Factors Affecting the Passive Hemagglutination Titration: Dilution Loops, Titration Trays, Vibration, Diluents

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Serial dilution with Takatsy loops resulted in exaggerated passive hemagglutination titers with most of the anti-bovine serum albumin sera tested. It appears that certain types of agglutinins adhere to the loop surface and are released only gradually. This adherence, or carry-over effect, was prevented by presoaking loops in gelatin or gelatin-rabbit serum-albumin solutions. Hemolysins did not adhere to loops. In general, hemagglutination reactions performed on plastic trays gave higher titers than those performed in glass test tubes. The quality of the hemagglutination pattern was dependent to a great extent on the type of plastic tray used. As much as a 100-fold difference in titers was obtained depending on the composition of the antiserum diluent. The increase in vibration, in terms of linear displacement (approximately twofold), resulted in an eightfold decrease in titers.

Although the passive hemagglutination (HA) test is one of the most simple and widely used immunological assays, it lacks reproducibility. Similar HA titrations performed in two laboratories are seldom comparable and, even in a single laboratory, titers often vary from day to day. The recent introduction of plastic HA trays and Takatsy dilution loops (6) further simplified the test but also contributed additional variables which are described in this report.

Recently we reported on a preparation of stabilized erythrocytes which could be coated with protein and polysaccharide antigens without the aid of coupling agents. Erythrocytes were stabilized by pyruvic aldehyde followed by formaldehyde (1). The antigen-coated cells, when stored in liquid nitrogen, gave reproducible HA titers for at least 4 months (3). Thus, the use of these cells eliminated one variable, i.e., that due to differences in cell preparations.

Using these stabilized cells, we evaluated the accuracy of loop dilutions and the effects of various types of titration trays, antiserum diluents, and vibration on HA patterns. We also studied the use of loops in hemolysin titrations.

MATERIALS AND METHODS

Antigens. Crystalline bovine serum albumin (BSA) from Armour Pharmaceutical Co., Kankakee, Ill., and sheep erythrocytes (SRBC) from a single sheep were used.

Antiserum. Anti-BSA serum 448 was obtained by intravenous injection of 20 mg of BSA into a 2-kg New Zealand white rabbit; the serum was collected 14 days later. Anti-BSA sera 1, 3, and 32 (kindly supplied by H. Ainis) were obtained by injecting rabbits subcutaneously in the four foot pads with a total of 40 mg of BSA in Freund's complete adjuvant. Sera were collected 28 days later. Hyperimmune anti-BSA sera 7, 8, and 385 were obtained by immunizing rabbits twice, several months apart, with 100 mg of BSA in Freund's complete adjuvant; intravenous, intraperitoneal, intramuscular, subcutaneous, and intradermal routes were employed. Sera were collected 7 to 14 days after the last injection. Anti-SRBC serum pools C and D were obtained from 12 rabbits 11 days after a single injection of 2 × 10^8 SRBC per kg and 10 days after the last of eight secondary injections of the same dose, respectively. By gel filtration analysis, at least 95% of the hemolytic activity in pool C was contained in the macroglobulin fraction, whereas about 85% of the hemolytic activity in pool D was associated with the γ-globulin (presumably 7S) fraction.

Preparation of erythrocytes coated with BSA. The procedure for preparation of erythrocytes coated with BSA is given in detail elsewhere (1). Briefly, rabbit erythrocytes were stabilized by treating the cells (4%) in a pyruvic aldehyde solution (1.5%) for 18 hr, followed by formaldehyde (1.5%) treatment for another 18 hr. The stabilized cells, which we call FPRE, were washed and suspended in 0.1 M phosphate buffer, pH 7.2. FPRE cells were coated with the antigen by stirring them for 2 hr in 0.1 M acetate buffer.
(pH 4) containing 0.1 mg of BSA/ml. The antigen-coated cells (BSA-FPRE) were washed and resuspended as 10% in 0.1 M phosphate buffer (pH 7.2), quick-frozen in liquid nitrogen, and stored at -20°C. BSA-FPRE cells were thawed and refrozen several times to increase sensitivity and stored in liquid nitrogen (3). Chicken erythrocytes were coated with BSA (BSA-FCE) in a similar manner except that these cells were stabilized by formaldehyde alone, and the coating was performed at pH 5 (4). For use in HA titrations, coated cells were diluted to 0.25% in the phosphate buffer.

Diluents for antisera. One diluent (Gel-PO₄) consisted of 1 mg of gelatin (Difco) per ml of 0.1 M phosphate buffer (pH 7.2). Another contained 10 mg of rabbit serum albumin per ml of phosphate buffer (RSA-PO₄). RSA was purchased from Hyland Laboratories, Los Angeles, Calif. (Cohn fraction V) or prepared by ammonium sulfate precipitation (between 75% and 70% saturation) of normal rabbit serum. These are designated as Hyland-RSA and ammonium sulfate-RSA, respectively. Gel-PO₄ diluent contained 1 mg of gelatin plus 0.5 mg of ammonium sulfate-RSA/ml of PO₄.

Hemolysin tests, the diluent for all reagents was Veronal-buffered saline with added Mg²⁺ and Ca²⁺ (5) and 1 mg of gelatin per ml (VB-Gel).

Titrations trays and test tubes. Disposable plastic trays with 96 V-shaped wells (0.3 ml capacity) were used. The V-shaped wells gave better cell buttons than the U-shaped wells. The trays tested were poly- styrene trays (no. IS-MVC-96, Linbro Chemical Co., New Haven, Conn., or no. 220-25A, Cooke Engineering Co., Alexandria, Va.) and polyvinyl chloride trays (Linbro no. S-MVC-96, or Cooke no. 220-25). These are designated as Linbro-styrene, Cooke-styrene, Linbro-vinyl, and Cooke-vinyl, respectively. Before use the trays were soaked in a mild detergent solution for about 2 hr, rinsed thoroughly with deionized water, and rinsed three times with glass-distilled water. Aside from the plastic trays, round-bottom Pyrex-brand glass test tubes (inside diameter, 7 mm) were also used.

Methods of antisera dilutions for HA. Antisera were diluted in serial twofold dilutions either by the use of 25-μl Takatsy “tulip type” loops (reference 6; Cooke Engineering Co., Alexandria, Va.) or by pipettes. In the latter case, dilutions were made in test tubes and 25-μl samples were transferred to titration trays or to glass test tubes.

Hemagglutination titration procedure. To 25 μl of antisera dilutions, 25 μl of an 0.25% of BSA-FPRE suspension was added; the trays were covered and shaken at 10-min intervals three times. To minimize carry-over effects, the trays were placed on a special platform consisting of a lead plate (thickness, 2 cm; 40 by 60 cm) over a 5-cm thick foam-rubber pad. The lead plate was covered with a cloth hand towel. The cells were allowed to settle for 18 hr at room temperature (24°C); then the HA titers were recorded.

Hemolysin titration procedure. Serial dilutions of antisera were prepared in Veronal buffer or VB-Gel by using either coil or tulip loops. To 25 μl of antisera dilutions in each well, 25 μl of a 1% suspension of thrice-washed SRBC was added. The trays were shaken, covered, and incubated at 37°C. After 0.5 hr, the trays were reshaken and 25 μl of a 1:30 dilution of complement (C’; fresh guinea pig serum absorbed at 0°C with SRBC) was added to each well. This amount of C’ approximated 12 CH₅₀ units (see below). The trays were shaken and incubated for another 0.5 hr at 37°C. Then 25 μl of 0.008 M sodium citrate in 0.15 M saline was added to inhibit further hemolysis. Unlysed cells were permitted to settle for 18 hr at 4°C. Titer was expressed as the highest dilution factor showing at least 50% hemolysis as judged by comparison with a graded series of standards equivalent to 0 through 100% hemolysis. Controls lacking antisemur or C’ were included with each set of tests.

RESULTS

Evaluation of the Takatsy loops in passive HA titrations. HA titers obtained by using Takatsy loops were compared with those obtained by twofold dilutions using pipettes. Quadruplicate titrations were performed on Linbro-styrene trays with Gel-PO₄ as antisemur diluent.

With six of the seven sera, the loop dilution method produced considerably higher titers compared to the pipette dilution; with one, the titers were the same (Table 1). The extreme high titers, such as 10⁸, are obviously in error. For instance, serum 385 contained 16 mg of total antibody protein per ml as estimated by the antibody-adsorbent method (2); hence, it was calculated that less than one antibody molecule per well should have existed at a dilution of 10⁶. This error probably resulted from excess carry-over of antibody during the dilution procedure.

When stock dilutions were prepared with pipettes prior to dilution with loops, the discrepancies in HA titers were not as great as when loop dilutions were used exclusively (Table 1).

The carry-over effect could have been exaggerated because of the extreme sensitivity of the test with BSA-FPRE. By using the BSA-FCE system with ½₀₀₀₅th the sensitivity, the discrepancy between HA titers obtained by loop and by pipette dilutions was diminished but nevertheless marked, especially when the starting dilution was 1:2 (Table 2).

In the following experiments we examined the possibility that some serum factors, other than antibody molecules themselves, were responsible for the exaggerated HA titers.

Effect of diluting antisemur in a normal rabbit serum prior to dilutions by gelatin solution. Antiserum 7 was initially diluted 1:10 or 1:100 by pipettes either in pooled normal rabbit serum or in Gel-PO₄. Subsequently, these were further diluted in Gel-PO₄ by loops. The HA titers were approxi-
TABLE 1. Comparison of antiserum dilutions by loops and by pipettes

<table>
<thead>
<tr>
<th>Dilution in first well</th>
<th>Antiserum no. 1</th>
<th>Antiserum no. 3</th>
<th>Antiserum no. 7</th>
<th>Antiserum no. 8</th>
<th>Antiserum no. 32</th>
<th>Antiserum no. 383</th>
<th>Antiserum no. 448</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.3 × 10⁴</td>
<td>3.2 × 10⁴</td>
<td>3.9 × 10⁻⁶</td>
<td>5.0 × 10⁻⁴</td>
<td>7.4 × 10⁴</td>
<td>1.2 × 10⁴</td>
<td>4.2 × 10⁴</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>4.8 × 10⁻⁶</td>
<td>1.4 × 10⁻⁸</td>
<td></td>
<td>1.9 × 10⁴</td>
<td>1.9 × 10⁴</td>
</tr>
<tr>
<td>200</td>
<td>1.6 × 10⁴</td>
<td>3.2 × 10⁴</td>
<td>5.5 × 10⁻⁶</td>
<td>2.7 × 10⁻⁸</td>
<td>9.1 × 10⁴</td>
<td></td>
<td>4.3 × 10⁴</td>
</tr>
<tr>
<td>2,000</td>
<td>1.6 × 10⁴</td>
<td>3.2 × 10⁴</td>
<td>1.7 × 10⁻⁶</td>
<td>1.3 × 10⁻⁸</td>
<td>9.1 × 10⁴</td>
<td>1.3 × 10⁴</td>
<td>1.6 × 10⁴</td>
</tr>
</tbody>
</table>

* Coated cells used: BSA-FPRE.
* Loops were presoaked in phosphate buffer.
* Antiserum dilutions were made in test tubes by using pipettes, and 25-µliter samples were transferred to the first well. Then, serial dilutions were carried out by loops.
* Note high degrees of loop carry-over effect in these sera.
* Geometric mean titers obtained by pipette dilutions throughout.

**TABLE 2. Comparison of antiserum dilutions by loops and by pipettes**

<table>
<thead>
<tr>
<th>Dilution in first well</th>
<th>Geometric mean HA titers obtained by loop dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antiserum no. 7</td>
</tr>
<tr>
<td>2</td>
<td>6.5 × 10⁴</td>
</tr>
<tr>
<td>20</td>
<td>8.5 × 10⁴</td>
</tr>
<tr>
<td>200</td>
<td>2.7 × 10⁴</td>
</tr>
<tr>
<td>2,000</td>
<td>2.0 × 10⁴</td>
</tr>
</tbody>
</table>

* Coated cells used: BSA-FCE.
* Loops were presoaked in phosphate buffer.
* Dilutions were prepared by pipettes and placed in the first well; then serial dilutions were carried out by loops.
* HA titers obtained by pipette dilutions throughout.

Ultimately, the same whether the antiserum was pre-diluted in normal rabbit serum or in Gel-PO₄. Hence, normal serum does not contain any substance which nonspecifically enhances antiserum titers in these tests.

**Prevention of carry-over effect by presoaking loops in protein solutions.** Loops were soaked in Gel-PO₄, Gel-RSA, or in preimmunization normal rabbit serum for 1.5 hr, blotted, and used for antiserum dilutions. Antiserum 8 was initially diluted 1:20 by pipettes and then diluted on trays by presoaked loops. The antiserum was diluted in Gel-PO₄.

HA titers obtained by loops presoaked in Gel-PO₄ or Gel-RSA were essentially identical to those obtained by pipette dilutions. Titers were about four wells higher with the use of serum-soaked loops. Thus, presoaking loops in appropriate protein solutions prevented the carry-over effect to a substantial degree. Nevertheless, the HA titers obtained by loop dilutions were not as precise as compared with pipette dilutions.

**Precision of the loop dilution procedure.** Antiserum 8 was initially diluted 40-fold in Gel-PO₄ by pipettes, and then further diluted in octuplicate by pipettes, by a single loop, or by eight different loops. Loops were presoaked in Gel-RSA before use. Geometric mean titers (× 10⁴) were 8.2 (range: 4.1 to 16.4), 7.2 (range: 3.3 to 52.4), and 21.9 (range: 3.3 to 210), respectively. It is clear that the pipette dilution procedure was the most precise of the three.

**Effect of the use of Takatsy loops on hemolysin titrations.** Quadruplicate titrations were performed for each of four starting dilutions for each serum: 1:2, 1:20, 1:200, and 1:2,000. The succeeding serial twofold dilutions on the trays were prepared by using tulip loops presoaked in VB-Gel.

The results show that the serum concentration in the initial well of the dilution series had no significant effect on the hemolytic end point for either serum (Table 3). In another experiment, a single coil loop was used instead of the tulip type. The results were virtually identical.

To test whether the composition of the buffer used for presoaking the dilution loops would influence the outcome of the hemolysin tests, as it did in the agglutinin system, the above experiments were rerun by using loops presoaked in VB without added gelatin. There were no significant differences in titer whether the loops were presoaked in VB or in VB-Gel.

**Effect of antiserum diluents on HA titers.** The composition of the medium used for diluting antiserum had considerable effect on the HA titers. Antisera were diluted by pipettes. The HA reactions performed in the gelatin solution gave...
the highest titers, whereas those performed in Hyland-RSA gave the lowest titers. For instance, with antiserum 385, quadruplicate titrations performed in Gel-PO₄, Gel-RSA, ammonium sulfate-RSA, and Hyland-RSA gave geometric mean titers of \(1.6 \times 10^4\), \(4 \times 10^4\), \(2 \times 10^5\), and \(2.6 \times 10^6\), respectively. Similar results were obtained repeatedly with several other sera. The bases for these differences are not as yet clear.

**Effect of vibration on HA titers.** Although it is generally accepted that vibrations lower HA titers, no quantitative data have been published. Thus, we examined the effect of vibrations at three different levels as measured by "vertical vibration displacements." An increase in vibration displacements from the ranges of 0.03 to 0.067 mils to 0.084 to 0.147 mils decreased the titer approximately 15-fold (Table 4).

**Effect of the use of plastic and glass vessels on HA titrations.** HA reactions were performed in four different types of plastic trays and in glass test tubes. The HA reactions were performed in septuplicates with seven separate trays of each type; two controls (diluent plus coated cells) were incorporated for each experimental row.

Table 3. *Anti-sheep hemolysin titers (geometric mean) obtained with the use of Takatsy loops*

<table>
<thead>
<tr>
<th>Dilution in first well⁵</th>
<th>Antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pool C</td>
</tr>
<tr>
<td>2</td>
<td>(1.0 \times 10^4)</td>
</tr>
<tr>
<td>20</td>
<td>(6.2 \times 10^4)</td>
</tr>
<tr>
<td>200</td>
<td>(1.3 \times 10^4)</td>
</tr>
<tr>
<td>2,000</td>
<td>(1.4 \times 10^4)</td>
</tr>
</tbody>
</table>

⁵ Loops were presoaked in VB-Gel.

Antiserum dilutions were prepared by pipettes and placed in first well; then serial dilutions were carried out by loops.

Table 4. **Effect of vibration on HA titers**

<table>
<thead>
<tr>
<th>Location of HA trays</th>
<th>Vibration displacement</th>
<th>HA titers read at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mil</td>
<td>3 hr</td>
</tr>
<tr>
<td>Inside hood without lead plate</td>
<td>0.084-0.147</td>
<td>1.9 (\times) (10^6)</td>
</tr>
<tr>
<td>Inside hood with lead plate</td>
<td>0.063-0.147</td>
<td>1.9 (\times) (10^6)</td>
</tr>
<tr>
<td>On bench with lead plate</td>
<td>0.032-0.067</td>
<td>1.9 (\times) (10^6)</td>
</tr>
</tbody>
</table>

⁶ Diluent for antiserum: 10 mg of ribonucleic acid per ml.

⁷ Vibration meter, International Research and Development Corp., Columbus, Ohio, was used; mil = inch/1,000. Vibration displacement in the range of 0.032 to 0.067 mils can be conveniently distinguished from that in the range of 0.063 to 0.147 mils by observing a specimen under a microscope at 400X. In the lower vibration range, the image is immobile; in the higher range, the image is in motion frequently.

**Discussion**

The passive HA tests done with antigen-coated aldehyde-treated erythrocytes were found to be influenced by the mode of antiserum dilution, antiserum diluents, vibrations, and the vessels in which titrations were carried out.

When serially diluted with Takatsy loops, the HA end point of most sera tested with BSA-PRE depended on the serum concentration in the first well of the titration plate. As the starting point dilution was increased (by preparing them separately by use of pipettes), the apparent titer decreased and approached the values obtained with tests made with pipetted dilutions through
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out. For example, a Takatsy loop serial dilution, starting from an initial 1:2 dilution of serum no. 8, yielded an apparent titer of $5.0 \times 10^{14}$, clearly absurd, whereas, starting from 1:2,000 it yielded a titer of $1.3 \times 10^9$. The latter titer, however, was still greater than that by pipette dilutions by a factor of 10. Only in the case of serum no. 3 did the titers obtained by both methods agree.

Since the end point varied with the initial serum concentration, it was possible that non-antibody serum factors were responsible for the exaggerated titers. This possibility was ruled out by showing that the results were the same when the various initial starting dilutions were prepared in normal rabbit serum.

The most likely explanation seems to be that there is an excess "carry-over" of antibody by the diluting loops. Antibody, as well as other serum components, is adsorbed to the loop surfaces and is then released only gradually throughout the well-to-well traverse. The amount initially adsorbed, and therefore the degree of the titer exaggeration, would seem to depend on the concentration of antibody to which the "clean" loop is initially exposed. Presoaking the loops in gelatin-fortified diluent apparently saturates the loop surfaces and effectively precludes adsorption of significant amounts of antibody to which it is subsequently exposed.

The expression of the carry-over effect depends also on the sensitivity of the test system (e.g., the BSA-FPRE HA versus the hemolytic assay), antibody concentration, the particular loop being used, and perhaps the species of immunoglobulin involved.

LITERATURE CITED


