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Particle counting immunoassay — an overview

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Abstract: The principles and applications of the Particle Counting Immunoassay technique (PACIA) are summarized, and the limit of detection of the method is estimated. The various serum interferences are discussed and solutions to the problems proposed.

Keywords: *Particle counting immunoassay; immunoglobulin G F(ab')2 fragments; polystyrene latex spheres; antigen, hapten and immunoglobulin assays; serum interferences; C1q complement protein; rheumatoid factor.*

In medical research, a simple, reliable, highly sensitive and rapid analytical technique is often the factor which decides whether a line of investigation can be followed. Some ten years ago available immunoassay techniques lacked the necessary sensitivity by some 4–5 orders of magnitude, or were slow, work-intensive and isotope-dependent. Experience with nephelometry [1, 2] showed that its sensitivity could not be extended beyond about 10^{-8} M. In a detailed review of all methods, latex agglutination, first described by Singer and Plotz [3], seemed to offer many advantages, being simple, sensitive, homogeneous, non-isotopic and rapid. Early work with the latex slide test for the determination of immune complexes [4, 5] confirmed the advantages but at the same time revealed a serious weakness. Strongly positive results were easy to read, but attempts to obtain quantitative results by serial dilution gave results which were very variable and highly dependent on the eye of the observer. An obvious method of quantitation was to count *only* the agglutinated or unagglutinated particles, using a conventional particle or blood cell counter. Experiments in this laboratory with antigen and albumin solutions were very promising. This early success has lead to 10 years of intensive research, mostly spent in solving the major problem of the latex agglutination technique — serum interference. It is the purpose of this paper to detail the present state of the art of immunoassay by particle counting. In summary, no more interferences now occur with particle counting immunoassay (PACIA) than with any well conceived radioimmunoassay (RIA). At the same time, the PACIA technique has retained all its original advantages. It has been extended to substances of all molecular weights, ranging from thyroxine to viruses in the concentration range 10^{-7}–10^{-15} M. The entire technology has been fully automated in the IMPACT® system (Acade SA, Brussels, Belgium).

Principle of the Assay

Antigens: $F(ab')_2$ fragments of antibodies are covalently coupled to polystyrene spheres of 0.8 μm diameter. When mixed with a sample containing the antigen and adjusted to the correct pH, the antigen will agglutinate some of the latex. The mixture is then passed through an optical cell counter (Fig. 1) where only the unagglutinated particles are counted. The concentration of unagglutinated particles is inversely proportional to the concentration of the antigen.

Antibodies are similarly determined by coupling antigen to the latex.

Haptens (univalent antigens) are coupled covalently to latex particles. Antiserum to the hapten and latex/hapten are added to the sample. Free hapten in the solution binds to the antibody and competes with the hapten on the latex surface. As IgG is a poor agglutinator, the agglutination is enhanced by using an IgM rheumatoid factor (RF) which couples IgG–hapten complexes through the Fc chain of IgG (Fig. 2). Free hapten inhibits agglutination. The concentration of free latex particles is therefore directly proportional to the hapten concentration.

The following simple calculation shows the sensitivity of the method to be at about 10^{-16} M. The counter counts between 1000 and 500 particles/s between full-scale (no reaction) and total reaction. A full-scale deflection is therefore equivalent to 30 000 particles/min which have been agglutinated. If, in the most improbable case, as many as 10 antigens are required to bind two latex particles, the count rate is 150 000 antigen molecules/min. The flow rate in the detector is about 2.0 ml/min. Hence, the number of antigen molecules/l counted is 8×10^7. Since Avogadro's number is 6×10^{23}, the technique at full-scale measures about 10^{-16} M levels of antigen. Table 1 shows the relative concentration of some serum substances.

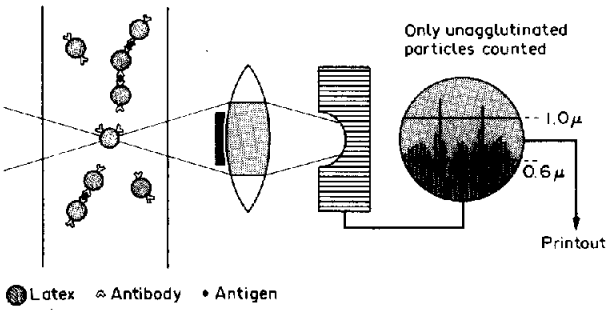

Figure 1

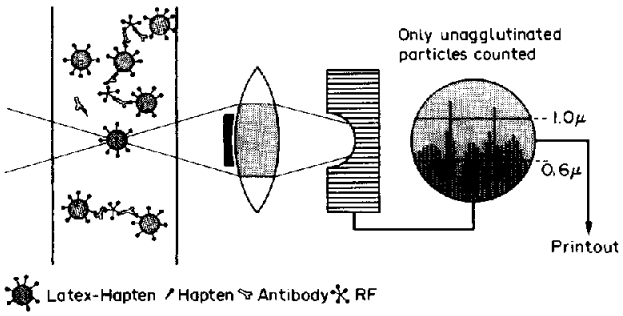

Figure 2

Table 1
Assays developed and used at Unit of Experimental Medicine, I. C. P., U. C. L., Brussels, Belgium

Assay	Sample	Range
Thyroid function tests		
*Thyroxine (T4)	Serum	1–320 $\mu g\ l^{-1}$
*Triiodothyronine (T3)	Serum	0.1–12 $\mu g\ l^{-1}$
*Thyroid stimulating hormone (TSH)	Serum	0.2–100 mIU l^{-1}
	Blood spots	10–100 mIU l^{-1}
*Thyroxine binding globulin (TBG)	Serum	1–80 $\mu g\ l^{-1}$
Thyroglobulin	Serum	4–500 $\mu g\ l^{-1}$
Immunoglobulins		
IgA	CSF	4–130 $\mu\ l^{-1}$
IgM	CSF	11.5–400 $\mu g\ l^{-1}$
IgE	Serum	1–200 IU ml^{-1}
	Cord blood	0.2–25 IU ml^{-1}
	CSF	0.2–40 IU ml^{-1}
Hormones		
Human chorionic gonadotropin (hCG)-β subunit	Serum	0–500 IU l^{-1}
Human growth hormone (hGH)	Serum	0.2–40 $\mu g\ l^{-1}$
*Human placental lactogen	Serum	0.1–16 $mg\ l^{-1}$
Proteins		
*Alpha-fetoprotein	Serum	1–650 KIU l^{-1}
*Ferritin	Serum	1–325 $\mu g\ l^{-1}$
	CSF	0.5–50 μg/L
*C-Reactive Protein	Serum	0.5–80 $mg\ l^{-1}$
	Cord blood	0.005–2 $mg\ l^{-1}$
	CSF	1–1000 $\mu g\ l^{-1}$
S-100	CSF	1–50 $\mu g\ l^{-1}$
	Amniotic fluid	1–100 $\mu g\ l^{-1}$
β_2-microglobulin	Urine	0.05–100 $\mu g\ l^{-1}$
	Serum	0–3 $mg\ l^{-1}$
SP-1 protein	Serum	0–5 $\mu g\ l^{-1}$
Infectious antigens		
Herpes simplex B antibody	Serum	Antigens in low pico- — or high femtomolar range.
Streptococcus B antigen/antibody	Serum	
Hemophilus influenzae antigen/antibody	Serum	
Pneumococcus antigen/antibody	Serum	
Bovine leukosis antibody	Serum	
Brucellosis antigen/antibody	Serum	
Toxoplasmosis antigen/antibody	Milk	–ditto–
Tetanus antibody	Serum	–ditto–
Miscellaneous		
Digoxin	Serum	0.2–6 $\mu g\ l^{-1}$
Immune complexes	Serum	Expressed as RF equivalent of aggregated IgG $\mu g\ ml^{-1}$ depending on reagent used.
	Cord blood	
	Aqueous humor	

*These analyses are commercially available.

Electrical conductivity or resistance counting has not been practical as the latex particles are extremely "sticky" and very quickly block the small capillary necessary. With optical counting, light is focused on a relatively large cross-section (75 × 150 μm) which is less prone to clog. However, even with optical counting, the flow cell is automatically reverse washed between each assay by a very rapid flow of buffer.

Elimination of Serum Interference

Latex coated with IgG and mixed with serum will be agglutinated by RF; this reaction is the basis of the well accepted test for RF. Since antibodies from antiserum are IgG molecules, admittedly from animals, one would expect naturally occurring RF to interfere with any assay based on an antigen–antibody latex agglutination reaction. There is also interference from the complement protein C1q. Consequently, the use of simple IgG antibodies on the latex surface will cause agglutination with over 60% of all serum samples, the extent of agglutination being highly variable.

Fortunately, the binding of both RF and C1q seems to be limited to the Fc portion of IgG, which is easily removed by pepsin digestion, leaving the immunologically active fragment $F(ab')_2$. Consequently, for all assays requiring antibody coupling to latex, the IgG is first precipitated with half saturated ammonium sulphate, isolated by chromatography on diethylamino-ethylcellulose, and digested with pepsin. The $F(ab')_2$ fraction is separated on an Ultrogel (AcA 4–4) column before being covalently bound to the carboxylated latex [6].

The amount of IgG $F(ab')_2$ fragment bound to the latex depends on the sensitivity of the assay [7]. The quantity used is not enough to cover the latex surface, so the "free" space must be filled with some protein neutral to the reaction, such as albumin. In certain pathological sera, this heavy loading of protein leads to false agglutination, presumably due to protein–protein interactions. Hence, for each assay a supporting electrolyte solution must be found to avoid this type of agglutination. Such solutions may be of high salt concentration, an optimum pH or contain chaotropic agents such as ammonium thiocyanate.

Human antibodies against the animal serum used to produce the antibodies are sufficiently frequent that the reagent must contain serum from non-immunized animals to absorb these antibodies. Furthermore, human antibodies reacting specifically with the $F(ab')_2$ fragment (not with the whole IgG molecule), can be expected in 1–2% of the population. To avoid this interference, glutaraldehyde aggregated $F(ab')_2$ from a non-immunized animal is added to the reagents [8].

One of the more significant developments in the technique to reduce interference in serum has been the use of pepsin in the assay of certain proteins, as well as haptens. Although treatment of serum with pepsin to destroy hapten carriers in the immunoassay of haptens has been described [9], its use in the assay for proteins is apparently original. In many cases the pepsin may destroy the protein antigen, but if the reaction time is controlled it will not destroy the antigenic determinant. In an assay for low levels of IgE, for example, the IgE molecule itself is digested with pepsin and the Fc″ fragment is separated and used as an immunogen to produce antiserum specific to this portion of the IgE molecule [10]. Subsequently, any serum sample containing IgE may be pepsin-treated, destroying interfering proteins, liberating the antigen Fc″, and therefore increasing the sensitivity of the assay some four times over that possible with RIA. Using this technique, the author's clinic now routinely screens the cord blood of neonates at

risk of allergy. The technique has been extended to the use of human chorionic gonadotropin and thyroxine stimulating hormone (TSH).

Clearly the latex agglutination technique is very dependent on highly specific antisera, which in recent years have been more readily available commercially. In the automated analysis system (IMPACT®), all analyses require 45 min incubation. Antiserum of high affinity is therefore also required. The development of a method for a new analyte is today more a question of trial and error than the discovery of fundamental new principles; most effort is usually devoted to finding or producing a suitable antiserum.

Applications of the PACIA method have been extensively reviewed [11]. The assays developed and used in the author's laboratory are listed in Table 1. The extreme rapidity of the particle counting immunoassay, combined with its very high sensitivity, make it an ideal assay for microbiology and virology. Current efforts are concentrated in this direction and femtomolar sensitivities (10^{-15}) for the capsular antigen (polyribose phosphate) distinctive of Hemophilus influenzae in serum samples have been achieved. In a medical research unit, particle counting immunoassay is an extremely useful tool for large-scale studies — a highly sensitive, rapid, quantitative, non-isotopic technique, free from any major interferences, and adaptable to the range of assays normally covered by RIA.

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