Improved Hemagglutination Inhibition Assay for Mammary Tumor Virus Antigen

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It is our experience that the use of sheep red blood cells pretreated with formaldehyde and pyruvaldehyde before sensitizing with viral antigen yields results that are comparable to that obtained by using the tannic acid method. The double aldehyde method provides the added advantage of being useful for assaying the viral antigen content in substances which contain high levels of interfering substances which would preclude the use of other assay methods.

The inhibition of passive hemagglutination has proven to be a useful and sensitive method for quantitating the amounts of viral antigen in various biological materials. The most commonly used method for preparing erythrocytes for the hemagglutination inhibition (HAI) test employs tannic acid treatment of the cells (1) prior to sensitization with antigen. However, the use of tannic acid-treated cells in the HAI assay is hampered by the fact that tannic acid-fixed cells are extremely sensitive to interfering substances that render the assays difficult to interpret. Interference may take the form of nonspecific agglutination or even, in extreme cases, lysis of the sheep red blood cells (SRBC). This kind of interference has proven to be particularly troublesome when assaying the murine mammary tumor virus (MTV) antigen content of various types of milk. We now report that the use of the double aldehyde method (3) for sensitizing SRBC for use in the HAI assay for MTV antigen virtually eliminates the problem of nonspecific interference. This modification of the HAI assay is both sensitive and reliable and may be successfully applied to a variety of biological materials that contain interfering substances.

MATERIALS AND METHODS

Sheep erythrocytes. SRBC, obtained from Truslow Farms, Md., were suspended in Alsever solution. Prior to use in the HAI assay, SRBC were washed five times with phosphate-buffered saline (PBS), pH 7.2, and prepared as an 8% (vol/vol) suspension. SRBC were used within 7 days.

Preparation of the SRBC. Equal volumes of 3% pyruvaldehyde solution in 0.11 M sodium phosphate, pH 7.2, and 8% SRBC were carefully mixed and then stirred by a magnetic stirrer for 17 h at room temperature. The cells were then washed five times with the 0.11 M phosphate solution and filtered through gauze. The pyruvaldehyde-treated SRBC were again made up to 8% in 0.11 M phosphate buffer, pH 7.2, and mixed with an equal volume of 3% formaldehyde in similar buffer. This mixture was agitated, washed, and filtered as described above. Samples of the stabilized SRBC were quick-frozen in liquid nitrogen until used. (For details of this method, see reference 3.)

Sensitization of the SRBC. Eight percent SRBC prepared as above were mixed with an equal volume of viral antigen (see below) and four volumes of PBS, pH 6.4. Adequate sensitization of the SRBC with viral antigen requires 15 min at room temperature. (The ideal concentration of viral antigen was determined for each new batch of virus.) The sensitized SRBC were then washed twice in PBS (pH 7.2) containing 1% normal rabbit serum.

Antiserum. Rabbit anti-MTV sera were prepared in the following manner. New Zealand rabbits weighing 2 to 3 kg were injected intramuscularly with Tween 80-diethyl ether-disrupted (3) MTV emulsified in Freund complete adjuvant. At 4-week intervals, the animals were boosted by a further injection of the viral preparation. Trial bleedings were collected after each booster dose of viral antigen. Blood was drawn for antiserum 1 week after the third booster of antigen. The anti-MTV serum was heat inactivated at 56 C for 30 min and stored at –70 C until used. The hemagglutination titer of our standard anti-MTV serum was >1,024.

MTV. Milk samples were obtained from mouse strains known to exhibit either high or low expression of MTV. High expressors of MTV were high parity C3H/He, originally obtained from the National Institutes of Health (NIH) laboratory, and RIII, originally obtained from Dan Moore, New Jersey Institute for Medical Research. Low virus expressers were BALB/c and NIH Swiss mice obtained from the NIH breeding colony. All milk samples were skimmed to remove fat prior to testing.

Standard MTV antigen was prepared by using gradient-purified Tween 80-diethyl ether-disrupted
MTV (4). Routine assays were controlled by the use of standard viral antigen preparations, milk samples containing known levels of MTV antigen, and negative milk samples.

**HAI test.** Disposable plastic microtiter plates (IS-MRC-96) obtained from Linbro Chemical Co., New Haven, were used. Twenty-five microliters of the unknown sample plus 25 μl of 0.1% gelatin (Difco) in PBS (pH 7.2) were mixed in the first well of the microtiter plate and serially diluted. Antiserum (50 μl) was then added to each well. The proper dilution of antiserum was determined prior to performing the test by using standard MTV antigen preparations. This mixture was thoroughly shaken and allowed to stand for 30 min at room temperature. Sensitized erythrocytes (25 μl) in 0.1% gelatin PBS were then added, the plate was covered, and the suspension mixture was again vigorously shaken. The plates were allowed to stand overnight at room temperature and were read the following morning. The HAI titer was four times the antigen dilution in the last well yielding a positive reading. This procedure takes into account the dilution of the antigen within the test system. Controls consisted of wells containing SRBC plus PBS, SRBC + 0.1% gelatin, and SRBC plus antiserum.

The tannic acid method for stabilization of SRBC was identical to that previously described by Sibal et al. (5).

**RESULTS**

Table 1 compares results obtained by using double aldehyde- and tannic acid-treated SRBC in the HAI assay. As can be seen, the two methods produce comparable sets of data. Samples containing varying levels of MTV antigen were found to give similar titers. Variation in individual readings were within the expected confidence range, i.e., plus or minus one dilution. It can be concluded that the use of double aldehyde-stabilized SRBC does not reduce the sensitivity of the HAI assay for MTV antigen.

Assaying milk samples for the presence of MTV antigen by using tannic acid-treated SRBC has been hampered by nonspecific interference by substances present in samples of many kinds of milk. Partial elimination of such interference can be accomplished by treatment of the milk samples with chymotrypsin (2), heat treatment (56°C for 30 min), and adsorption with SRBC. However, procedures used to remove interference destroy significant amounts of viral antigen. Use of double aldehyde-stabilized SRBC in the HAI test was found to eliminate the need for pretreatment of the milk samples other than skimming to remove fat from the milk. Table 2 illustrates the comparative titer of MTV antigen in milk and tissue culture samples from various sources. Reliable HAI titers were obtained most consistently by using the double aldehyde-treated SRBC. Therefore, the double aldehyde method appears to be the method of choice for testing biological materials containing interfering substances.

An additional advantage of aldehyde-treated

| Sample no. | Method of sensitizing SRBC |
|------------|---------------------------|
|            | Tannic acid | Double aldehyde |
| 1          | 16          | 128 |
| 2          | N*          | 16  |
| 3          | 8           | 16  |
| 4          | 16          | 128 |
| 5          | 64          | 64  |
| 6          | 32          | 128 |
| 7          | 16          | 128 |
| 8          | 16          | 32  |
| 9          | 128         | 128 |
| 10         | 32          | 32  |
| 11         | 16          | 64  |
| 12         | 16          | 16  |
| 13         | 128         | 512 |
| 14         | 64          | 128 |
| 15         | 4           | 32  |
| 16         | 128         | 32  |
| 17         | 128         | 128 |
| 18         | 64          | 128 |
| 19         | 64          | 256 |
| 20         | 32          | 64  |
| 21         | 16          | 4   |
| 22         | N           | N   |
| 23         | N           | N   |
| 24         | N           | N   |
| 25         | N           | N   |
| 26         | N           | N   |
| 27         | N           | N   |
| 28         | N           | N   |
| 29         | N           | N   |
| 30         | 8           | 16  |
| 31         | N           | N   |
| 32         | N           | N   |
| 33         | N           | N   |
| 34         | N           | N   |
| 35         | N           | N   |
| 36         | 64          | 16  |
| 37         | N           | N   |
| 38         | 64          | 32  |
| 39         | 16          | 8   |
| 40         | N           | N   |
| 41         | N           | N   |
| 42         | N           | N   |
| 43         | N           | N   |
| 44         | N           | N   |

* Each sample represents amounts of C3H milk obtained from first parity C3H/He mice.

N, Negative.
Table 2. Representative assays demonstrating the elimination of interference by use of double aldehyde-treated MTV-sensitized SRBC

| Assay            | Sample no. | Method of sensitizing SRBC | Tannic acid | Double aldehyde |
|------------------|------------|----------------------------|-------------|-----------------|
| RIII milk        | 1          | I*                        | 2,048       |                 |
|                  | 2          | 1,024                     | 2,048       |                 |
|                  | 3          | I                         | 1,024       |                 |
|                  | 4          | I                         | 512         |                 |
| C3H milk         | 1          | 16                        | 32          |                 |
|                  | 2          | 64                        | 64          |                 |
|                  | 3          | 8                         | 16          |                 |
|                  | 4          | 64                        | 32          |                 |
|                  | 5          | 8                         | 8           |                 |
|                  | 6          | 128                       | 256         |                 |
| NIH Swiss milk   | 1          | I                         | 8           |                 |
|                  | 2          | I                         | 16          |                 |
| BALB/c milk      | 1          | I                         | 64          |                 |
|                  | 2          | I                         | 32          |                 |
|                  | 3          | I                         | 64          |                 |
|                  | 4          | I                         | 8           |                 |
| Human milk       | 1          | I                         | N           |                 |
|                  | 2          | I                         | N           |                 |
| Tissue culture medium from MTV + culture | 1 | 8 | 16 | |
|                  | 2          | 4                         | 4           |                 |
|                  | 3          | 16                        | 32          |                 |

* I, Interference of sufficient intensity so as to prevent testing of the material.
* N, Negative.

SRBC is the ability of such SRBC to withstand storage in the frozen state. Therefore, large batches of SRBC can be produced at one time for routine use in the HAI assay. This procedure eliminates the need for treatment of SRBC prior to each HAI assay, thereby saving time and adding to the precision of the assay.

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