Improved Method for Detecting Hemagglutination by Centrifugal Analysis

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Centrifugal analysis can be used to detect hepatitis B surface antigen, antibody to rubella virus, and fibrin-related antigen. The procedure is performed with the same reagents used in conventional hemagglutination studies. Positive and negative reactions are distinguished by the rates of erythrocyte clearance in the centrifugal field (ΔA/Δ time); positive cells move more rapidly than negative cells, and this difference varies directly with the concentration of detectable antigen or antibody. This phenomenon is thought to be a result of the greater adhesion of negative cells to the cuvette's surface. Sensitivity and specificity are greater in the centrifugal analysis technique than in the more conventional hemagglutination tests. False-positive reactions are eliminated and the quantitative data are accurate and reproducible.

**Additional Keyphrase:** hepatitis B surface antigen

Hemagglutination is used as the end point of various immunossays. Erythrocyte antigens can be detected directly by the agglutination of the cells in the presence of specific antibodies. Antibodies against these antigens can be assayed by their agglutination of standardized cell suspensions. Most erythrocyte serology is performed by this method. The technique can be extended to the detection of various other antigens and antibodies by binding a specific protein to the cell membrane, a reaction that is facilitated by pretreating the cells with tannic acid (1) or with one of several aldehydes (2). These cells will then agglutinate in the presence of antibodies directed against the immobilized antigen. This reaction, termed "passive hemagglutination," is very useful for determining serological immunoassays. Conversely, cells coupled to a specific antibody will agglutinate in the presence of antigen. This reaction, called "reversed passive hemagglutination," is sensitive to the presence of extremely low concentrations of antigens and is now widely used, particularly for the detection of hepatitis virus (3).

In hemagglutination inhibition, a third variation of the same approach, immobilized and free antigen compete for a common antisera. An erythrocyte-bound antigen and a standardized antisera are used as reagents. Addition of a specimen containing free antigen results in neutralization of the antisera and prevents agglutination of the cells.

These techniques have several shortcomings. Interpretation of the end point requires subjective recognition of various sedimentation patterns; at times, these patterns are somewhat ambiguous. Both the subjectivity and ambiguity curtail the reproducibility and accuracy of the procedures. In addition, the reversed passive hemagglutination reaction is subject to interference from the presence of antibodies in the specimen directed against antigens on the cells other than those that are intentionally bound to them. This interference requires that positive reactions be confirmed by neutralizing the antigen with soluble antibody, and many positive reversed passive hemagglutination reactions fail to so confirm (3, 4). These "false-positive" reactions delay the tests' final determination.

We previously described a modified method for detecting hepatitis B surface antigen (HBsAg) by reversed passive hemagglutination and the use of a centrifugal analyzer (5). This technique is superior to the conventional test in several ways: it provides an objective end point, eliminates false-positive reactions, and the incubation time is substantially shortened.

This technique is based on the observation that, under carefully defined conditions, agglutinated cells move more rapidly than nonagglutinated cells in a centrifugal field. Serum samples that are positive for HBsAg are readily distinguished from negative samples by centrifugal analysis. The rate of change in absorbance for positive controls is consistently greater than for negatives. Assay of 14 control sera produced values of 0.43 ± 0.03 (SD) A/min for positives and 0.14 ± 0.04 A/min for negatives (5).

In this paper we report the results of studies that help clarify some of the reasons for these effects and describe how we have extended the approach to passive hemagglutination and hemagglutination inhibition procedures.

**Materials and Methods**

**Apparatus**

The centrifugal analyzer we used was built at the Oak Ridge National Laboratory, Oak Ridge, TN 37830. Unlike other centrifugal analyzers, this device has a one-piece rotor that contains sample and reagent compartments and cuvettes; it is removed for loading and placed on the centrifuge–spectrophotometer for mixing and reading. The rotors we used had 17 cuvettes with 0.5-cm light paths that could accommodate 125 to 150 μL total reaction volumes (6).

The rotors were loaded by use of the loading station (7), which includes two modified Automatic Pipettes (Micromed Systems, Inc., Horsham, PA 19044) for dispensing and diluting specimens and reagents.

**Reagents**

Reversed passive hemagglutination studies were performed with a commercial kit for the detection of HBsAg (Aussell; Abbott Labs., North Chicago, IL 60064), which contains lyophilized human erythrocytes coupled to a guinea pig anti-

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Received April 26, 1979; accepted June 5, 1979.
body to HBsAg (anti-HBs); phosphate buffer (80 mmol/L, pH 7.2), used to reconstitute the cells; and phosphate buffer (0.1 mol/L, pH 7.2) supplemented with gelatin, which is used as a specimen diluent.

Passive hemagglutination studies were performed with Rubecell® (Abbott Labs.), a diagnostic test for the detection of antibody to rubella virus. The kit contains lyophilized human erythrocytes coupled to rubella antigen and the phosphate buffers used for reconstitution and dilution, as above.

Hemagglutination inhibition studies were performed with materials supplied by Dr. C. Merskey (Dept. of Lab. Med., Albert Einstein College of Medicine, Bronx, N.Y. 10461). These materials are used to detect fibrin-related antigen (6) in the sera of patients with conditions such as disseminated intravascular coagulation. The reagents consist of "tanned" (i.e., tannic acid-treated) erythrocytes coupled to human fibrinogen and hyperimmune antiserum containing antibodies to fibrinogen. The antiserum cross reacts with fibrinogen, fibrin, and many of the larger products of fibrin degradation.

The various human sera we used were clinical specimens, from "proficiency panels" compiled by the Bureau of Biologics and from apparently normal donors. Positive and reagent controls are provided in the kits. In the hemagglutination inhibition studies, human plasma containing a measured quantity of fibrinogen was used as a positive control and serum prepared by coagulating plasma in the presence of excess ε-aminocaproic acid was used as a negative control.

Rotors were siliconized by treatment with a water-soluble silicone concentrate (Silicol; Clay Adams, Parsippany, NJ 07054).

Methods

Assays for HBsAg were performed as described previously (5). Cells were reconstituted to an initial hematocrit of 0.5%, and sera were diluted eightfold before handling by the automated loading station. The station was programmed to deliver 40 μL of the diluted serum and 60 μL of the cell suspension to the specimen and reagent wells of the rotor. A "flush" of 30 μL of 0.15 mol/L saline was used to avoid specimen carryover. The total solution volume in each cuvette was 150 μL.

To start the reaction, the rotor was accelerated to 4000 rpm and immediately braked; this transferred the reagents and specimens to the cuvettes and mixed them. The rotor was then removed from the centrifuge and incubated under previously defined conditions (5). After this incubation the rotor was returned to the centrifuge and accelerated to 450 rpm. The absorbance of each cuvette was monitored at 415 nm every 5 s for 90 s. As shown previously (5), the most useful data were obtained during the first 30 s.

Analytical Variables

Sensitivity. We assessed the sensitivity of the method by determining the highest dilution of positive control sera and clinical specimens that produced changes in absorbance two standard errors greater than those produced by negative controls. The conventional subjective end points were confirmed by two independent observers.

Temperature. The effect of temperature was evaluated with reversed passive hemagglutination–hepatitis reagents by analyzing serial dilutions of positive sera after 30 min of incubation at 4, 22, or 37 °C. Similar studies were performed with positive hemagglutination–rubella, and hemagglutination inhibition–fibrin-related antigen reagents, incubated at the same temperatures for 90 min. Sensitivity was estimated from the highest dilution to produce positive data, as above.

Specificity. False-positive results are most frequently encountered with the reversed passive-hemagglutination test.

Therefore, the following studies were performed with the reversed passive hemagglutination–hepatitis test only.

Sera known to produce strong false-positive reactions were supplemented with increasing quantities of serum containing HBsAg. Each supplemented specimen was evaluated by both the conventional reversed passive-hemagglutination neutralization procedure and by our technique. Specificity was estimated from the greatest dilution of HBsAg that could be detected.

Effect of tanned cells: We conducted hemagglutination experiments, using our procedure and the conventional sedimentation procedure with fresh group O, Rh−-positive blood. These cells had not been exposed to either tannic acid or aldehydes. Positive reaction mixtures were prepared by augmenting normal serum with a sufficient quantity of anti-RhD to produce agglutination by standard serological techniques. Negative controls were prepared by augmenting the identical serum with the diluent used in preparing the anti-RhD reagent. Incubation and monitoring were the same as in the other experiments.

Effect of cuvette surface: Serial dilutions of an HBsAg-positive specimen, as well as negative specimens, were analyzed in two rotors, the cuvettes of the first rotor being treated with an aqueous solution of silicone and those of the second with distilled water. We compared changes in absorbance of these identical reaction mixtures, to assess the role of the rotor's plastic surface in the centrifugal reaction.

Effect of diluent viscosity: Various diluents were substituted for the phosphate buffer in both techniques. Various diluents were used to study identical reaction mixtures of sera and reagent cells. These diluents included 0.15 mol/L saline, commercial specimen dilution buffer, and a 60 g/L solution of hydroxyethyl starch; these were used alone and in various combinations. The resulting relative viscosities ranged from 1 for saline to 4 for hydroxethyl starch.

Effect of incubation and sedimentation. Positive and negative specimens were incubated with reagent cells in test tubes, instead of in the rotor's cuvettes, for 30 minutes at ambient temperature. The reaction mixtures were then transferred to the rotor and monitored in the usual manner. The data obtained were compared with those obtained for identical reaction mixtures that were incubated in the cuvettes.

In a related experiment, positive and negative mixtures were allowed to incubate in the usual manner, except that at
the end of 30 min, but before monitoring, the rotor was shaken to resuspend sedimeted cells.

Results

Sensitivity

The sensitivity of the centrifugal techniques consistently exceeded that of the conventional sedimentation methods. Specimens positive for HB$_3$Ag were detected when diluted 8000-fold by the conventional method, but in 32 000-fold dilution by centrifugal analysis. Anti-rubella antibody detectable at 32-fold by the conventional passive hemagglutination test was detectable at 128 by our technique. The sensitivity of our technique for detecting fibrin-related antigen by hemagglutination inhibition was comparable to the previously published techniques (9), and consistently detected fibrin-related antigen in concentrations of 5 mg/L or greater.

Incubation at 4 °C markedly decreased the sensitivity of our technique (Figure 1). The sensitivities were comparable at incubation temperatures of 22 and 37 °C, but at 22 °C the rate of change of absorbance was most closely related to the quantity of antigen.

Specificity

The centrifugal technique detected one part of HB$_3$Ag-positive serum in 2000 parts of a serum that produced strong false-positive results. The conventional neutralization technique could not confirm the presence of HB$_3$Ag in a mixture containing equal parts of the positive and false-positive sera.

After the customary 30-min incubation, positive sera were easily distinguished from negative sera. There was no difference between the reactions of the negative and the false-positive sera. After 90 to 150 min of incubation, the typical incubation period of the conventional reversed passive hemagglutination test, the rate of change of absorbance for the false-positive reactions resembled those of the positive reactions. The negative reactions were essentially unchanged (Figure 2).

Unlike the reversed passive hemagglutination procedure, passive hemagglutination and hemagglutination inhibition tests were best read after 90 min of incubation. These tests are not subject to the same false-positive reactions, and therefore their specificity is unaltered by the longer incubation.

Mechanism

**Effect of non-tanned cells:** The rate of change of absorbance during centrifugation of group O Rh$_o$-positive fresh erythrocytes was the same whether or not antibody was present. Under the same conditions, the appropriate differences in sedimentation patterns were observed.

**Effect of cuvette surface:** The rate of change of absorbance during centrifugation of specimens positive for HB$_3$Ag was dramatically increased by treating the rotor with silicone (Figure 3). The rate of change for negative specimens was similarly increased, so that the relation between positive and negative did not change. Starting absorbances are also higher for both groups of specimens in the siliconized rotor than in the nonsiliconized rotor.

**Effect of diluent viscosity:** Although different media caused differences in the rate of change of absorbance, the effect on positive and negative specimens was relatively the same (Figure 4).

**Effects of incubation and sedimentation:** Incubating the mixture outside the cuvette and then pipetting it into the cuvette abolished the difference in the change in absorbance between positive and negative specimens, as did agitation of the rotor before centrifugation.

Discussion

Hemagglutination reactions in this technique are measured as the movement of cells out of the light path of the photometer during centrifugation. The rate of movement, as measured by the rate of change of absorbance, is a function of the antigen-antibody reaction, which under similar but not identical conditions results in hemagglutination.

The mechanism by which mixtures containing the ingredients necessary for agglutination behave differently in the centrifugal field is uncertain. In attempting to explain the differences we noted that:

- The behavior of tanned or aldehyde-treated cells in the centrifugal field is different from that of fresh cells.
- The movement of both positive and negative specimens was influenced by the surface of the rotor. Siliconization of the cuvettes results in an increased rate of movement. Cells in identical concentrations absorbed more light after incubation in siliconized rotors, which suggests that fewer cells were adhering to the periphery of the siliconized cuvettes and thus more cells were present in the light path.
- Sedimentation is necessary for the effect, but changing the suspending medium so as to change the rate of sedimentation has little influence. Saline, gelatin-fortified buffer, and
hydroxyethyl starch did not produce significant changes in the relative behavior of positive and negative specimens. Change of diluent in the conventional techniques can cause positives and negatives to be indistinguishable.

Distinctive patterns form in the positive and negative cuvettes during the undisturbed incubation periods. If the cuvettes are agitated before centrifugation, positives and negatives cannot be distinguished.

These observations lead us to speculate that during the brief period of incubation before centrifugation, tanned or aldehyde-treated erythrocytes become weakly attached to the plastic surface of the cuvettes; cells whose surfaces are immunologically coated with additional antigens or antibodies, however, are inhibited from forming such attachments. Presumably, centrifugal force moves those cells that are not attached to the surface more easily than those that are attached; therefore the movement of positive cells is more rapid than that of negative cells. We postulate that cells that are attached to the surface can be loosened by reaction with antibody; this would explain how cell suspensions that are negative after 30 min can become false positive after 90 min or more of incubation. Longer incubation periods are required for reactions that are governed by larger, less-mobile determinants, such as immunoglobulin, than for reactions governed by smaller particles, such as soluble antigens. Thus passive-hemagglutination, hemagglutination inhibition, and reversed passive-hemagglutination false-positive tests require longer incubation than is needed for the HBAg technique.

The sensitivity for the reversed passive-hemagglutination test for HBAg and the passive-hemagglutination test for rubella were increased by centrifugal analysis. The sensitivity of the hemagglutination inhibition test for fibrin-related antigen was comparable to that by our technique.

Poor specificity is a major limitation of the conventional reversed passive-hemagglutination procedure. Specimens that appear to be positive on initial screening must be confirmed by neutralization of the hepatitis antigen, which is time-consuming and may result in false-negatives. A serum that contains a concentration of HBAg lower than the concentration of the substance that causes a false-positive reaction may not be confirmed as positive. Results with centrifugal analysis after 30 min of incubation are not affected by the factors that result in false positivity. Consequently, this technique can detect concentrations of antigen in serologically false-positive sera that are too small to be detected by the customary test. This enhanced sensitivity is a function of the shortened incubation time. With incubation times of 90 min or more, false and true positives become indistinguishable.

We thank Mrs. Arlene Saunders for her clerical assistance.

References


1 Package insert, Raphadex B: Reversed passive hemagglutination test for confirmation of the presence of HBAg. (Ortho Diagnostics, Oct. 1978).