Comparison of Methods for Removal of Nonspecific Inhibitors of Arbovirus Hemagglutination

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Human sera were treated with kaolin, acetone, and dextran sulfate to determine the best method for removing nonspecific hemagglutination inhibitors. Results indicated that on surveys for group A, group B, and some group C arbovirus HI antibodies, dextran sulfate treatment of sera could be used effectively. This method, however, has limited usefulness for detecting HI antibody for a number of arboviruses, particularly some members of the Bunyamwera supergroup since nonspecific inhibitors for these antigens were not completely removed. HI antibodies in sera drawn early after dengue and Venezuelan equine encephalitis infection were detected more readily after dextran sulfate treatment than after kaolin treatment. Kaolin, but not dextran sulfate, was shown to remove antibody from IgM fractions of sera.

Human sera are known to contain substances that nonspecifically inhibit in vitro arbovirus hemagglutination. These inhibitory substances are present in the low-density lipoprotein fractions of serum and may be removed by absorption with specific beta lipoprotein antiserum (1). In routine hemagglutination-inhibition (HI) tests, however, arbovirus nonspecific inhibitors are usually removed by extraction with acetone or absorption with kaolin. Unfortunately, acetone extraction is tedious, and both methods may remove significant amounts of specific antibody (5, 9).

Precipitation of serum lipoproteins with sulfated polysaccharides (4) has been suggested as an alternative method for removing arbovirus nonspecific inhibitors (NSI; 1). Recently Liebhahler (8) reported use of dextran sulfate and CaCl₂ (DS-C) to remove rubella inhibitors without the removal of specific antibody. In our laboratory, a similar method of DS-C treatment of serum has been developed for use in arbovirus serology. This paper describes the technique and compares it with the methods of kaolin and acetone treatment for removal of inhibitors from human, equine, and primate sera.

MATERIALS AND METHODS

**Human sera.** Sera used in serological tests were collected in 1968 during a survey of Seminole Indians inhabiting the Big Cypress Reservation in south Florida. Sera had been stored in glass ampoules at −20°C.

**Monkey sera.** Rhesus monkeys were experimentally infected with dengue type II virus strain isolated from a human case during the Puerto Rican epidemic of 1969. Serial serum samples were obtained from the monkeys, some of which were subjected to gel filtration as described below.

**Horse sera.** As part of another study (Henderson, Chappell, Johnston, and Sudia, unpublished data), serial bleedings were available from horses inoculated with the FE3-7C strain of Venezuelan equine encephalitis (VEE) virus.

**Immunoglobulin fractionation.** Selected antisera were fractionated by filtration through a Sephadex G-200 column (2.5 by 100 cm), by the techniques of Flodin (6). The elution buffer contained 0.1 M tris-(hydroxymethyl)aminomethane-hydrochloride and 1.0 M NaCl (pH 8.1 to 8.2). Peaks were analyzed by immunoelectrophoresis, with commercially prepared goat antihresus sera to immunoglobulin (IgM) and IgG (Microbiological Associates, Bethesda, Md). Fractions containing peak IgM and IgG activity were pooled and concentrated to the original serum volume (2 to 3 ml) by dialysis against carboxymethyl cellulose.

**Removal of inhibitors.** Sera were treated with kaolin and acetone by standard methods (3). Preliminary experiments were conducted by using various concentrations of dextran sulfate (analytical grade, Nutritional Biochemicals, Cleveland, Ohio) and CaCl₂ to precipitate lipoproteins from clear and grossly lipemic sera. Concentrations of dextran sulfate and CaCl₂ in serum were independently varied from 0.025% (w/v) dextran sulfate-0.025 M CaCl₂ to 0.5% dextran sulfate-0.1 M CaCl₂. With several combina-
tions of reagent concentrations, the time allowed for lipoprotein precipitation at 4 C was also varied from 5 to 60 min. Tests were conducted with arbovirus antigens from groups A, B, C, Bunyamwera, California, and Phlebotomus fever. Optimal results were obtained with the following procedure. To 0.5 ml of unheated whole serum or immunoglobulin fraction in a test tube (15 by 85 mm) were added 0.3 ml of borate-saline (pH 9.0), 0.1 ml of 1% dextran sulfate, and 0.1 ml of 0.75 M CaCl₂. The mixture was shaken once and incubated at 4 C for 20 min. After centrifugation (600 × g for 10 min), the supernatant fluid was decanted and absorbed with packed goose erythrocytes. The treated serum was then diluted 1:5 with borate-saline (pH 9.0) before use in the HI test; thus the final dilution of serum was 1:10.

At concentrations of DS-C higher than those used, erythrocytes did not settle normally; instead, they gave the appearance of hemagglutination. Repeated absorptions with goose erythrocytes or treatment with 0.15 M ethylenediaminetetraacetic acid did not decrease this effect. At the concentrations used, however, only occasional difficulty was encountered in obtaining normal settling patterns in serum controls. Serum controls were routinely performed at several twofold dilutions; except in rare instances, interpretation of HI test results at the 1:10 dilution was not affected.

**HI tests.** HI tests were performed by the technique of Clarke and Casals (3) adapted for use in a microtiter system. Antigen were prepared by sucrose acetone extraction of infected suckling mouse brains or from suspension cultures of BHK-21 cells (2).

**N tests.** Screening neutralization (N) tests were performed with a plaque-reduction technique in BHK-21/C13 cell cultures, by methods previously described (Monath, Nuckolls, Berall, Bauer, Chappell, and Coleman, Amer. J. Epidemiol, in press).

**Quantitative Ig assays.** Ig assays were made by radial immunodiffusion in Partigen plates (Behring-werke, Certified Blood Donor Service, Woodbury, N.Y.).

**RESULTS**

Comparison of acetone, kaolin, and DS-C; Seminole Indian sera. Thirty-six sera were tested for HI activity after treatment to remove inhibitor activity. HI tests were done with untreated sera against each antigen to measure the level of inhibitors. After treatment with acetone, kaolin, or DS-C, most sera contained no detectable HI activity to members of arbovirus groups A, B, C, Bunyamwera, or Patois (Table 1). The N test was more sensitive than the HI for the detection of antibodies to VEE, St. Louis encephalitis (SLE), and Gumbo Limbo viruses, regardless of the method used to remove inhibitors.

Seven sera had detectable HI activity to VEE (Table 2). No significant difference in HI titer between sera treated with acetone, kaolin, or DS-C was found. One serum gave an apparent false-positive test after kaolin absorption.

Fourteen sera had detectable HI activity to group B arbovirus antigens (Table 3). Of these, four were confirmed by N test. All three methods adequately removed NSI for SLE, Ilheus, yellow fever, Cowbone, and dengue II antigens. Seven

**Table 1. Sera with no detectable HI antibody**

| Arbovirus group | Antigen                  | No. sera | Reciprocal titer with untreated serum | No. N positive |
|-----------------|--------------------------|----------|--------------------------------------|----------------|
|                 |                          |          | Range                               | Geometric mean |
| A               | Venezuela equine encephalitis | 26       | 40–640                               | 155.5          | 1              |
|                 | Eastern equine encephalitis | 30       | <10–80                               | 17.0           | ND             |
|                 | Western equine encephalitis| 30       | <10–20                               | 6.5            | ND             |
| B               | St. Louis encephalitis    | 29       | <10–160                              | 39.0           | 2              |
|                 | Ilheus                   | 28       | 80–320                               | 168.0          | ND             |
|                 | Yellow fever             | 28       | 10–160                               | 36.5           | ND             |
|                 | Cowbone                  | 30       | <10–40                               | 17.0           | ND             |
|                 | Modoc                    | 21       | 40–160                               | 83.0           | ND             |
|                 | Dengue II                | 27       | 20–160                               | 45.5           | ND             |
| C               | Gumbo Limbo              | 30       | <10–40                               | 11.0           | 1              |
| Bunyamwera      | Tensaw                   | 27       | 10–80                                | 28.5           | ND             |
| Patois          | Pahayokee                | 27       | ND                                   | 0              |
| California      | La Crosse                | 1        | 40                                    | 0              |

* HI, hemagglutination inhibition.

* Reciprocal titers for all test sera after treatment with acetone, kaolin, or dextran sulfate-CaCl₂ were < 10. ND, not done.
TABLE 2. Sera with detectable Venezuelan equine encephalitis antibody

| Serum no. | Untreated sera | Reciprocal HI titer after treatment<sup>a</sup> | N test |
|-----------|----------------|-----------------------------------------------|--------|
|           |                | Ac    | Kao  | DS-C |        |
| 18        | ND<sup>b</sup> | 80    | 80   | 80   | +      |
| 19        | 320            | <10   | 10   | <10  | 0      |
| 22        | 160            | 20    | 20   | 20   | +      |
| 30        | ND             | 20    | 40   | 40   | +      |
| 31        | 160            | 40    | 40   | 40   | +      |
| 34        | 320            | 80    | 80   | 80   | +      |
| 35        | 320            | 20    | 20   | 10   | +      |

<sup>a</sup> Abbreviations: HI, hemagglutination inhibition; Ac, acetone; Kao, kaolin; DS-C, dextran sulfate-CaCl<sub>2</sub>.

<sup>b</sup> Not done.

TABLE 3A. Sera with group B antibody confirmed by N test

| Arbovirus          | Serum no. | Untreated sera | Reciprocal titer after treatment with<sup>b</sup> | N test |
|--------------------|-----------|----------------|-----------------------------------------------|--------|
|                    |           |                | Ac    | Kao  | DS-C |        |
| St. Louis encephalitis | 3        | 80             | 10    | 20   | 20   | +      |
|                    | 18        | ND             | 20    | 10   | <0   | +      |
|                    | 30        | ND             | <10   | 10   | 1    | +      |
| Yellow Fever       | 5         | 40             | 20    | 20   | 20   | +      |

<sup>a</sup> Abbreviations: Ac, acetone; Kao, kaolin; DS-C, dextran sulfate-CaCl<sub>2</sub>; ND, not done.

sera gave low-titer positive reactions to Modoc virus after DS-C treatment but were not confirmed by N test.

Five sera had detectable HI activity to Tensaw virus (Table 4), and four of these were confirmed by N test. Of the N-positive sera, HI activity was detected in three after DS-C, in three after acetone, and in four after kaolin treatment.

HI activity to Pahayokee was detected in nine sera after treatment with DS-C but not after acetone or kaolin (Table 5). Of these, only two were confirmed by N test. To investigate the possibility that the observed HI activity was due to infection with Shark River, a related virus known to occur in south Florida, further N tests were performed. One additional serum contained N antibody to this virus. Further study revealed a loss of HA units with Pahayokee antigen due to a pH change of the phosphate buffer in the presence of DS-C.

With California La Crosse (LAC) BHK-21 cell suspension and La Crosse mouse brain antigens, 35 out of 36 sera contained HI activity after DS-C. None was HI positive after kaolin or acetone treatment. HI titers ranged from 1:10 to 1:40. The titers of the untreated sera ranged from 1:10 to 1:80. N tests for antibodies to LAC and two California viruses known to occur in south Florida (Trivittatus and Keystone) were performed. Of the 35 HI-positive sera, only 13 contained N antibodies to one or more California viruses tested.

HI antibodies in sera of monkeys infected with dengue virus. The results of HI tests on sera from eight monkeys drawn 1, 2, and 3 weeks after inoculation of dengue II virus are presented in Table 6. Antibodies to the homologous virus...
Table 5. Sera with detectable Pahayokee HI antibody

| Serum no. | Reciprocal HI titers after treatment with | N test |
|-----------|------------------------------------------|--------|
|           | Ac | Kao | DS-C | PAH | SR |
| 4         | <10 | <10 | 10   | 0   | 0  |
| 5         | <10 | <10 | 10   | 0   | +  |
| 6         | <10 | <10 | 10   | 0   | 0  |
| 7         | <10 | <10 | 10   | 0   | +  |
| 8         | <10 | <10 | 10   | 0   | 0  |
| 10        | <10 | <10 | 10   | 0   | 0  |
| 12        | <10 | <10 | 10   | 0   | 0  |
| 17        | <10 | <10 | 10   | 0   | 0  |
| 18        | <10 | <10 | 10   | 0   | +  |

* Untreated sera not tested. Abbreviations: HI, hemagglutination inhibition; Ac, acetone; Kao, kaolin; DS-C, dextran sulfate-CaCl₂.

Table 6. HI antibody responses of monkeys to experimental dengue II infection

| Monkey no. | Dengue I antigen | Dengue II antigen |
|------------|------------------|------------------|
|            | Titer after kaolin treatment | Titer after DS-C treatment | Titer after kaolin treatment | Titer after DS-C treatment |
|            | Week 1 | Week 2 | Week 3 | Week 1 | Week 2 | Week 3 | Week 1 | Week 2 | Week 3 |
| 02         | <10    | <10    | 20     | <10  | 20     | <10  | 160    | <10   | 160 |
| 03         | <10    | <10    | 20     | <10  | 20     | <10  | 80     | <10   | 80  |
| 04         | <10    | 10     | 80     | <10  | 20     | 80   | <10    | 160   | 640 |
| 05         | <10    | <10    | 20     | <10  | 10     | 40   | <10    | 40    | 160 |
| 06         | <10    | <10    | 20     | <10  | 20     | 40   | <10    | 20    | 80  |
| 07         | <10    | 10     | 20     | <10  | 40     | 20   | <10    | 160   | 160 |
| 08         | <10    | <10    | 10     | <10  | 10     | 20   | <10    | 10    | 320 |
| 09         | <10    | <10    | 20     | <10  | 20     | 40   | <10    | 80    | 160 |

* Abbreviations: HI, hemagglutination inhibition; DS-C, dextran sulfate-CaCl₂.

Table 7. Dengue HI antibody in immunoglobulin fractions

| Monkey no. | Immunoglobulin fraction | Dengue I | Dengue II | Dengue III | St. Louis encephalitis |
|------------|-------------------------|----------|-----------|------------|------------------------|
|            |                         | Kao | DS-C | Kao | DS-C | Kao | DS-C | Kao | DS-C |
| 04         | IgM                     | 20  | 10   | 10  | 10   | 10  | 10   | <10 | <10  |
|            | IgG                     | 40  | 80   | 40  | 80   | 80  | 80   | 20  | 80   |
|            | WS                      | 160 | 320  | 640 | 640  | 80  | 80   | 320 | 320  |
| 06         | IgM                     | <10 | 20   | <10  | 10   | <10  | <10  | <10 | <10  |
|            | IgG                     | 10  | 40   | 10  | 20   | 20  | 20   | 10  | 10   |
|            | WS                      | 80  | 80   | 80  | 160  | 40  | 40   | 40  | 80   |
| 08         | IgM                     | <10 | 20   | <10  | 80   | <10  | <10  | <10 | <10  |
|            | IgG                     | 10  | 80   | 40  | 80   | 10  | 20   | 10  | 20   |
|            | WS                      | 40  | 80   | 320 | 320  | 20  | 40   | 40  | 40   |

* Abbreviations: HI, hemagglutination inhibition; Ig, immunoglobulin; Kao, kaolin; DS-C, dextran sulfate-CaCl₂; WS, whole serum.
horses, antibodies were detectable in sera treated with DS-C 1 day earlier than in kaolin-treated sera. In the subsequent sera, there was no consistently significant difference in HI titers between DS-C and kaolin-treated sera.

**Removal of immunoglobulins by DS-C and kaolin.** Twelve human sera treated with DS-C and kaolin were assayed for Ig content (Table 9). Significant amounts of IgM were removed by kaolin but not by DS-C. Kaolin also removed more IgA than DS-C. IgG level after kaolin and DS-C correlated more closely.

**DISCUSSION**

The formation of insoluble complexes between dextran sulfate and beta-lipoproteins in the presence of Ca^{2+} ions has been extensively studied and reviewed (4, 8). The optimal conditions for precipitation of beta-lipoproteins may differ for individual sera, especially if grossly lipemic. Although in preliminary experiments we undertook to determine the optimal component concentrations of DS-C, other parameters affecting precipitation of inhibitors, such as pH and ionic strength, were not studied extensively because of limitations of the HI test. Awareness of possible changes in pH at the 1:10 dilution of DS-C-treated sera is important. Known positive and negative sera should be included for each antigen to detect the change in pH which results in lowering of required hemagglutinating units, giving false positives at the 1:10 dilution. For some sera, optimal conditions for beta-lipoprotein precipitation probably were not met. Quantification of beta-lipoproteins in precipitates and supernatant fluids was beyond the scope of this study.

Variation occurs in the susceptibility of different arbovirus antigens to HI by nonspecific lipoprotein substances (1, 7, 10). In general, we found poor correlation between the titer of inhibitors in untreated sera and the efficiency with which inhibitors were removed by the three methods used. DS-C precipitated VEE inhibitor activity in all sera and Ilheus inhibitor activity in all but one serum, despite very high titers of inhibitors in untreated sera. However, California virus inhibitors were inadequately removed by DS-C although untreated sera had inhibitor titers of only 1:10–1:80. NSI for California viruses possibly were present in a portion of the low-density-lipoprotein spectrum not precipitated under the conditions employed. Bidwell and Mills (1) have shown differences in inhibitor levels by separating ultracentrifugally low-density-lipoprotein components for various arbovirus antigens.

DS-C would appear to have limited usefulness in serological surveys with the HI test, since

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**Table 8. HI antibody response to inoculation of horses with Venezuelan equine encephalitis (VEE; Fe3-7C)**

| Horse no. | Time post-inoculation | Reciprocal HI titer to VEE (FE3-7C) |
|-----------|------------------------|-----------------------------------|
|           |                        | Kao | DS-C                  |
| 72        | days                   |     |                      |
| 0         | <10                    | <10 |                      |
| 6         | <10                    | <10 |                      |
| 7         | <10                    | 20  |                      |
| 8         | 10                     | 20  |                      |
| 9         | 20                     | 20  |                      |
| 10        | 160                    | 320 |                      |
| 11        | 640                    | 640 |                      |
| 12        | 640                    | 320 |                      |
| 34        | 80                     | 160 |                      |
| 73        | 0                      | <10 | <10                   |
| 6         | <10                    | <10 | <10                   |
| 9         | <10                    | <10 |                     |
| 10        | <10                    | 20  |                     |
| 11        | 20                     | 40  |                     |
| 12        | 40                     | 80  |                     |
| 34        | 40                     | 80  |                     |
| 74        | 0                      | <10 | <10                   |
| 6         | <10                    | <10 | <10                   |
| 7         | <10                    | 20  |                     |
| 8         | 20                     | 20  |                     |
| 9         | 80                     | 160 |                     |
| 10        | 160                    | 160 |                     |
| 11        | 320                    | 320 |                     |
| 12        | 320                    | 320 |                     |
| 34        | 80                     | 80  |                     |

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**Table 9. Effect of kaolin and DS-C on human serum immunoglobulin (Ig) levels**

| Serum | IgM | IgA | IgG |
|-------|-----|-----|-----|
|       | %   | %   | %   |
| 1     | 14  | 31  | 45  |
| 2     | 0   | 62  | 100 |
| 3     | 17  | 75  | 75  |
| 4     | 22  | 60  | 67  |
| 5     | 25  | 54  | 67  |
| 6     | 0   | 64  | 67  |
| 7     | 23  | 50  | 67  |
| 8     | 22  | 49  | 76  |
| 9     | 0   | 60  | 90  |
| 10    | 17  | 48  | 99  |
| 11    | 48  | 100 | 100 |
| 12    | 0   | 73  | 100 |

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*Abbreviations: HI, hemagglutination inhibition; Kao, kaolin; DS-C, dextran sulfate-CaCl2.
inhibitors for a number of arbovirus antigens (particularly members of the Bunyamwera supergroup) were not completely removed. In surveys for group A, most group B, and some group C arbovirus HI antibodies, however, the DS-C method could be used effectively and is somewhat easier to perform than kaolin absorption. Removal of IgM antibody by kaolin absorption, not an important consideration in surveys for past infection, may be of great importance in diagnostic serology. HI antibodies in sera drawn early after dengue infection were detected more readily after DS-C than after kaolin treatment. Moreover, kaolin, but not DS-C, was shown to remove antibody from IgM fractions. Further studies are needed to confirm the increased sensitivity of DS-C-treated whole serum in detecting early antibody. Because of the low levels of serum IgM, HI antibody, and the variability in removal of IgM by kaolin, comparison of HI titers in DS-C- and kaolin-treated whole serum probably will not yield useful and reproducible information. In experimental studies, however, DS-C may be useful for treatment of whole sera before column chromatography or gel filtration or of Ig fractions themselves.

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