Improved Dextran Sulfate-Calcium Chloride Method for the Removal of Nonspecific Inhibitors with Modifications for Nonspecific Agglutinin Removal in the Rubella Hemagglutination Inhibition Test

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The dextran sulfate precipitation method for removal of nonspecific inhibitors from sera to be tested in the rubella hemagglutination-inhibition test was modified by shortening the dextran sulfate-serum mixture incubation period from 2 hr to 30 min. A comparative study using both treatment times showed no significant difference in rubella antibody titers. Reducing the treatment time even further also gave comparable results, indicating the select 30-min incubation period is not marginal for undertreatment. It was shown that 41.5% of the 159 sera tested had nonspecific agglutinins at levels from 1:8 to as high as 1:64. In each specimen, all demonstrable nonspecific agglutinin was removed by adsorption with 10%, 0- to 2-day-old chick red blood cells rather than the usually recommended 50% adult chicken erythrocytes.

The rubella hemagglutination-inhibition [HAI] test as introduced in March 1967 by Stewart et al. (10) and then modified by the National Communicable Disease Center (5) (renamed the Center for Disease Control; CDC) used kaolin for the removal of nonspecific inhibitors from sera to be tested. It soon became apparent that the kaolin method of serum treatment was not always satisfactory because it had a tendency to remove significant amounts of immunoglobulin (1, 9), and it may also fail to remove some of the beta-lipoprotein inhibitor.

As early as 1955, it had been reported that certain sulfated polysaccharides will selectively precipitate beta lipoproteins from serum in a certain pH range, at a specific ionic strength, and, for some, at specific metal ion concentrations (3). Two of these, heparin (9) and dextran sulfate (7), have been used for serum treatment for removal of inhibitors in virus serology. Modifications of these two techniques have now been recommended to replace the earlier kaolin methods for the rubella HAI test (2).

Shortly after these new techniques were published, we at the Wisconsin State Laboratory of Hygiene began work with both to determine which might be better utilized in our diagnostic service and research. We have found the method of heparin and manganous chloride to be unsatisfactory because of apparent nonspecific hemagglutinins at low serum dilutions. This appears to be a specific problem of compatibility with the rubela HAI test, since this procedure works well with rubella HAI. The alternate method described in the CDC guide (2), using dextran sulfate and calcium chloride, proved to be very reliable, and it has been adopted as the routine test in this laboratory. A major disadvantage to this method is the total time required, over 3 hr, for the recommended serum treatment before testing. This paper reports the results of our efforts to reduce the time requirement, as well as our findings regarding the incidence of nonspecific inhibitors and agglutinins found in our test sera.

MATERIALS AND METHODS

The reagents and methods used for the dextran sulfate-calcium chloride serum treatment method are essentially those of Liebhaber (6), which the
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One hundred fifty-nine test sera were selected from specimens submitted to the virus section of the State Laboratory of Hygiene over a 6-month period starting in November 1970. Thirty sera were from 26 children less than 10 years of age, 99 were from 60 patients between the ages of 15 and 33, (39 of these were from 19 serologically positive rubella cases), and 30 sera from 29 patients between the ages of 40 and 86. All of the sera from the 15 to 33 age group were submitted for rubella serology, and a few of the children's sera were also rubella diagnostic requests. None of the children's sera showed positive serological changes for rubella. All of the older age group sera were submitted to this laboratory for problems clinically unrelated to rubella. Most of the children's sera and those of the older age group were stored frozen at −30°C for 1 week to 6 months, whereas most of those from the 15 to 33 age group were tested on receipt before freezing, or after being frozen for less than 2 weeks.

Screening for total inhibitory effect and nonspecific agglutinin levels of untreated sera was done by a simple 1:8 dilution of the serum in HSAG buffer. Subsequently, serial twofold dilutions were made from 1:8 through 1:4096, with HSAG as a diluent. It was necessary to dilute sera to 1:4096 (10 twofold dilutions) to reach the endpoint of inhibitory activity, which was found to be as high as 1:2,048 in some sera.

RESULTS

Non-specific inhibitors were demonstrable in most of the sera tested and many were present at high levels (Table 1). All untreated sera showed a total inhibitory effect in the range of 1:256 to 1:2048. When compared with titers obtained after treatment, 80 to 100% of the sera in each age group, except the convalescents of positive cases, showed a drop of fourfold or greater. The specific antibody titer of many of the convalescent sera of positive cases were probably as high or higher than the non-specific inhibitor, so that a reduction in total inhibitory effect was not demonstrated with these sera.

The amount or titer of non-specific inhibitor does not appear to be related to or directly proportional to the specific antibody titer. The distribution of true rubella antibody titers shows no pattern of clustering (Table 2). As many individuals with no detectable true antibody (<1:8) have a total inhibitor titer at 1:1024 dilution as those who show an antibody titer of 1:256. When the specific antibody level is elevated, the effect of the non-specific inhibitor may be partially or entirely masked.

A preliminary study on serum pools was carried out by using 30-, 60-, 90-, and 120-min incubation times for the dextran sulfate-calcium chloride treatment. Since no significant
differences were detected, duplicate tests were carried out on all 159 sera with treatment times of 2 hr for one test and 30 min for the other. Incubation was at 4 C for the precipitation of the nonspecific inhibitors. No major difference in results was observed (Table 3). In no case was there more than a twofold variation in the antibody level. In 125 sera (78.6%), the titers were identical for both treatment times; 12 (7.6%) showed titers one twofold dilution higher after 30 min of treatment, and 22 (13.8%) had titers one twofold dilution lower. The geometric mean titers were nearly identical; 1:34.4 with the 2-hr treatment and 1:34.2 for the 30-min incubation period.

To test the possibility that 30 min of treatment prior to addition of red blood cells might represent a marginal time to effectively remove inhibitor, 43 sera were selected for retesting (10 each with total inhibitor levels of 1:256, 1:512, and 1:1024 and all 13 at 1:2048). Where possible, four sera were used with true antibody levels less than 1:8, three with intermediate levels, and three with a level slightly below the total inhibitor value. The dextran sulfate, calcium chloride, and red cells were added consecutively without an incubation period before addition of the red cells. Total reaction time was 30 min at 4 C. Again, no significant differences were seen; 32 of the 43

| Table 1. Levels of total inhibitor* in untreated serum by age group |
|-----------------------------|-----------------------------|-----------------------------|
| Age group (years) | Type of case | Sera tested | Reciprocals of HAI titers | No. of sera with titers ≥ four times higher than the antibody level after dextran SO₄-CaCl₂, treatment |
|-----------------------------|-----------------------------|-----------------------------|
| <10 | | 30 | 4/30 3/30 8/36 0/30 | 28/30 93.3% |
| 15-33 | Acute sera-positive | 19 | 11/19 6/19 2/19 0/19 | 19/19 100.0% |
| | Convalescent sera-positive | 20 | 1/20 6/20 9/20 4/20 | 6/20 30.0% |
| | Single sera and pairs not showing rise in titer | 60 | 9/60 21/60 24/60 6/60 | 48/60 80.0% |
| 40-86 | | 30 | 0/30 11/30 16/30 3/30 | 30/30 100.0% |
| | All age groups | 159 | 25/159 62/159 59/159 13/159 | 131/159 100.0% |

* Total inhibitory levels of antibody and nonspecific inhibitor, or both.

| Table 2. Levels of total inhibition* in untreated sera compared with inhibitor levels in sera treated for 2 hr with dextran SO₄-CaCl₂ |
|-----------------------------|-----------------------------|-----------------------------|
| Reciprocals of total inhibitor in untreated sera | No. of specimens | Reciprocals of HAI titers after treatment (rubella antibody) |
|-----------------------------|-----------------------------|-----------------------------|
| 256 | 25 | <8 8 16 32 64 128 256 512 1,024 |
| 512 | 62 | 12 1 2 3 2 3* 1 1* 0 |
| 1,024 | 59 | 16 1 5 11 8 9 8 4 0 |
| 2,048 | 13 | 10 1 0 5 15 12 9 3 4 |
| Total | 159 | 38 3 7 20 27 26 19 11 8 |

* Total inhibition caused by antibody or nonspecific inhibitors, or both.
* Italicized titers, 28 of 159 (17.6%), did not show a ≥ fourfold drop in level after treatment.
* This serum which is a twofold dilution higher than the total inhibitor level before serum treatment is considered to be within the normal limits of reproducibility.
sera (74.4%) showed the same titer with this method and the 2-hr treatment, seven (16.3%) gave a single twofold higher titer at 0 minutes incubation, and four (9.3%) gave a twofold lower titer. The geometric mean titer of sera treated for 2 hr was 1:41.0 and for those treated 0 minutes it was 1:40.8.

Tests on the untreated sera showed a range of nonspecific agglutinins from <1:8 to 1:64 (Table 4). Nonspecific agglutinins were present in 66 of 159 (41.5%) sera. However, 37 of these 66 (56.1%) sera had a titer of only 1:8. There appears to be no definite age effect, since both the youngest and oldest patients each showed 30%, and the 15 to 33 age group had 48% with demonstrable hemagglutinin. There also is no apparent correlation between the antibody level and the level of nonspecific agglutinin. Of the three children with the highest levels of nonspecific agglutinin, the two with 1:32 titers had antibody levels of <1:8, and the other with a titer of 1:64 had an antibody level of 1:32. Conversely, two of five convalescent sera with antibody levels of 1:1024 had agglutinin levels of <1:8. In no case was there failure to remove nonspecific agglutinins from any of these sera with the 10% concentration of 0- to 2-day-old chick cells used.

**DISCUSSION**

Nonspecific inhibitors in concentrations of fourfold or more above the titers of the specific

**Table 3. Comparison of HAI titers obtained by using treatment times of 2 hr and 30 min for precipitation of nonspecific inhibitors with dextran sulfate and calcium chloride**

| Reciprocal of titers–30-min treatment | Reciprocal of titers–2-hr treatment |
|--------------------------------------|------------------------------------|
| <8                                   | 38                                 |
| 8                                    | 2                                  |
| 16                                   | 3                                  |
| 32                                   | 2                                  |
| 64                                   | 1                                  |
| 128                                  | 2                                  |
| 256                                  | 2                                  |
| 512                                  | 2                                  |
| 1,024                                | 2                                  |
| 512 <8                               | 38                                 |
| 8                                    | 2                                  |
| 16                                   | 3                                  |
| 32                                   | 2                                  |
| 64                                   | 1                                  |
| 128                                  | 5                                  |
| 256                                  | 12                                 |
| 512                                  | 6                                  |
| 1,024                                | 1                                  |

* Geometric mean titers: 2-hr treatment = 1:34.4; 30-min treatment = 1:34.2.

**Table 4. Distribution of nonspecific agglutinin levels in untreated serum by age group and type of specimen**

| Age group (years) | Type of case | Reciprocal of nonspecific agglutinin titer |
|-------------------|--------------|------------------------------------------|
| <10               | Acute*       | <8: 21/30 70.0% 8: 4/30 13.3% 16: 2/30 6.7% 32: 2/30 6.7% 64: 1/30 3.3% |
| 15–33             | Acute*       | <8: 9/19 47.4% 8: 8/19 42.0% 16: 1/19 5.3% 32: 1/19 5.3% 64: 0/19 0.0% |
|                   | Convalescent*| <8: 9/20 45.0% 8: 6/20 30.0% 16: 3/20 15.0% 32: 2/20 10.0% 64: 0/20 0.0% |
|                   | Single       | <8: 18/38 54.0% 8: 10/38 26.0% 16: 9/38 23.6% 32: 0/38 0.0% 64: 1/38 1.8% |
|                   | Paired*      | <8: 15/22 68.0% 8: 1/22 4.5% 16: 2/22 9.1% 32: 4/22 18.4% 64: 0/22 0.0% |
| 40–86             |              | <8: 21/30 70.0% 8: 8/30 26.7% 16: 0/30 0.0% 32: 1/30 3.3% 64: 0/30 0.0% |
| Total: all ages   |              | <8: 93/159 58.5% 8: 37/159 23.3% 16: 17/159 10.7% 32: 10/159 6.2% 64: 2/159 1.3% |

* Sera from serologically proven positive cases of rubella.

* Sera with either no demonstrable titer or no rise in titer between specimens where two specimens were submitted from the same patient.
antibody inhibitors were found in 82.4% of the sera tested. The high concentration and high frequency of these inhibitors point out the necessity for a treatment which will dependably remove the beta-lipoprotein inhibitor in all cases without affecting the immunoglobulin fraction. Liebhaber (7) has shown quite effectively that his dextran sulfate-calcium chloride method can in fact remove virtually all the nonspecific inhibitor without significantly changing the concentration of immunoglobulins G, A, and M. These studies demonstrate that reducing the incubation period of the dextran sulfate-calcium chloride treatment does not affect the efficacy of the Liebhaber method. In our tests, only 28 of 159 sera showed no significant reduction in total inhibitor level after treatment. Titers of these sera ranged from 1:128 to 1:1,024. It is possible, but highly unlikely, that these sera could represent a failure to remove all nonspecific inhibitors. However, 14 of these 28 sera were from patients in the convalescent phase of clinically diagnosed rubella, and all 14 developed complement-fixing antibody in addition to HAI antibody. Also, since results obtained with all incubation times used were essentially the same, any failure to remove inhibitor at 30 min would also have had to have occurred with the full 2-hr treatment.

In these tests, the same pooled serum controls were used for all procedures. In all tests run, the negative serum never showed a positive reaction, and the two positive sera never varied more than one twofold dilution from the expected titer. No more than a twofold increase or decrease, the range of acceptable equivalent titers, was encountered in any serum between the variations of dextran sulfate-calcium chloride-serum incubation periods used. The first comparison of efficiency of incubation times using the standard 2 hr compared with 30 min resulted in geometric mean titers for the 159 sera of 1:34.4 and 1:34.2, respectively. An additional study of 43 sera showed no loss of efficiency for the removal of nonspecific inhibitors when the incubation period was reduced to virtually 0 min prior to red blood cell adsorption. In view of the results presented, it would appear that a total incubation time of 30 min prior to red cell adsorption is fully satisfactory.

It should be emphasized that there are major chemical and reaction variations in dextran sulfate compounds from different sources. All of the tests reported in this paper were done with dextran sulfate obtained from Nutritional Biochemicals Co., which was designated as "Dextran sulfate, analytical grade for serum lipoprotein determination." A rubella HAI test kit, utilizing the dextran sulfate method from Flow Laboratories, proved to be satisfactory with a 30-min procedure. We have since learned that the dextran sulfate in that kit was from the same source as ours. In a 5% aqueous solution, both preparations were pale yellow, with a pH in the range of 6.55 to 6.7. The manufacturer gives the molecular weight as 12,000 ± 3,000 and the sulfur content as 17%. Dextran sulfate obtained from another source just prior to completion of this study was found to be totally unusable although the molecular weight and sulfur content listed for that product were within the range recommended in the CDC guide (2) (molecular weight = 8,000-20,000; and sulfur content = 14.38%). A 5% aqueous solution of this product was dark brown in color and had a pH of 8.8. No significant effect on nonspecific inhibitors could be demonstrated with incubation periods of 30 min or 2 or 16 hr. Adjustment of the pH to 6.2 resulted in no improvement.

Nonspecific agglutinins we encountered in these studies could be easily removed by the use of chick red cells in a 10% concentration. As shown in Table 4, 147 of 159 (92.5%) had nonspecific agglutinins at a level ≤1:16. Feldman (4), working with a population 50 years of age or more, found that 95% had nonspecific agglutinins at these levels. Over the past 4 years, less than 1% of our sera have had to be readsorbed after treatment for 30 min with 10% red cells from 0- to 2-day-old chicks because of incomplete removal of agglutinins. In addition, application of this type of cell in the technique for both adsorption of the sera and as a stock concentration from which the 0.25% cell suspension for the test proper can be made eliminates the need to have available cells from adult chickens for adsorption of the sera and the necessity for two separate red blood cell concentrations.

The procedure used here presents a method that has the advantage of the heparin-manganous chloride method in that it is a specific chemical precipitation of the nonspecific inhibitors. It also retains the short, total processing time of both that technique and the kaolin methods while avoiding the reported problems of both techniques.

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