Kinetics and proposed mechanism of the reaction of an immunoinhibition, particle-enhanced immunoassay

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We report kinetic studies on the reaction of a latex agglutination immunoassay used to quantify phenytoin in serum. In this assay, polystyrene particles with a covalently attached analog of phenytoin react with an antiphenytoin monoclonal antibody to form light-scattering aggregates, with the rate of this reaction being decreased by addition of phenytoin from sample. In the absence of free (sample) phenytoin, this reaction did not exhibit a maximum rate of agglutination in the presence of excess antibody, i.e., an equivalence point. Furthermore, agglutination was inhibitable by free phenytoin even when the latter was added after agglutination of particles with antibody had begun. Most significantly, the immunoagglutination proceeded in an identical fashion with monovalent F(ab) fragment. These data are consistent with low-affinity immunospecific particle–antibody complexation, which then induces colloidal aggregation, without requiring immunospecific bridging by antibody molecules. The described mechanism is not generalizable to all latex agglutination immunoassays, although disturbance of colloidal stability may be a component in most assays.

Latex particles have been used for many years in the development of a wide range of immunoassays, from rapid and simple semiquantitative assays to very precise and sensitive assays used with automated photometric analyzers [1–5]. The latex particles serve two purposes: (a) to enhance the light scattering achieved on reaction of antigen with antibody, and (b) to act as a core particle in the development of an immunoinhibition assay. The latter has facilitated the development of light-scattering assays for haptens and other relatively low-molecular-mass analytes (<5000 Da and only providing one viable antibody binding site per molecule) by using the principle of immunoinhibition [5].

The reaction between a polyvalent antigen and a bivalent antibody is generally assumed to lead to aggregate formation through bridging by the antibody between antigen molecules, the amount of light scattering reflecting aggregate formation and the amount of antigen and (or) antibody present. In the case of an immunoinhibition assay for a hapten, the polyvalent hapten latex particles form an immunoaggregate in the presence of antibody, quantification of sample analyte being achieved by the amount of inhibition of aggregate formation by free antigen.

The development and routine use of these assays has advanced with the production of more uniform latex particles, the ability to conjugate hapten (or antibodies) to particles, and a greater knowledge of the chemistry of the particle surface as well as the antigen–antibody reaction.

In 1961 Oreskes and Singer characterized the binding of human γ-globulins (hGG) to polystyrene latex particles [6]. They proposed that the mechanism of agglutination of hGG-coated particles in certain serological assays for macroglobulins could be due to either cross-linking by multivalent globulins or destabilization of the particle colloidal suspensions upon binding of globulins to hGG on particle surfaces. Later Singer et al. [7] and von Schulthess et al. [8] investigated an assay in which anti-

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3 Nonstandard abbreviations: hGG, human γ-globulin, NHS, N-hydroxysuccinimide; and HSA, human serum albumin.
body-modified particles were agglutinated by a protein to which the antibody was directed. These workers developed mathematical descriptions of a proposed bridging mechanism and showed agreement of observed behavior with their theories.

A colloidal suspension of latex particles is maintained predominantly by electrostatic repulsion between highly charged surfaces [9, 10]. Many workers have studied influences of protein adsorption, pH, ionic strength, and detergents on the stability and surface charge of latex particles [9–12]. Reduction of the surface charge or zeta potential induces aggregation, and this can be achieved by increasing ionic strength, increasing protein concentration, and decreasing detergent concentration. Thus, to develop viable diagnostic assays with latex particles, paying careful attention to factors altering colloidal stability is essential.

While many analysts and educators would claim that all light-scattering immunoassays involving latex particles are based on bridging between polyvalent antigen molecules to achieve aggregate formation, the possibility of colloidal instability as the likely mechanism for aggregation still exists. In 1984 Craig proposed that the behavior of the reactions of certain diagnostic tests under development was consistent with particle destabilization upon immunospecific binding of antibodies to the particles [13].

We now report on studies of a latex particle immunoinhibition assay for phenytoin (diphenyl hydantoin) designed to elucidate the mechanism of the reaction. The assay involves hapten-labeled particles and a monoclonal antibody. Analytical performance of this assay, now in use on a commercial system, has been reported previously [14]. Using a monovalent F(ab) fragment, we report evidence that a bridging mechanism is not necessarily involved in the aggregation. Rather, we propose that under the specific conditions used, a rapid highly reversible immunospecific reaction of antibody with particle-bound analog is followed by a relatively slow colloidal aggregation that is not a result of immunoreactions, but results from the changed colloidal nature of the particle surfaces bearing protein.

**Materials**

Particle reagents were from a commercial phenytoin diagnostic test manufactured by Dade International. The particles of these reagents had a polystyrene core and a polyglycidylmethacrylate shell covalently modified with a conjugate of phenytoin, and were 65 ± 6 nm in diameter. Particle preparation techniques used have been published [1]. Density of phenytoin analog is ~1800 molecules per particle, on the basis of the stoichiometry used in preparation.

A monoclonal antiphenytoin antibody (IgG) and the F(ab')2 fragment from this antibody were also from Dade. The F(ab')2 and particle reagent are part of the aforementioned diagnostic product. The antibody was derived from a hybridoma cell line resulting from fusion of spleen cells of Balb/c mice immunized with keyhole limpet hemocyanin (KLH)-conjugated phenytoin and mouse myeloma cells, with standard techniques [15]. The F(ab')2 preparation was stored in 9.6 mmol/L PBS, pH 7.4. From the same whole antibody, F(ab) fragments were prepared by standard procedures [16], with papain, d-cysteine, and EDTA from Sigma. The digestion mixture was dialyzed against PBS and then purified with a column containing protein A-Sepharose fast flow resin from Pharmacia equilibrated with 3 mol/L NaCl and 1 mol/L glycine, pH 8.8. This process removed Fc fragments and undigested IgG. After further dialysis of the eluted F(ab) against PBS, a final purification was performed by HPLC by using gel filtration Zorbax, GF-250 from DuPont, and a mobile phase of 0.2 mol/L sodium phosphate, pH 7.0. Analysis of the product was performed with the HPLC system and column described above and Wavescan/Nelson integrator analysis software from Nelson Analytical. This gave approximate molecular masses of 150 000, 90 000, and 45 000 Da for the IgG, F(ab')2, and F(ab) fractions, respectively. Preparations of the F(ab) fraction were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (nonreducing conditions) along with molecular mass calibrators with a Mini 2-Gel Device from Integrated Separation Systems with a precast 4–20% gradient gel run at 30 mA for 2 h. Gels stained with Coomassie blue showed only one band, which migrated to the same position as a 39 000-Da calibrator.

Poly(oxy-1,2-ethanediyl), α-(nonylphenyl)-α-hydroxyphosphate, trade named Rhodofac (previously Gafac, and herein referred to as such) was obtained from Rhone-Poulenc. Sodium and potassium phosphate salts, NaOH, and NaCl were from VWR Scientific. Phenytoin serum-based calibrators were from Dade. Water used throughout the studies was purified and deionized with an activated-carbon and mixed-bed resin treatment system from U.S. Filter.

**Experimental Procedures and Results**

All reactions were monitored at 37 °C with a Cobas Bio centrifugal analyzer (Roche Diagnostic Systems), unless otherwise stated below. Measurements of absorbance were made at 340 nm 5 s after initiation and at 10-s intervals for 295 s; the cuvette pathlength was 1 cm. Data was captured by using Red Ryder interface software (Freesoft Co.) on a Macintosh Plus computer (Apple Computer). All reactions were performed in a sodium phosphate buffer (77 mmol/L) containing sodium chloride (11 mmol/L) and Gafac (9 g/L), pH 6.95, at 37 °C. These and the concentrations indicated below pertain to final reaction conditions. Antibody concentrations were derived from absorbance measurements at 280 nm, assuming an absorptivity of 1.4 L g−1 cm−1. Particle concentrations refer to particles themselves (not phenytoin label).
AGGLUTINATION REACTION WITH F(ab’)$_2$ AND F(ab) ANTIBODIES

A 3-μL aliquot of human serum-based calibrator was mixed with reaction buffer and particle reagent to a total volume of 194 μL; after temperature equilibration for 30 s, the agglutination reaction was initiated by adding 18 μL of either F(ab’)$_2$, or F(ab) at final concentrations of 0.15 and 0.50 μmol/L respectively, followed by 20 μL of water as flush. The final particle concentration was 6.2 nmol/L, equivalent to 11 μmol/L analog phenytoin in the final reaction mixture, assuming that all of the label was available for reaction. The progress curves are shown in Fig. 1. No agglutination was observed when the phenytoin antibody fragments were replaced by a nonspecific F(ab) at the same concentration.

DETERMINATION OF RATE DEPENDENCE ON ANTIBODY CONCENTRATION

These experiments were undertaken with the protocol outlined above, without the addition of sample and varying the F(ab’)$_2$ concentration in the cuvette between 0 and 0.19 μmol/L. The initial rates were determined from the progress curves by extrapolating the d(absorbance)/dt vs t plot to the initiation point and plotting the initial rate obtained against antibody concentration as shown in Fig. 2.

DETERMINATION OF RATE DEPENDENCE ON PARTICLE (ANTIGEN) CONCENTRATION

A volume of buffered particle reagent (150 μL) was diluted with water and temperature-equilibrated for 30 s, and then F(ab’)$_2$ antibody was added to a final volume of 231 μL. The effect of varying the particle concentration between 0.5 and 4.0 nmol/L was studied and the first-, second-, and third-order plots of the initial rates (determined as above) are shown in Fig. 3.

EFFECT OF HIGH ANTIBODY/PARTICLE ANTIGEN RATIO

To study agglutination rates at very high concentration ratios of antibody binding sites, a volume of buffered particle reagent (150 μL) was diluted with water and temperature-equilibrated for 30 s, and then F(ab’)$_2$ antibody was added to give a particle concentration of 0.81 nmol/L in the final reaction mixture; a range of F(ab’)$_2$ concentrations between 0.05 and 1.5 μmol/L was studied (selected so that the influence of a large excess of antibody over particle antigen reagent could be explored). The initial rates were plotted against antibody concentration and are shown in Fig. 4.

INHIBITION OF IN-PROGRESS AGGLUTINATION BY FREE PHENYTOIN

These experiments were performed on a Dimension® clinical chemistry system (Dade International) [17]; this analyzer can perform the particle-enhanced phenytoin assay used in this study with the additional facility (albeit nonstandard) for manual addition of a reagent during the progress of a reaction. In this case, buffered particle reagent
reagent (6.2 nmol/L in the final mixture) and water diluent were temperature-equilibrated for 65 s, after which the agglutination reaction was initiated by addition of F(ab\(^9\))\(^2\) antibody (0.15 \(\mu\)mol/L) to give a total volume of 500 \(\mu\)L. The sample, in this case phenytoin serum calibrator, was added (4 \(\mu\)L) with a 20-\(\mu\)L adjustable pipette exactly 20 s after antibody initiation; mixing was effected by rapid in-and-out flow from an adjustable 100-\(\mu\)L pipette. The pathlength was 0.5 cm. The progress curves from a phenytoin-free serum calibrator and a 40 mg/L phenytoin calibrator (final concentration 1.3 \(\mu\)mol/L) are shown in Fig. 5.

**DETERMINATION OF RATE CONSTANTS FOR BINDING OF ANTIBODY TO PHENYTOIN**

Measurements were made on a BIAcore\(^{\text{TM}}\) surface plasmon resonance detector from Biacore. The carboxymethyl dex-modified gold sensor chips [18] used with this instrument were modified to contain phenytoin analog on their surfaces as follows, with a running buffer of 10 mmol/L HEPES-buffered saline (150 mmol/L NaCl, 3.4 mmol/L EDTA, 0.05 mL/L P-20 nonionic surfactant) at 5 \(\mu\)L/min flow rate: (a) The gold sensor chip (certified CM 5) was preactivated with 35 \(\mu\)L of a solution of 0.2 mol/L \(\text{N-ethyl-N'-(dimethylaminopropyl)-carbodiimide (EDC)}\) and 0.05 mol/L \(\text{N-hydroxysuccinimide (NHS); (b) thirty-five microliters of 100 mmol/L human serum albumin (HSA) in 10 mmol/L sodium formate, pH 3.6, was injected; (c) thirty-five microliters of 1 mol/L ethanolamine hydrochloride, pH 8.5, was injected (to block remaining NHS ester groups); (d) thirty-five microliters of 2 \(\mu\)mol/L...
1-(4-carboxybutyl) 4,4-diphenylhydantoin succinimidyl ester in 100 mmol/L sodium borate, pH 8.3, was injected and allowed to react with the immobilized HSA to create conjugated phenytoin; (c) ten microliters of 50 mmol/L NaOH/200 mL/L acetonitrile was injected 20 times to condition the sensor chip.

Association rate constants were determined at 37 °C in duplicate in 100 mmol/L sodium phosphate, 1.3 g/L Gafac, pH 7.0. Five concentrations of F(ab')2, from 84 to 840 nmol/L were injected at a rate of 10 μL/min. Regeneration of the phenytoin-labeled sensor chip was accomplished after each run by injection of 10 μL of 50 mmol/L NaOH/200 mL/L acetonitrile, followed by equilibration with the above buffer solution. Dissociation rate constants were determined by injection of 45 μL of 10 mmol/L phenytoin in the above buffer solution after injection of 20 μL of 2.6 μmol/L F(ab')2. Analysis was carried out by using a biphasic fit to the integrated rate equation [19]. This enabled the whole of the binding curve to be analyzed and enabled identification of separate binding components. The association rate constant analysis showed a monophasic binding with a bimolecular rate constant of (7.4 ± 3.0) × 10⁴ (mol/L⁻¹ s⁻¹). Dissociation rate analysis gave a predominantly monophasic dissociation with a calculated rate constant of (6.5 ± 2.2) × 10⁻³ s⁻¹. The association equilibrium constant was thus 1.1 × 10⁷ (mol/L)⁻¹.

**Discussion**

The inhibition of the agglutination reaction by free (i.e., sample) phenytoin as shown in Fig. 1 is the basis of the immunoinhibition assay principle, namely, in this case, inhibition of reaction between antibody with phenytoin-modified particles to produce initially dimers and then aggregates of increasing size containing several antigen and antibody molecules. Early work by Singer et al. [7] involved the study of agglutination of polystyrene particles induced by protein adsorption, and von Schulthess et al. [8] examined a system in which anti-IgA antibody-coated polystyrene particles were agglutinated by IgA. Both studies led to observations that a maximum occurred in the curve relation of agglutination vs antibody concentration. This is analogous to the relation observed by Heidelberger and Kendall [20] relating agglutination to antigen concentration, illustrating the typical bell-shaped curve. These authors and many others subsequently have interpreted these findings with a proposed mechanism of immunospecific bridging between particles by the bivalent antibody molecules. Binding partner concentrations were found that were high enough to approach saturation of half the binding sites on particles during a rapid equilibration with antibody, with the result that further increases in concentration led to less aggregation.

The results described in Fig. 4 do not show this behavior. The concentration of latex particles was 0.81 nmol/L; for the 64-nm diameter particle, the average surface area per particle is calculated to be ~12 900 nm², and, assuming a surface coverage by each antibody molecule of 110 nm², the maximum density of antibody parking sites (i.e., coupled antigen molecules) is estimated to be 120 (i.e., without steric hindrance existing). The 0.81 nmol/L particle concentration thus corresponds to a concentration of available binding sites of ~97 nmol/L; the highest antibody concentration used was 15 times this figure (1.5 μmol/L). Despite this excess of antibody, no maximum of agglutination signal (the Heidelberger–Kendall bell-shaped relation) was reached; this suggests some other mechanism than bridging by bivalent antibody.

This proposal is substantiated in the data of Fig. 1 (right), showing that the agglutination reaction proceeds with monovalent F(ab) fragments, which offer no possibility of bridging. We used somewhat higher concentrations of F(ab) than F(ab')2 to achieve comparable agglutination rates in the experiments; this requirement might be expected from (a) the need to use a concentration of monovalent F(ab) twice that of the divalent F(ab')2 to ensure the same concentration of antibody binding sites and (b) the well-known alteration of affinity constants of parent antibody molecules by enzymatic fragmentation. It is also evident from Fig. 1 that the agglutination reaction with the monovalent antibody is inhibitable by free (sample) phenytoin, just as in the case with the bivalent antibody. Other experiments have shown that nonspecific F(ab) and F(ab')2 fragments did not produce any aggregation of phenytoin particles. Clearly, an immunospecific reaction is taking place in the case of both F(ab) and F(ab')2 antibody fragments.

Observation of the reaction kinetics sheds further light on the processes involved. We could not detect any lag phase after inhibition of the reaction. The first step of the reaction, the binding of antibody with antigen sites on particles, thus appears to be much faster than the ensuing agglutination, which can be taken to be the rate-limiting step. This behavior might be expected since the frequency of collision of particles would be slower than collision of faster diffusing antibody molecules with particles. The influence of reactant concentrations on agglutination rate shown in Figs. 2 and 3 reveals that the initial reaction rates are first order in antibody and approximately second order in particle reagent. This is consistent with the formation of dimer during the initial reaction phase, as induced by prior binding of an antibody molecule to phenytoin analog. We used the initial reaction rate as the basis for these determinations to avoid the interpretive problems associated with changes in relative light scattering of higher order of aggregates than dimers, absorbance outside the range of photometric linearity, and the tendency of such systems not to reach a clear equilibrium condition but to proceed to full flocculation of immunoprecipitate.

The association equilibrium constant for the F(ab')2 was found to be >10⁷ (mol/L)⁻¹ and an association rate constant of 7.4 × 10⁴ (mol/L⁻¹ s⁻¹), indicating an antibody with acceptable affinity for hapten [21]. However,
the data in Fig. 5 show that agglutination was arrested by addition of free phenytoin, but without the reduction of light-scattering signal associated with the breakdown of aggregate. We interpret this to mean that binding between particle and antibody is readily replaced by free drug, with the result that no further agglutination occurs. Since the turbidity does not decrease after introduction of phenytoin, even at very high concentration (which would have been expected with a dissociation constant indicating a $K_d$ of $10^{-5}$ s), the formation of aggregate is deemed not to be reversible, indicating that aggregate formation is not solely due to antigen–antibody binding and involves some other mechanism that is not inhibited by free antigen. This stage of the reaction is proposed to reflect a deterioration in the colloidal stability of the particle as a consequence of binding of protein that induces agglutination and eventually precipitation as suggested by Nakamura et al. [9] and Hidalgo-Alvares and Galisteo-Gonzalez [10].

Colloidal instability of protein-coated latex particles is a well-known phenomenon [7, 11, 12]. As proteins are coupled or adsorbed to the surface, the surface charge (assessed as the zeta potential) decreases and with sufficient protein, the repulsive charge collapses and the particles agglutinate. Thus, the binding of increasing amounts of antibody to phenytoin, through a specific immunological mechanism, could cause sufficient alteration in the surface charge to induce agglutination. While the initial binding would be inhabitable by free antigen, the colloidal instability phase would not. The degree of change in the colloidal state is presumably to be slight, otherwise the agglutination progress would not be arrested by mopping up free antibody binding sites after initiation. The specific binding may induce a metastable colloidal mixture where hydrophobic interactions predominate, the relatively high detergent concentration (9 g/L) presumably playing an important role in regulating this reaction. Further experiments with nonionic and other anionic detergents might allow this hypothesis to be explored.

In summary, the observations reported here indicate that in this particular system, the formation of an immunoglutinate takes place in two stages, a first immunospecific reaction followed by a slower agglutination of particles that is induced by their altered colloidal state, thereby not requiring antibody bridging. All particle-enhanced immunoassay reactions are not expected to operate in this way, although one can reasonably expect that all particle-enhanced immunoprecipitation/agglutination reactions will depend to a certain degree on colloidal instability.

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References