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Comparison of Two Enzyme-Linked Immunosorbent Assays for Antigen Quantitation: Direct Competition and Antibody Inhibition

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Two enzyme-linked immunosorbent assays for antigen quantitation, direct competition and antibody inhibition, were used to measure rabbit immunoglobulin G in polystyrene microtiter plates and were compared for sensitivity and reproducibility. Both procedures repetitively detected this antigen in the 1- to 100-ng/ml range. Both procedures were predictably reproducible, with direct competition procedures having steeper slopes in ranges tested. Antibody inhibition does not require conjugated antigen and can detect sample antigens if available stock antigens can be passively bound to a solid-phase polystyrene plate.

Most enzyme-linked immunosorbent assay (ELISA) procedures measure antibody. Various antigens, including polysaccharides, glycoproteins, and lipopolysaccharides, are bound to polystyrene plates or tubes in appropriate buffers and pH. In the indirect antibody ELISA, sample antibody is attached to solid-phase-bound antigen, and enzyme-conjugated antiglobulin and substrate are added stepwise. Substrate is reduced to a colored endpoint in direct proportion to the amount of bound antibody. Current literature documents more than 100 such systems.

The direct competition (DC) antigen ELISA, developed by Engvall et al. in 1971 (3), quantitates antigen and has been used to detect staphylococcal enterotoxins (6), carcinoembryonic antigen (4), insulin (5), and other protein antigens. Sensitivity and specificity are comparable to solid-phase radioimmunoassay (2, 7). The method is simple and inexpensive. In DC, hyperimmune antibody is bound to a solid polystyrene phase. Sample antigen competes with enzyme-labeled stock antigen for bound antibody. Subsequent substrate conversion is inversely proportional to the concentration of sample antigen (Fig. 1A).

Predictable conjugation of antigen to enzymes is decisive for proper use of DC. Antigens not readily conjugated because of size, instability, or poor affinity attenuate the sensitivity of DC. We were repeatedly unsuccessful in preparing high-quality enzyme-conjugated lipopolysaccharide by several methods. We therefore assessed the usefulness of an antibody inhibition (AI) method. Rabbit immunoglobulin G (RIgG) was chosen as antigen to allow direct comparison with the classical ELISA DC procedure (3). Microtiter plates were chosen for handling ease and to assess internal and interplate variability of this widely used solid phase.

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MATERIALS AND METHODS

Plates. Flat-bottom polystyrene substrate plates (Cooke Laboratory Products) were used. Flexible polyvinyl transfer plates (Cooke Laboratory Products) were used with substrate plates in the AI procedure. Transfer plates have orifices in the bottom of each well. Wells retain contents while the plate is in a carrier, but release them to fluid in substrate plates by loss of surface tension and capillary action (Fig. 1B).

Antigen. Chromatographically pure RIgG (Cappel) was used as sample antigen. Samples were diluted threefold to reproduce test conditions of previous DC work (3).

Antibody. Goat anti-RIgG (Cappel) was used. The protein concentration was 8.2 mg/ml.

Conjugates. For the DC, method RIgG was coupled to alkaline phosphatase (Sigma type VII) by the one-step glutaraldehyde method of Avrameas (1). For the AI method, goat anti-RIgG was coupled similarly. Conjugates were used at a working dilution determined by checkerboard titration. Conjugate was serially diluted and incubated with its plate-bound ligand. The highest conjugate dilution producing an absorbance of 0.6 in 1 h after substrate addition was chosen for further use. Typical conjugate dilutions were 1:15,000 for DC and 1:25,000 for AI, based upon enzyme-to-final working volume ratios. Conjugates were stored in 1% bovine serum albumin-tris(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 8.0) at 4°C.

Dilutions. Phosphate-buffered saline (PBS) (0.05 M; pH 7.2) plus 0.05% Tween 20 (PBS-Tween) was used for washes and dilutions unless otherwise stated.

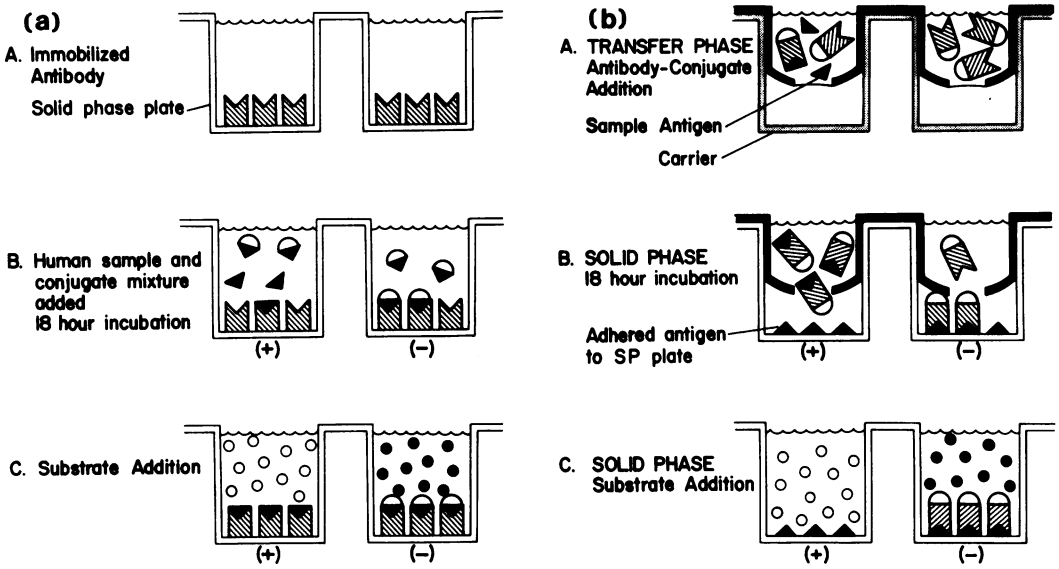

FIG. 1. *(a) DC method. Substrate plates sensitized with anti-RIgG antibody (notched rectangles), RIgG (triangles), or conjugated RIgG (triangles with semicircles) compete for substrate antibody; when conjugated molecules are competitively inhibited, sample antigen concentration is inversely proportional to colored substrate (small circles). (b) In AI, sample antigen binds to conjugated antibody (notched rectangles with semicircles) in the transfer plate. Remaining antibody conjugate binds to substrate plate antigen, and substrate reduction is inversely proportional to sample antigen.*

Substrate. *p*-Nitrophenyl phosphate (disodium salt, 1 mg/ml) (Sigma) in 0.05 M carbonate buffer (pH 9.8), containing 0.001 M $MgCl_2$, was used as the substrate. Color production was stopped with 25 μl of 4 N NaOH.

Washes. Wash steps were 5 min each with PBS-Tween, using a washer-aspirator (Dynatech). Washes were done in triplicate and used between every ELISA step unless otherwise stated. After washing, plates were forcefully evacuated to remove residual fluid.

Incubations. Incubations were carried out at 37°C for 1 h in a humidity chamber unless otherwise stated.

DC testing. A minor modification of the DC procedure as described by Engvall et al. (3) was used in microtiter plates. Substrate plates were coated with 200 μl, containing 2 μg of goat anti-RIgG per ml in 0.1 M carbonate buffer (pH 9.6), overnight at 37°C. A 100-μl sample of RIgG was added to 20 μl of the working dilution of conjugated antigen. Incubation was for 18 h at 26°C. Substrate was added, and color production was stopped with 4 N NaOH at 60 m. Well contents were read spectrophotometrically at 405 nm on an ELISA Reader (Dynatech).

AI testing. Volumes of 125 μl of various concentrations of RIgG were placed in transfer plate wells, and 25 μl of conjugated antibody at a working dilution in PBS was added. The transfer plate was incubated for 18 h at 26°C in a carrier. Substrate plates were coated with 200 μl, containing 2 μg of RIgG per ml, and stored at 4°C. During the transfer phase, substrate plates were washed with PBS. Movement of transfer plate contents into substrate plate wells containing 50 μl of PBS-Tween created final substrate plate volumes of 200 μl. Incubation followed. Substrate was added, color production was stopped, and absorbance was determined as above.

Samples. RIgG was diluted threefold from 1,093.5 ng/ml to 0.5 ng in PBS-Tween for DC studies. Similar dilutions for AI used PBS. Both tests generated quadruplicate samples on three different test dates. Controls without sample, conjugate, and substrate plate-bound ligand were used. Standardized incubation times, reagents, and proper performance of control wells were used as criteria for acceptance of all data.

RESULTS

RIgG was measured by AI and DC at threefold dilutions from 0.5 to 1,093.5 ng/ml on three separate test dates.

Absorbance by AI at 60 min after substrate addition ranged from 0.48 ± 0.12 at 1,093-5 ng/ml to 1.27 ± 0.13 at 0 ng/ml. Coefficient of variation was 25% at 1,093.5 and 10.6% at 0 ng of RIgG per ml. This interexperimental variance did not significantly alter slope (Fig. 2A).

When the mean absorbance of each of three successive runs was used to generate a linear regression analysis from 1.5 ng/ml to 121.5 ng/ml, the coefficient of determination was high (r^2 = 0.92) (Fig. 2A). In the 0- to 40-ng/ml portion of the curve, r^2 fell to 0.79, implying that AI was less accurate at RIgG concentrations below 40 ng/ml. The lower limit of significance appeared to be 40 ng/ml ($P < 0.001$, Student's independ-

ent t test, 35 df). At 13 ng/ml the discernment of RIgG decreased ($P < 0.575$).

Linear regression analysis of the AI method in the 1.5- to 121.5-ng/ml RIgG range, correlating absorbance with the $\log_{10}$ of RIgG, disclosed a slope of -0.2205, y intercept of 1.41, and $r = 0.95$ (Fig. 3). The addition of experiments containing samples to 9,337 ng/ml changed the slope to -0.336, changed the y intercept to 1.52, and changed r to 0.980.

Internal AI absorbance variability was assessed by calculating the coefficient of variation for each mean of each RIgG sample in every experiment. The overall mean coefficient of variation due to the internal variability of absorbance at all dilutions studied was 5 ± 3.7%.

Absorbance by DC at 60 min ranged from 0.07 ± 0.02 at 1,093 ng of RIgG per ml to 1.62 ± 0.23 at 0 ng of RIgG per ml. The coefficient of variation at these two extremes was 28.5 and 14%.

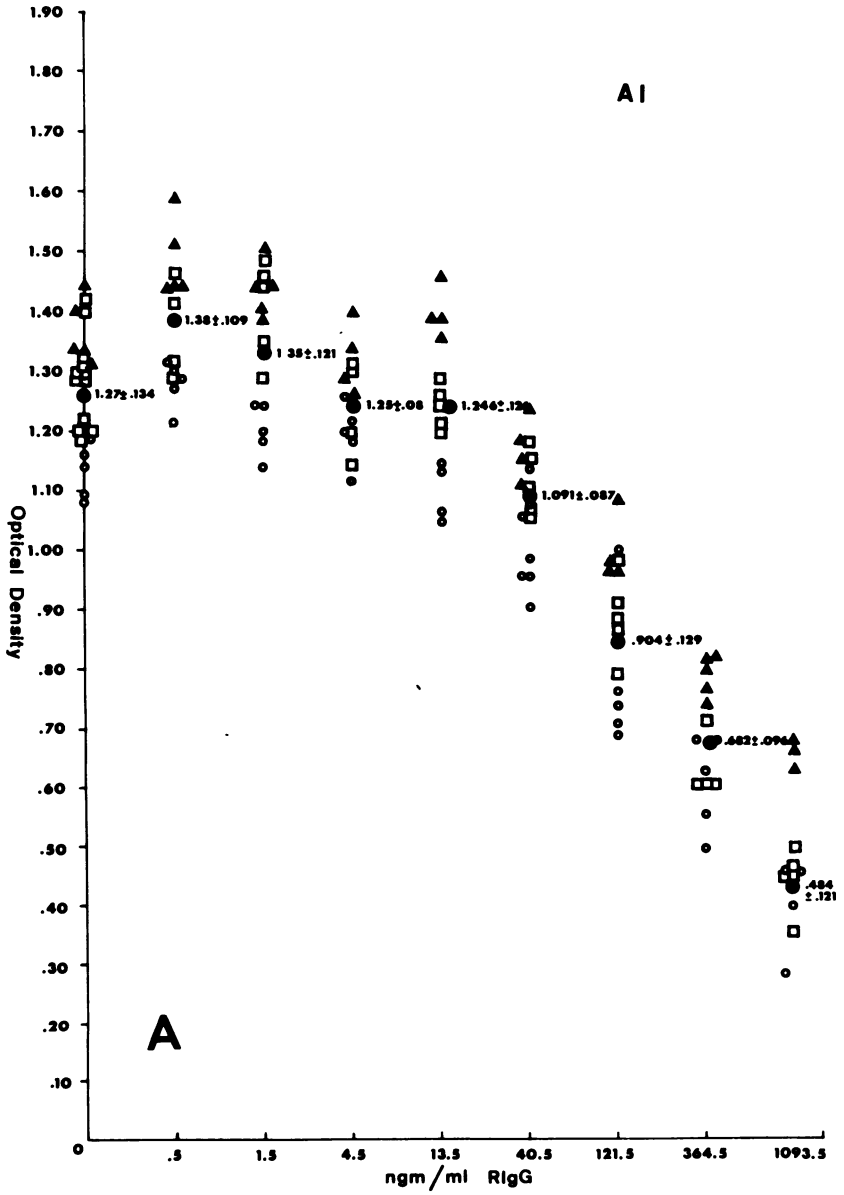

FIG. 2. *Scattergrams of absorbances generated by indicated RIgG concentrations. Similar geometric figures represent each day's run; large solid circles depict the overall mean ± 1 standard deviation.*

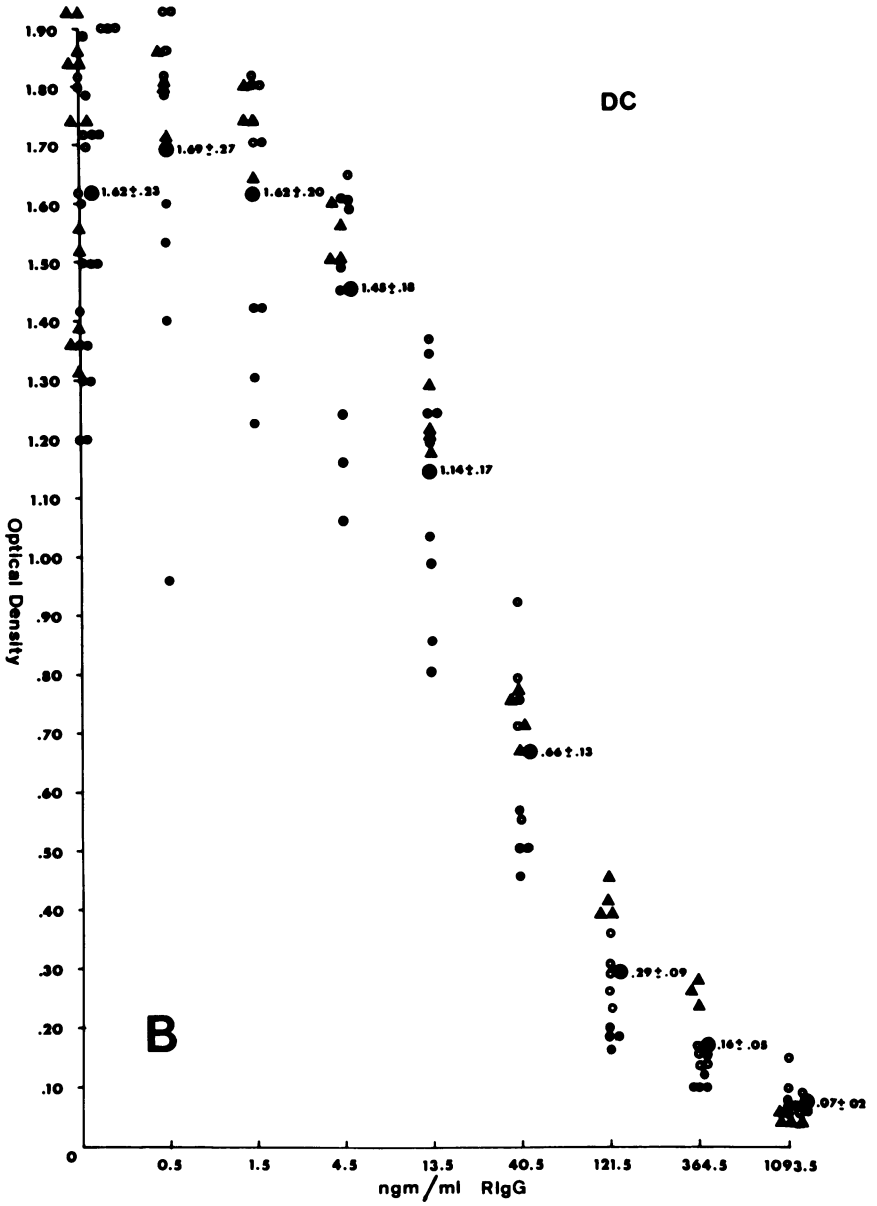

FIG. 2B

This variation did not significantly alter slope (Fig. 2B).

From 1.5 to 121.5 ng of RIgG per ml, r^2 was 0.83 as determined from interexperimental sample means (Fig. 2B). From 0 to 13.5 ng/ml, r^2 increased to 0.97, implying higher correlation in this low range. The lower limit of discernment appeared to be 13.5 ng of RIgG per ml ($P <$ 0.0001, Student's independent t test, 35 df); 4.5 ng/ml was not statistically discerned ($P < 0.102$).

Linear regression analysis of the DC method in the range of 1.5 to 121.5 ng of RIgG per ml, using absorbance as a function of the $\log_{10}$ of RIgG, disclosed a slope of -0.73, y intercept = 1.849, and $r = 0.987$ (Fig. 3).

Internal absorbance variabilities using the coefficient of variation for each quadruplicate sample set were assessed as for AI. The DC method's absorbances showed a mean internal variability of 8 ± 5.4%.

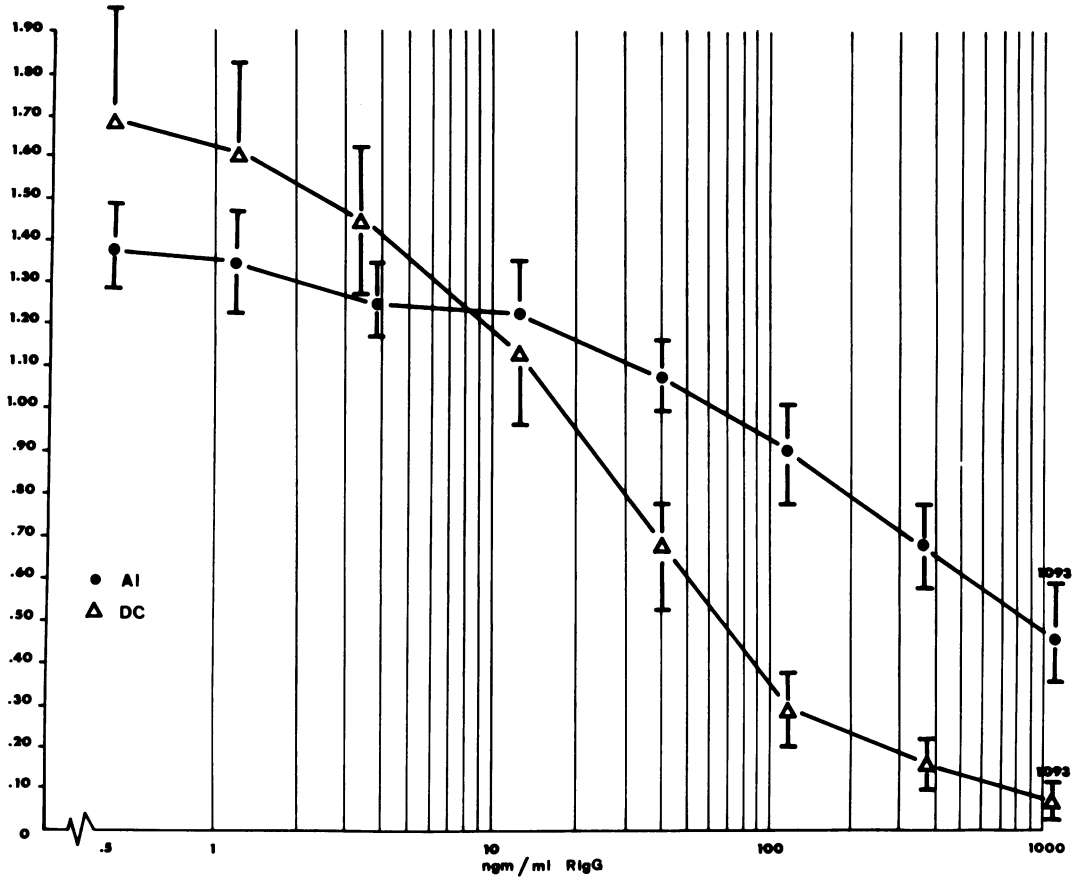

FIG. 3. *Mean absorbance ± standard deviation for AI and DC for indicated concentrations of RIgG.*

DISCUSSION

The DC method appears to be more sensitive to change in antigen concentration in the 1.5- to 121.5-ng/ml range of RIgG than does AI; the slope was at least three times larger. DC appears to be the better ELISA procedure. The overall range of color was 30% greater than that of AI, but visual color interpretation was possible on both DC and AI plates.

Excessive free AI conjugate molecules raise background absorbance, which lowers slope and decreases sensitivity. Further dilutions of antiglobulin conjugate increased the slope of the AI method (data not presented). This alteration, however, reduced the amplitude of peak color, and a delicate equilibrium existed where conjugate was limited but sufficient to produce strong yellow color. Checkerboard titration procedures are therefore required to find working dilutions of all AI antiglobulin conjugates.

An alternate explanation for increased AI background absorbance is simultaneous antiglobulin adherence to soluble and solid-phase antigen. We currently use bovine serum albumin to reduce nonspecific binding and polyethylene glycol 6000 to precipitate immune complexes of antiglobulin conjugate and antigen (M. Berdischewsky, L. S. Young, and P. Stevens, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., abstr. no. 395, 1978).

There are instances where the AI method is preferred to DC. The AI method is not dependent on successful conjugation of antigen to enzyme. When test antigens are difficult to couple to enzymes, an AI procedure might be worth trying. We have recently been successful in using the AI ELISA for bacterial lipopolysaccharide and are currently detecting nanogram amounts.

Microtiter plate methods were reproducible in this study. The internal coefficient of variation was only 5% for AI and 8% for DC. What portion of this error was due to factors other than the

microtiter plate is unknown. It is interesting that sensitivity of DC in microtiter plates was in the same range as that described by Engvall et al. (3), who used polystyrene test tubes.

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