homogeneous SPIA

All homogeneous SPIAs are based on agglutination or agglutination inhibition of antibody-coated gold particles. In such homogeneous assays, separation of the bound and free labelled immunocomponents is not necessary. These types of immunochemical reactions are well known from haemagglutination (inhibition) and latex agglutination (inhibition) immunoassays.

Various methods of detection can be used in combination with agglutination (inhibition) SPIAs, based on the use of Au_{50}—Ab dispersions. As mentioned before, such dispersions in a buffer have a magenta colour. However, when the properly dispersed particles are brought to aggregate, the magenta colour of the dispersion is reduced or even completely disappears, depending on the degree of aggregation. This is a typical characteristic of gold sols [35] and is based on a change in the reflection and transmission of light falling onto the dispersion. This colour change can be easily detected by eye. One method to force the Au_{50}—Ab particles to aggregate is to add a suitable amount of the proper antigen to the dispersion. As aggregation of particles in an immunochemical reaction is called agglutination, this technique is therefore called agglutination SPIA [36, 37].

The absorbance of the reaction mixture of an agglutination SPIA can also be measured by a colorimeter. By means of a calibration curve, the concentration of the antigen can be measured [36, 37].

Haptens, e.g. steroids and many drugs, are immunochemically monovalent, and cannot be detected by a direct agglutination assay. By coupling hapten molecules to a carrier, e.g. bovine serum albumin (BSA), an immunochemically multivalent hapten complex may be obtained which, like bi- or multivalent antigens, agglutinates gold particles coated with antibodies against the hapten. The agglutination of the gold particles coated with anti-hapten antibodies by the hapten—BSA complex may then be inhibited by free hapten molecules from a sample or a standard solution.
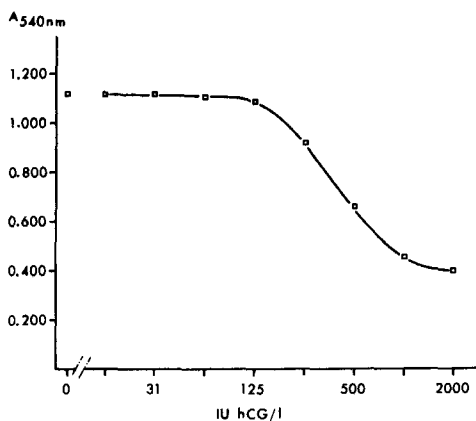

Fig. 4. An example of a dose–response curve obtained in an agglutination SPIA by measuring after a fixed incubation period the absorbances at 540 nm of reaction mixtures containing various amounts of hCG.

The degree of agglutination of the anti-hapten antibody-coated gold particles is related to the concentration of the hapten, and reflected in a change of the colour of the reaction mixture and its absorbance at a particular wavelength [38]. A typical dose—response curve is shown in Fig. 4. It is evident that the agglutination (inhibition) SPIA can also be used to detect antibodies against a particular immunogen.

Both types of homogeneous SPIA have a high practicability: only mixing of buffered reagent(s) and a sample aliquot followed by an incubation period of < 30 min are necessary. The result of the tests can be assessed either by means of a colorimeter or by eye, for tests performed in test tubes or in micro-titration plates [37]. Similar tests can be run on centrifugal analysers. With these analysers, incubation periods as short as 5 min can be used (unpublished results). Therefore, these simple techniques have two important future applications: qualitative (yes/no) tests which are suitable for use both at home and in the laboratory, and semi- or fully automated assays for use in laboratories only. Detection limits obtained with these simple test systems are shown in Table I. These results have been obtained in urine samples or in extracts from sera. The problem of interference owing to undiluted serum samples is not yet completely solved.

TABLE I

DETECTION LIMITS OBTAINED WITH HOMOGENEOUS SPIA (IN URINE)

Type of SPIA	Method of detection	Analyte	Detection limit (pmol/l)
Agglutination	Eye	hCG	750
	Colorimeter	hCG	160
Agglutination Inhibition	Colorimeter	Total oestrogens[*]	260

[*]After extraction.

IMMUNOASSAYS USING COLLOIDAL ORGANIC DYE PARTICLES

Hydrophobic colloidal dye particles have been used as labels in immuno-assays: disperse dye immunoassay or DIA [39—41]. These particles can be used as labels in two essentially different ways: (i) in an agglutination assay where the immunoreaction results in the formation of aggregated dye particles which can be detected visually or optically and (ii) in a sandwich assay, utilizing particularly the colour properties of the dye particles, which can be detected directly, or preferably after their dissolution into an organic solvent. Further experimental evaluation of agglutination assays based on dye sol particles [39] indicated, however, that gold sol particles were to be preferred in this type of assay. This is due to the considerably higher specific density of gold as compared to organic dyes, and to the fact that the aggregation of gold sol particles yields a characteristic change in colour.

The particular properties of colloidal dye particles are far better utilized, however, by applying them as label in sandwich-type immunoassays (Fig. 5).

$$\text{—Ab} + \text{Ag} \rightleftharpoons \text{—AbAg} + \text{Ab—}\oslash \longrightarrow$$

$$\text{—AbAgAb—}\oslash \xrightarrow[\text{solvent}]{\text{organic}} \begin{array}{l}\text{coloured solution,}\\ \text{measure absorbance}\end{array}$$

: solid phase (microtitration plate)   Ab: antibody

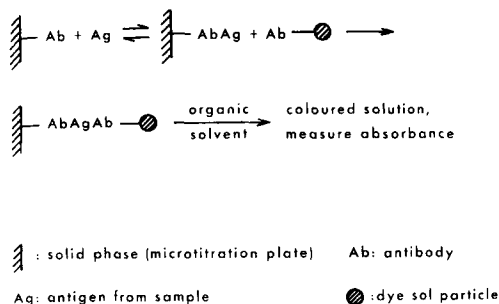Ag: antigen from sample   ⊘ : dye sol particle

Fig. 5. Reaction principle of a sandwich disperse dye immunoassay.

The specific advantage lies in the amplification of the detection signal (absorbance) achieved by the final dissolution of the dye particles, yielding a monomolecular dye solution. The advantage of dye sol labelling over molecular dye labelling (by covalent coupling of individual dye molecules to antibodies) is obvious. The number of dye molecules per immunocomplex is considerably higher (by at least 1000 times) in the former case, and the immunoreactivity of the antibodies is not impaired by the chemical modification [39]. In colorimetric, sandwich-type immunoassays, dye particles have an advantage over gold sols owing to the substantially higher molar absorbances of the organic dyes.

The organic—synthetic, chemically well defined, stable and non-radioactive dye particle label can be easily prepared from a wide range of commercially available disperse dyes. The labelling of antibodies is performed by simple physical adsorption, and several detection methods can be applied to the final determination of the label: visual observation, colorimetry, fluorimetry, (carbon rod) AAS (in the case of metal--complex dyes). Experimental aspects, generally important for the development of labelled sandwich immunoassays, equally apply to DIA: the type of solid phase (microtitration plate), the quality of the antibodies (specificity, affinity, source, poly-/monoclonal) and general assay conditions (incubation intervals, temperature, pH, concentration of reagents, sample interference). Particularly essential features of DIA are the properties of the dyes: particle size distribution and shape, solubility in appropriate organic solvents, value of the molar absorbance, colloid-chemical stability of the dye sol, conditions for adsorptive antibody immobilization, binding capacity of the dye particles for IgG, stability of the adsorptive dye particle--antibody bond [39, 40].

Commercially available disperse dyes [42] are taken as starting material for the preparation of the dye sols. These sols were prepared by centrifugal fractionation of the aqueous dye dispersions. The labelling of antibodies was performed by their adsorptive immobilization on the dye particles, followed by a secondary coating with BSA, yielding finally so-called "conjugates". The conjugates are stored in the lyophilized state. Aqueous conjugates should be used within six days of preparation.

Sandwich immunoassays are performed using antibody-coated polystyrene microtitration plates: sample incubation (30 min, 37°C), conjugate incubation (2 h, 37°C), dissolution of the bound dye label into an organic solvent,

measurement of absorbance. All experimental procedures, materials and reagents have been described in detail [39, 40]; recent investigations showed dimethyl sulphoxide to be a better alternative to 1-propanol.

Disperse dyes suitable for conjugate preparation can be found only by screening. The particular selection may also depend on the type of antibody preparation that is used. Good representatives are: Foron® Brilliant Blue SR (Sandoz), Terasil® Brilliant Flavin 8GFF (Ciba-Geigy), Palanil® Luminous Red G and Palanil Yellow 3GE (BASF). Optimal conjugates are obtained using dye sols with a particle size distribution corresponding to a modal effective diameter of $\approx$ 200 nm or a median value of $\approx$ 300 nm [40]. The shape of the particles should be as spherical as possible [39]. The pH during conjugate preparation is rather critical when mouse monoclonal antibodies are used, but is of minor importance in the case of polyclonal rabbit antibodies; the optimal pH is also dye-dependent. The type of dye and antibody also determine the value of the pH to be applied during conjugate incubation to achieve maximum response and a minimum blank [40].

The assay protocol for DIA (cf. Fig. 5) also depends upon the type of antibody used. Separate, sequential incubation of sample and conjugate is necessary in the case of polyclonal antibodies (DIA-I). Sample and conjugate can be incubated simultaneously when the conjugate is based on monoclonal antibodies (DIA-II), yielding a simplified assay procedure (Table II).

TABLE II

PROCEDURES FOR DISPERSE DYE IMMUNOASSAY (DIA)

DIA-I	DIA-II
Add sample	
Incubate: 30 min, 37°C	
Aspirate and wash	
Add conjugate	Add conjugate and sample
Incubate: 2 h, 37°C	Incubate: 2.5 h, 37°C
Aspirate and wash	Aspirate and wash
Add organic solvent	Add organic solvent
Measure absorbance	Measure absorbance

Representative results of both assay protocols for the hCG/anti-hCG system, using hCG-containing samples in buffer, (pooled) urine, (pooled) serum and diluted serum randomized over twelve microtitration plates, are given in Figs. 6 and 7. The detection limit (DL) for hCG in buffer was ca. 2 I.U./l in DIA-I (DL defined as the hCG concentration yielding a response equal to blank + 3 standard deviation).

Serum interference impairs the results, particularly in the case of DIA-II. There is also a considerable effect on the response when individual sera, spiked with the same hCG concentration, are used instead of pooled serum (Fig. 8). These effects are reduced by using $F(ab')_2$ fragments instead of total IgG for the preparation of coated microtitration plates and conjugates.

Most of the exploratory and developmental investigations have been performed using hCG/anti-hCG as a model system. Sandwich DIAs were also
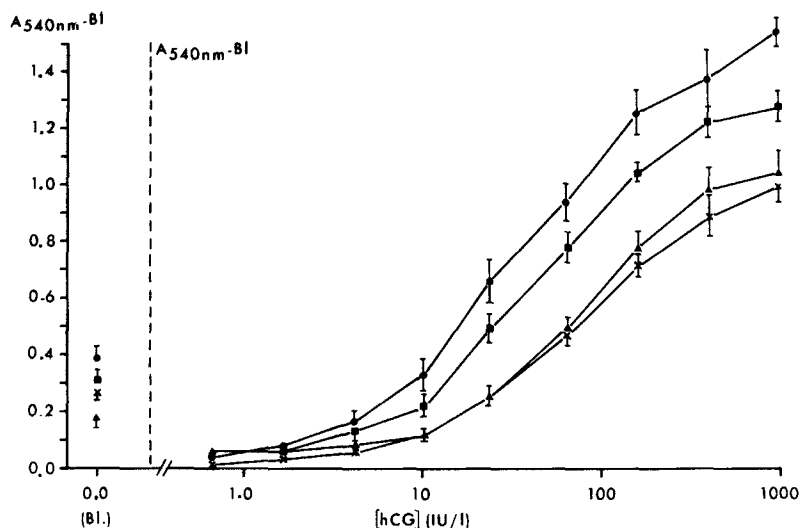
Fig. 6. Sandwich DIA-I for hCG using sequential incubation of conjugate and sample. Plate wells are coated with rabbit anti-hCG; conjugate is mouse monoclonal anti-hCG/Palanil Luminous Red G. All points represent the mean values for samples determined in six-fold. (●) Buffer; (▲) urine; (×) serum; (■) 10% (v/v) serum.
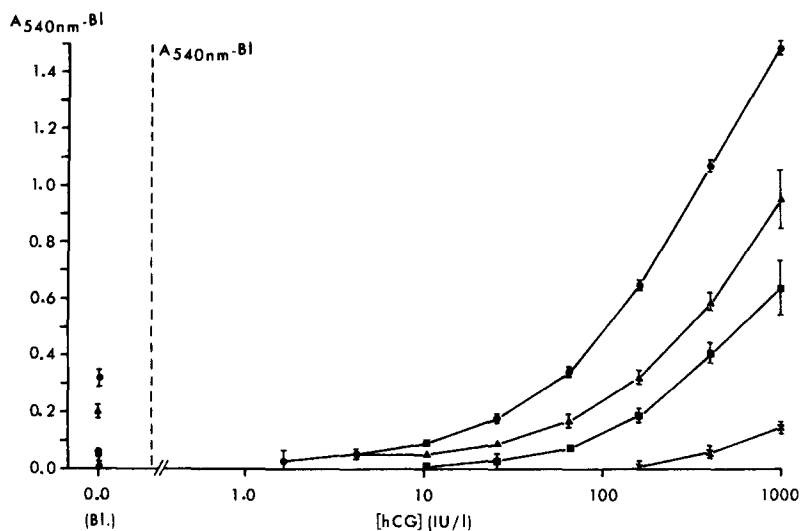

Fig. 7. Sandwich DIA-II for hCG applying simultaneous incubation of conjugate and sample. Conditions as in Fig. 6.

applied, however, for the determination of human placental lactogen (hPL), human prolactin and testosterone [39]. The determination of α-fetoprotein has been investigated using spiked, pooled human sera. A detection limit of 5 μg/l can be obtained, but the assay requires further optimization.

An attractive feature of the sandwich DIA is the possibility of determining two different antigens simultaneously, using the corresponding antibodies labelled with differently coloured dye particles, which are clearly
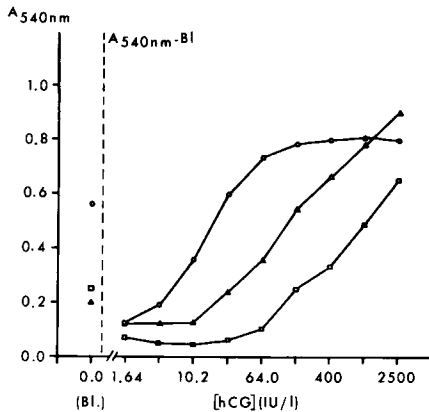
Fig. 8. Sandwich DIA-II for hCG with the simultaneous incubation of conjugate and sample; effect of serum interference. Plate wells coated with mouse monoclonal anti-hCG; conjugate is mouse monoclonal anti-hCG/Palanil Luminous Red G (different types of monoclonal anti-hCG were used for plate well and conjugate). (○) Buffer; (△) normal human serum 8307/82-19; (□) normal human serum 8433/82-312.

distinguishable spectrophotometrically. Blue and yellow chromophores, yielding various shades of green upon mixing, are particularly suitable in this respect. The high sensitivity of the human eye for various shades of green could possibly enable the development of eye-reading tests for the qualitative determination of antigen ratios. An example of the simultaneous determination of hCG and hPL has been described [39, 41].

DISCUSSION

The present applications of particle immunoassays, especially those based on the use of erythrocytes and latex particles, are in the field of eye-reading tests. Their sensitivities are adequate for use as screening tests, and because of their ease of operation they have obtained a dominant position in this area. The tests using erythrocytes are also suitable for semi-quantitative tests, since their sedimentation patterns allow statistical evaluation. A general problem of homogeneous assays is the presence of interfering factors, from the sample aliquot, which appear in the same solution where the final response is read. Therefore, they can affect both the primary reaction between antibody and antigen and the final reaction, agglutination and/or sedimentation of the particles. The use of antibody fragments, instead of whole antibodies, and the use of chaotropic ions can reduce the extent of sample interference.

Recent developments, summarized for hCG tests in Table III, have shown that improvements and innovations in the field of particle immunoassays are still possible. For example, the introduction of monoclonal antibodies in reverse agglutination tests led to a considerable improvement in sensitivity (i.e. to 75 I.U. hCG/l for tests using erythrocytes and to 250 I.U. hCG/l for tests depending on latex particles). The replacement of variable antisera by these new reagents also improved the reproducibility of manufactured tests. Developments in the field of latex particle assays have also taken place: (i) in the construction of sophisticated equipment to measure agglutination in the initial phases of the process, which has increased the sensitivity of the method and

TABLE III

PARTICLE IMMUNOASSAY SYSTEMS FOR hCG

Particle	Method	Sensitivity	Remarks
Erythrocyte	Reverse passive haemagglutination	75 I.U./l of urine	Simple procedure; assay time 2 h
Latex	Agglutination, eye reading	250 I.U./l of urine	Simple procedure; assay time 3 min
Latex	Agglutination, instrument reading	2 I.U./l of urine	Instrumentation necessary; complicated; expensive
Gold particle	Agglutination, eye-reading	450 I.U./l of urine	Simple procedure; assay time 0.5 h
Gold particle	Agglutination, colorimeter	100 I.U./l of urine	Simple instrumentation; assay time 0.5 h
Gold particle	Sandwich colorimeter	4 I.U./l of buffer	Simple instrumentation; assay time 4 h
Gold particle	Sandwich carbon rod atomic absorption spectrophotometry (CRAAS)	4 mI.U./l of buffer	Expensive instrumentation; overnight incubation
Disperse dye: Palanil Luminous Red G	Sandwich colorimeter	3.5 I.U./l of urine	Simple instrumentation; assay time 3 h

(ii) in finding measures to reduce the effect of interfering substances in biological samples, which again improved the practical sensitivity.

A recent innovation was the use of colloidal gold particles with a diameter of ca. 50 nm, coated with antibody, as an immunoreagent in SPIA. Various methods for assessing the result of a homogeneous agglutination SPIA are possible: the reduction of the specific absorbance of gold sols, brought about by agglutination, can be read by eye (yes/no tests) and by a spectrophotometer (quantitative tests). A practical advantage of the homogeneous SPIA is that after mixing reagent and sample it needs neither the rocking action of the latex slide test nor the disturbance-free settling period of the erythrocyte test. It is probably the easiest test to perform. By measuring the absorbance (at 540 nm) of the reaction mixture, a quantitative test for hCG in urine can be obtained with a sensitivity of 100 I.U./l. Sample interference is a problem in the agglutination SPIA. Applied in a sandwich-type assay, a detection limit of 1 I.U. hCG per l buffer (5 pmol/l) can be reached when colorimetry is used. Using AAS, a concentration of hCG in buffer of 4 mI.U./l (20 fmol/l) can be measured.

A new principle was introduced in particle immunoassay when disperse dyes were used for the preparation of conjugates. The high sensitivity of DIA is accomplished by dissolving the dye particles into an organic solvent in the final step of the assay. A sensitivity similar to or better than that reached with the

sandwich SPIA (4 h incubation) using colorimetry can be obtained. However, assays using different serum samples clearly showed that, again, serum interference reduces the practical sensitivity of this assay type. It seems that in assays using latex or gold sols, or disperse dyes, this problem must be solved before the sensitivity can be improved further.

In conclusion, we expect the agglutination SPIA to be the most promising new technique in the field of yes/no tests. The process which most clearly requires further improvement in the preparation of both sensitized erythrocytes and latex particles is the reproducible binding of antibody or antigen to the particle. Binding methods that prevent leakage of antibody or antigen from the particles, and which do not alter the immunochemical activity of the substance bound, may improve the sensitivity and reproducibility of particle immunoassays.

REFERENCES

1   K. Landsteiner, Wien. Klin. Wochenschr., 14 (1901) 1132.
2   R.M. Binns, S.T. Licence, B.W. Gurner and R.R.A. Coombs, Immunology, 47 (1982) 717.
3   S.V. Boyden, J. Exp. Med., 93 (1951) 107.
4   D. Pressman, D.H. Campbell and L. Pauling, J. Immunol., 44 (1942) 101.
5   N.R. Ling, Immunology, 4 (1961) 49.
6   L. Gyenes and A.H. Schon, Immunochemistry, 1 (1964) 43.
7   H.M. Johnson, K. Brenner and H.E. Hall, J. Immunol., 97 (1966) 791.
8   S. Avrameas, B. Tadou and S. Chuilon, Immunochemistry, 6 (1969) 67.
9   C.J. Sanderson, Immunology, 18 (1970) 353.
10 G.L. Stewart, P.D. Parkman, H.E. Hopps, R.D. Douglas, J.P. Hamilton and H.M. Meyer, N. Engl. J. Med., 276 (1967) 554.
11 G.N. Vyas and N.R. Shulman, Science, 170 (1970) 332.
12 I. Cayzer, D.S. Dane, C.H. Cameron and J. v. Denning, Lancet, 7 (1974) 947.
13 A. Schuurs and J. Kacaki, in M. Matthes and V. Nagel (Editors), Forschungsergebnisse der Transfusionsmedizin und Immunhaematologie, Medicus, Berlin, 1974, p. 645.
14 H. Strausser, N.J. Diss. Abstr., 20 (1959) 430.
15 L. Wide and C.A. Gemzell, Acta Endocrinol. (Copenhagen), 35 (1960) 261.
16 J. Polderman, Pharm. Weekbl., 97 (1962) 529.
17 A.H.W.M. Schuurs and C.J. van Wijngaarden, Acta Endocrinol. (Copenhagen) Suppl., 141 (1969) 13.
18 H. van Hell and J. Helmich, Int. Biotechnol. Lab., 2 (1984) 22.
19 R.R.A. Coombs, in A. Voller, A. Bartlett and D. Bidwell (Editors), Immunoassays for the 80s, MTP Press Limited, Lancaster, 1981, p. 17.
20 J.M. Singer and C.M. Plotz, Am. J. Med., 21 (1956) 888.
21 C.L. Christian, R.M. Bryan and D.L. Larson, Proc. Soc. Exp. Biol. Med., 98 (1958) 820.
22 J.L. Robbins, G.A. Hill, B.N. Carle, J.H. Carlquist and C. Marcus, Proc. Soc. Exp. Biol. Med., 109 (1962) 321.
23 L. Fleck and Z. Evenchik, Nature, 194 (1962) 548.
24 G.B. Benedek, Polarization, Matter and Radiation, Presses Universitaires de France, 1969, p. 49.

25   R.J. Cohen and G.B. Benedek, Immunochemistry, 12 (1975) 349.
26   K.G. von Schulthess, R.J. Cohen and G.B. Benedek, Immunochemistry, 13 (1976) 963.
27   K.G. von Schulthess, M. Giglio, D.S. Cannel and G.B. Benedek, Mol. Immunol., 17 (1980) 81.
28   C.L. Cambiaso, A.E. Leek, F. de Steenwinkel, J. Billen and P.L. Masson, J. Immunol. Methods, 18 (1977) 33.
29   D. Collet-Cassart, C.G.M. Magnusson, J.G. Ratcliffe, C.L. Cambiaso and P.L. Masson, Clin. Chem., 27 (1981) 64.
30   J.N. Limet, D. Collet-Cassart and C.G.M. Magnusson, J. Clin. Chem. Clin. Biochem., 20 (1982) 141.
31   D. Collet-Cassart, J.C. Mareschal, C.J.M. Sindic, T.P. Tomassi and P.L. Masson, Clin. Chem., 29 (1983) 1127.
32   J.H.W. Leuvering, P.J.H.M. Thal, M. van der Waart and A.H.W.M. Schuurs, J. Immuno-assay, 1 (1980) 77.
33   J.H.W. Leuvering, PhD Thesis, University of Nijmegen, 1984.
34   J.H.W. Leuvering, P.J.H.M. Thal and A.H.W.M. Schuurs, J. Immunol. Methods, 62 (1983) 175.
35   R. Zsigmondy and P.A. Thiessen, Das Kolloide Gold, Akademische Verlagsgesellschaft, Leipzig, 1925.
36   J.H.W. Leuvering, P.J.H.M. Thal, M. van der Waart and A.H.W.M. Schuurs, J. Immunol. Methods, 45 (1981) 183.
37   J.H.W. Leuvering, B.C. Goverde, P.J.H.M. Thal and A.H.W.M. Schuurs, J. Immunol. Methods, 60 (1983) 9.
38   J.H.W. Leuvering, P.J.H.M. Thal, A.H.W.M. Schuurs and D.D. White, J. Immunol. Methods, 62 (1983) 163.
39   T. Gribnau, F. Roeles, J. van der Biezen, J. Leuvering and A. Schuurs, in T.C.J. Gribnau, J. Visser and R.J.F. Nivard (Editors), Affinity Chromatography and Related Techniques, Anal. Chem. Symp. Ser., Vol. 9, Elsevier, Amsterdam, 1982, Ch. V, p. 411.
40   T. Gribnau, A. van Sommeren and F. van Dinther, in I.M. Chaiken, M. Wilchek and I. Parikh (Editors), Affinity Chromatography and Biological Recognition, Academic Press, New York, 1983, Ch. V, p. 375.
41   H. van Hell, J.H.W. Leuvering and T.C.J. Gribnau, in W.P. Collins (Editor), Alternative Immunoassays, Wiley, Chichester, 1985, Ch. 4, p. 39.
42   J.M. Straley, in K. Venkataraman (Editor), The Chemistry of Synthetic Dyes, Vol. III, Academic Press, New York, 1970, p. 385.