Method for Extracting Viral Hemagglutination-Inhibiting Antibodies from the Nonspecific Inhibitors of Serum

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Various methods are used to remove nonspecific inhibitors from sera before titering viral hemagglutination-inhibiting antibodies. These methods have several undesirable features; some are tedious and time-consuming, some remove antibody along with nonspecific inhibitors, and different techniques are usually required to remove the nonspecific inhibitors for different viruses. This communication describes a single method that uses diethylaminoethyl-Sephadex to extract the immunoglobulin G antibodies for several viruses from nonspecific inhibitors. The procedure is fast, simple to perform, and removed the nonspecific inhibitors for influenza, Western equine encephalitis, dengue-2, and rubella viruses.

Since its description for the diagnosis of influenza (14), the hemagglutination-inhibition (HI) test has become one of the more useful serological tools of modern virology. Rapid and easy to perform, this test is used to measure antibodies to many human pathogens, including the myxoviruses, certain arboviruses, poxviruses, adenoviruses, enteroviruses, and rubella virus. Most sera, however, contain nonspecific inhibitors for viral hemagglutinins that must be removed before specific antibody can be measured. Little is known about these unwanted inhibitors, except that they are multiple and are associated with both proteins and lipids. Consequently, the methods devised to extract them are more or less empirical, relatively crude, and remove the nonspecific inhibitors for different viruses with varying effectiveness.

The present work describes a different approach to this problem. Instead of trying to remove only the nonspecific inhibitors, all proteins except the immunoglobulin G (IgG) globulins were extracted from serum with diethylaminoethyl (DEAE)-Sephadex. This technique effectively removed the nonspecific inhibitors for influenza, Western equine encephalitis (WEE), dengue-2, and rubella viruses and theoretically should eliminate these factors for all types of viruses.

MATERIALS AND METHODS

Preparation of DEAE. DEAE-Sephadex A50 (Pharmacia Fine Chemicals, Piscataway, N.J.) was processed and equilibrated with low-ionic-strength buffer as described by Baumstark et al. (3). Specifically, 100 g of DEAE was slowly added to 9 liters of tap water in a cylindrical glass jar (16 by 45 cm) and allowed to stand for at least 1 hr. The fines were next removed by suspending the DEAE, allowing settling for 20 min, and aspirating all of the supernatant fluid to the barely discernible line separating the rapidly sedimenting beads from those that settled more slowly. Tap water was then added to 9 liters, and the procedure was repeated eight times or until essentially all of the DEAE settled rapidly and completely within 20 min. The DEAE was then added to a Buchner funnel and successively washed with 2 liters of 0.5 N NaOH, distilled water until the pH was below 10, 2 liters of 0.5 N HCl, and finally with distilled water until the pH was above 4. The DEAE was then resuspended in 3 liters of 0.01 M (pH 6.5) phosphate buffer (0.388 g of K$_2$HPO$_4$ and 1.06 g of KH$_2$PO$_4$ per liter of distilled water), brought to pH 6.5 with 1 N NaOH, and washed three to five times with the 0.01 M (pH 6.5) buffer in the manner used to remove fines. This and the initial steps to remove fines are crucial; extracted sera that contain fines will agglutinate red cells in serum controls of the HI test.

Extraction with DEAE. The suspension was arbitrarily standardized by pouring it into a 1-liter graduated cylinder and allowing the beads to settle for 1 hr. At this time, buffer was either removed or added until the settled DEAE occupied 66% of the total volume.
of liquid in the cylinder. For each serum to be extracted, 8 ml of evenly suspended DEAE was added to a 40-ml round-bottom centrifuge tube. To prevent unnecessary dilution of serum during extraction, excess phosphate buffer was removed in the following manner. After allowing the DEAE to settle for 10 min, a glass tube 1.5 cm in diameter, with a 0.1-cm-thick disc of high porosity sintered-glass fused into its lower opening (available from Warren R. Foster Laboratory Glassware, Gaithersburg, Md.), was placed into the centrifuge tube and suction was applied. As buffer was drawn into the tube, the sintered-glass filter was lowered onto the surface of the DEAE beads. As soon as air was aspirated, the suction apparatus was removed and transferred to the next centrifuge tube. Although this procedure required a small amount of practice to produce consistent results, it removed excess buffer more rapidly and completely than centrifugation.

A 0.5-ml amount of each serum to be extracted was then added to the DEAE and the resulting slurry was vigorously stirred on a Vortex-Genie (Scientific Industries) every 15 min for 1 hr. The specimens were centrifuged at 1,400 × g (4 C) for 10 min, a Pasteur pipette was placed on the bottom center of each centrifuge tube, and the supernatant fluid containing purified IgG globulins was aspirated by a rubber bulb. The small amount of DEAE remaining in these supernatant fluids was finally removed by centrifugation at 1,400 × g (4 C) for 15 min. Immunoelcrophoresis (12) utilized an antiserum prepared by immunizing a rabbit with multiple injections of whole human serum. Concentrations of IgG globulins were measured by radial diffusion (10) in commercially produced agar plates containing monospecific anti-human IgG (Hyland Laboratories, Los Angeles, Calif.). Human IgG purified by DEAE cellulose column chromatography and standardized on a Beckman DU-2 spectrophotometer (17) was the reference standard for this assay.

**Measurements of antibody to viruses.** The serological responses to influenza were measured in the acute and convalescent (2 week) sera of eight airmen who developed a febrile illness during a recent influenza epidemic at a U.S. base in Thailand. During the acute phase, influenza virus (A/Thai/303/68) was isolated from six of these eight individuals. Complement-fixing (CF) and HI antibodies were measured in microtiter plates (Limbro Chemical, New Haven, Conn.) utilizing periodate or DEAE to remove nonspecific inhibitors (26). Neutralizing antibody was measured in primary rhesus monkey kidney cell cultures (26).

Nineteen sera taken from 11 patients suspected of having arbovirus disease were selected from the Walter Reed serum files for assay of HI antibodies to WEE and dengue-2 arboviruses. Prior serology indicated 11 sera had dengue-2 titers of 1:10 or less, whereas the remaining 8 contained higher levels of this antibody. HI titrations were performed as described by Clarke and Casals (9) by using microtiter plates. WEE antigen was prepared from the supernatant fluid of infected chick embryo cells, whereas dengue-2 was extracted with sucrose and acetone from infected suckling mouse brain. Nonspecific serum inhibitors were removed with DEAE or by mixing 0.1 ml of serum with 14 ml of acetone at room temperature. The acetone was removed by centrifugation and overnight drying at 37 C. All dilutions of DEAE- and acetone-extracted sera were made in pH 9 borate-saline buffer.

Rubella antibody was assayed by plaque reduction (PR) neutralization and HI tests in single sera taken from 11 women during their first 20 weeks of pregnancy. The HI test was performed in microtiter plates by the techniques of Stewart et al. (22). Nonspecific inhibitors were absorbed with DEAE or by incubating 1 volume of serum with 3 volumes of 25% acid-washed kaolin for 30 min at 4 C.

The rubella PR test was standardized and performed by John A. Stephenson at Walter Reed. Rubella virus, grown in baby hamster kidney cells, was diluted to contain approximately 75 plaque-forming units/0.1 ml. A 0.2-ml amount of equal parts of virus and serum dilutions was added to 25-cm² tissue culture flasks (Falcon Plastic, Los Angeles, Calif.) containing a monolayer of LLC-MK² line of monkey kidney cells. After 1 hr at 37 C, each flask was overlaid with 5 ml of medium 199 containing 10% heat-inactivated fetal bovine serum, 1% glutamine, 0.3% sodium bicarbonate, 0.02% DEAE-dextran, penicillin, streptomycin, and 1% Difco purified agar. After 7 days at 37 C, the flasks were overlaid with 4 ml of the above diluent, minus bicarbonate, containing 0.01% neutral red stain. Plaques were counted after 24 hr at room temperature.

Viruses and serum controls were included in all HI titrations. Except as noted, appropriate sera were absorbed for natural goose or chick red cell agglutinins before arbovirus and rubella HI tests were performed.

**RESULTS**

**DEAE extraction technique.** The proper proportions of DEAE-Sephadex and serum and the timing of the extraction procedure to produce pure IgG at a usable concentration were first defined. When 0.5 ml of serum was extracted for 1 hr with the DEAE aspirated from 4 ml of 66% suspension, traces of non-IgG serum components were identified in the supernatant fluids by immunoelectrophoresis. At a ratio of 8 to 0.5 ml of serum, impurities were found after 30 min but not after 1 hr of extraction. Therefore, the final method was to extract 0.5 ml of serum for 1 hr with the DEAE remaining after aspiration of 8 ml of 66% suspension. Figure 1 compares the immunoelectrophoretic patterns of four representative sera before and after this extraction. Such treatment consistently yielded immunoelectrophoretically pure IgG globulins.

This extraction consistently diluted the IgG globulins of individual sera to approximately 20% of their original levels. Table 1 illustrates that the concentrations of IgG extracted from 15 of the 16
influenza sera were between 1:4.7 and 1:6 of their levels in the unextracted sera. A similar analysis of other sera confirmed that this value was constant. Therefore, this DEAE extraction method was assumed to dilute the IgG globulins of sera 1:5 for all serological titrations.

Serological diagnosis of influenza infections. DEAE extraction was a useful adjunct for the serological diagnosis of influenza infections. Table 2 compares the CF, neutralizing, and HI titers in acute and convalescent sera from eight patients with “flu-like” illnesses. The CF and HI titers of untreated and periodate-treated sera were of limited value. Only three of eight patients had a fourfold CF response, whereas one of eight untreated serum pairs and two of eight treated with periodate had similar diagnostic HI antibody rises. The HI titers of acute sera were relatively high in both untreated and periodate-treated specimens. Although this activity might have been due to either nonspecific inhibitors or preexisting specific antibody, the neutralizing data of Table 2 suggest that nonspecific factors were responsible; only

![Image](image.png)

**FIG. 1.** Immunelectrophoretic demonstration of the purity of IgG globulins extracted from four sera by DEAE. Each slide contains a whole serum (upper well), the IgG extracted from that serum (lower well), and rabbit anti-whole human serum (trough). Slides, from top to bottom, examine bleedings from influenza patients Kt 7 acute, Kt 7 convalescent, Kt 15 acute, and Kt 15 convalescent.

| Table 1. Dilution of IgG globulins after treatment of whole serum by DEAE-Sephadex |
|-------------------------------------|------------------|-----------------|------------------|
| Patient   | Acute sera (mg of IgG/100 ml) | Convalescent sera (mg of IgG/100 ml) |
|           | Whole serum | DEAE supernatant fluid | Dilution factor | Whole serum | DEAE supernatant fluid | Dilution factor |
| Kt 2      | 900        | 185                | 4.9             | 775         | 160                | 4.8             |
| Kt 6      | 1,250      | 250                | 5.0             | 1,300       | 240                | 5.4             |
| Kt 7      | 700        | 150                | 4.7             | 750         | 140                | 5.4             |
| Kt 10     | 800        | 160                | 5.0             | 750         | 150                | 5.0             |
| Kt 11     | 1,450      | 280                | 5.2             | 1,500       | 250                | 6.0             |
| Kt 15     | 1,450      | 290                | 5.0             | 1,500       | 300                | 5.0             |
| Kt 16     | 1,350      | 185                | 7.3             | 1,075       | 215                | 5.0             |
| Kt 17     | 1,400      | 255                | 5.5             | 1,300       | 215                | 6.0             |

* IgG of whole serum/IgG left after extraction.

| Table 2. Serological diagnosis of influenza infections by various titration procedures |
|-------------------------------------|------------------|------------------|------------------|
| Patient   | CF Un-treated | Perio-date-treated | DEAE-treated | Neutralization |
| Kt 2      | 10/5*        | 40/40             | 20/20         | <5/5 <5/5 <5/5 |
| Kt 6      | 5/20         | 40/80             | 80/80         | <5/10 <5/20 <5/10 |
| Kt 7      | 20/40        | 40/80             | 40/40         | <5/10 <5/20 <5/10 |
| Kt 10     | 5/5          | 40/40             | 20/20         | <5/5 <5/5 <5/5 |
| Kt 11     | 10/5         | 40/80             | 20/80         | 5/40 5/40 5/160 |
| Kt 15     | 10/80        | 40/320            | 40/160        | 5/80 <5/10 <5/10 |
| Kt 16     | 5/40         | 40/40             | 40/80         | <5/20 <5/40 <5/20 |
| Kt 17     | 10/20        | 80/80             | 80/40         | <5/20 <5/20 <5/20 |
| No. with four-fold or greater response | 3 | 1 | 2 | 6 |

* Reciprocal titer in acute serum/reciprocal titer in convalescent serum.

two acute sera had neutralizing activity, and in both cases titers were 1:5.

The HI titers of the DEAE-extracted IgG in acute and convalescent sera agreed very closely with the neutralizing data. In both instances, acute sera had little or no activity and both tests identified the same six patients as having significant serological responses. It appears that DEAE extraction was superior to periodate treatment for the diagnosis of influenza HI responses because nonspecific inhibitors were removed by the former treatment that were not eliminated by periodate.

Removal of nonspecific inhibitors of arbovirus
hemagglutination. The nonspecific inhibitor soft arbovirus hemagglutinins are associated with serum lipids and are effectively removed by acetone (8). But since this takes considerable time, the following experiments were done to determine whether these inhibitors could also be removed by the more rapid DEAE-Sephadex procedure.

DEAE removed these nonspecific inhibitors as well as acetone (Table 3). The nonantibody activity for the group A (WEE) virus and the dengue-2 inhibitors in the two sera that had little or no antibody to this group B organism were drastically reduced by both forms of extraction. Further, DEAE did not remove significant amounts of specific activity from the eight sera that possessed group B antibody; the HI titers of these sera were essentially equal after DEAE and acetone extraction.

Table 3. Removal of nonspecific inhibitors of arbovirus hemagglutinins by acetone and DEAE

| Sera with low or absent group B titers | Reciprocal HI titer to group A (WEE) | Reciprocal HI titer to group B (dengue-2) |
|--------------------------------------|-------------------------------------|------------------------------------------|
|                                      | Un-treateda | Acetoneb | DEAEb | Acetoneb | DEAEb |
|--------------------------------------|-------------|--------|-------|--------|-------|
| DF 73                                | 1,024 <10 <10 | <10 | <10 | <10 | <10 |
| DF 74                                | 1,024 <10 <10 | <10 | <10 | <10 | <10 |
| DF 75                                | 256 <10 <10 | 10 | <10 | <10 | <10 |
| DF 43                                | 256 <10 <10 | <10 | <10 | <10 | <10 |
| DF 203                               | 256 <10 <10 | <10 | <10 | <10 | <10 |
| DE 333                               | 1,024 <10 <10 | 10 | <10 | <10 | <10 |
| DE 294                               | 2,048 <10 <10 | <10 | <10 | <10 | <10 |
| DE 295                               | 1,024 <10 <10 | 10 | <10 | <10 | <10 |
| DE 288                               | 2,048 <10 <10 | <10 | <10 | <10 | <10 |
| DF 314                               | 256 <10 <10 | 10 | <10 | <10 | <10 |
| DF 315                               | 1,024 10 <10 | <10 | <10 | <10 | <10 |
| Sera with group B antibody            |             |       |       |       |       |
| DF 51                                | 256 <10 <10 | 320 | 320 |       |       |
| DF 52                                | 256 <10 <10 | 640 | 640 |       |       |
| DF 53                                | 256 <10 <10 | 640 | 640 |       |       |
| DF 94                                | 256 <10 <10 | 80 | 40 |       |       |
| DF 48                                | 256 10 <10 | 80 | 20 |       |       |
| DF 27                                | 1,024 <10 <10 | 320 | 320 |       |       |
| DF 28                                | 2,048 <10 <10 | 320 | 320 |       |       |
| DF 42                                | 512 <10 <10 | 320 | 640 |       |       |

* Highest dilution of untreated serum that prevented complete (+ or ++) agglutination by WEE virus.

b Highest dilution of serum that totally prevented virus hemagglutination (0 reaction).

table 4. Reciprocal titers to rubella virus measured by various serological procedures

| Patient | HI in untreated sera | HI in kaolin-treated sera | HI in DEAE-treated sera | Plaque reduction titera |
|---------|-----------------------|----------------------------|----------------------------|------------------------|
| B 204   | 80                    | 10                         | 40                         | 10                     |
| D 34    | 320                   | 40                         | 160                        | 36                     |
| D 192   | 80                    | <5                         | <5                         | 4                      |
| E 129   | 5                     | <5                         | 10                         | 4                      |
| E 285   | 40                    | <5                         | <5                         | 4                      |
| F 226   | 40                    | <5                         | 10                         | 5                      |
| F 199   | 40                    | <5                         | <5                         | 0                      |
| F 228   | 80                    | <5                         | <5                         | 0                      |
| F 239   | 80                    | <5                         | <5                         | 0                      |
| F 400   | 20                    | <5                         | <5                         | 0                      |
| H 98    | 160                   | <5                         | 5                          | 0                      |
| Negative control | 40                 | <5                         | <5                         | 0                      |
| Positive control | 320                 | 40                         | 80                         | 0                      |

a Dilution of serum producing a 50% reduction in rubella plaques.

The treatment of arbovirus sera with DEAE possessed an advantage over acetone extraction in addition to being more rapid. Before arbovirus HI activity can be titered, acetone-treated sera must be absorbed with goose red cells to remove natural hemagglutinins. In contrast, DEAE extraction removed goose cell agglutinins and nonspecific inhibitors simultaneously. The DEAE-treated sera of Table 3 were not absorbed with these cells, and in all 19 cases serum controls were negative.

Detection of rubella antibody. Rubella antibody was assayed in sera of 11 pregnant women by measuring the HI activities in DEAE- and kaolin-extracted sera and by PR titrations (Table 4). The PR test detected specific antibody in six of these sera. Using this data as the standard for comparison, the kaolin-extracted HI results agreed with PR titrations indicating whether antibody was present in 7 of 11 cases, and the titers after DEAE extraction agreed in 8 of 11 instances. In all situations in which the methods failed to agree, titers were at the limits of detection in the test, indicating antibody was present. The HI titers of DEAE-treated fractions were always higher than those after kaolin treatment, suggesting that the latter technique removed specific antibody. A similar observation has been made by others (7, 20, 21).

DISCUSSION

That nonspecific inhibitors have been a considerable impediment to the HI test can be illustrated by some of the problems encountered in such titrations of influenza antibodies. Serum
possesses at least three nonantibody inhibitors for this virus: one substance is heat-labile and consequently easy to eliminate, whereas two heat-stable factors are difficult to deal with (13). Among those substances used to remove the latter inhibitors have been the receptor-destroying enzyme of cholera filtrates, KIO₄ with and without trypsin, dry ice, and kaolin (1, 4, 13). The fact that many procedures have been used implies that none is entirely satisfactory; indeed, Ananthanarayan et al. compared the ability of the first four treatments to dispense with nonspecific inhibitors directed to several strains of influenza (1). Since the treatments varied in their abilities to remove inhibitors for the same virus in different sera and for different strains of influenza in the same sera, no single technique was completely suitable.

Because of such problems, an attempt was made to remove these inhibitors more effectively with DEAE-Sephadex. Three objectives were considered important in developing this procedure. First, it was deemed desirable to develop a single technique potentially capable of removing the nonspecific inhibitors for all viruses. DEAE, an anion exchanger, seemed ideally suited to this purpose since it is commonly used to separate the IgG globulins from other serum proteins in a chemically defined manner without denaturing antibody. But DEAE is usually used in column chromatography, a cumbersome time-consuming procedure that requires specialized equipment. To satisfy the second objective, that the process be simple and rapid to perform, DEAE was used by the "batch" technique (3). The third objective was to procure IgG globulins of different sera in concentrations that were as great as possible and at dilutions that were constant from sample to sample: as presented, the DEAE-Sephadex consistently diluted this immunoglobulin to one-fifth of its level in whole serum.

The obvious criticism to this method is that it extracts only IgG antibodies from serum. Certainly viral antibodies occur in other serum immunoglobulins, and, in fact, immunoglobulin M (IgM) activity usually appears first after stimulation with new antigens. But in most cases this will probably not compromise DEAE extraction for the purposes of diagnostic serology. When IgM antibodies to viruses have been found, they usually contribute only a small portion of total antibody activity (5) or IgG antibodies appear within a few days of the first specific IgM (2, 16, 18, 19). There are exceptions to this, and Ishii et al., for example, could not demonstrate HI antibodies of the IgG class until as late as 5 weeks after the onset of Japanese encephalitis in unvaccinated patients (15). At the other extreme, however, it is relatively difficult to find substantial amounts of IgM or IgA antibodies directed to influenza virus (6, 24, 25). If the acute and convalescent sera from patients are separated by the usual 2 to 3 weeks, diagnostic responses to most viruses will probably not be missed. The exclusion of other antibodies may even prove an asset for serological diagnosis. If IgM titers have risen to high levels when the acute serum is obtained, a subsequent rise in the IgG antibody of the convalescent serum might increase total antibody titers very little. In such cases, the titration of only IgG globulins may identify more distinctive differences between the acute and convalescent sera of infected individuals.

The practical value of DEAE extraction for the purposes of routine diagnostic serology remains to be established by a larger trial. The technique does have two minor disadvantages. First, the initial processing of DEAE-Sephadex requires the greater part of 1 day since much time is spent waiting for the DEAE to settle while removing fines. The DEAE, however, can be prepared in large batches and a single batch can be used for a period of weeks. We found that eventually serum controls in the HI tests became positive when aged stocks were used. Presumably this was due to the release of "fines" from the Sephadex beads since reprocessing alleviated this problem. A preliminary agglutination test consisting of supernatant buffer from the stock suspension, NaCl to 0.9%, and test red cells will indicate whether rewashing of aged DEAE preparations is necessary. A few days of practice may also be necessary to become adept at the extraction method, and the consistency of dilutions and purity of extracted IgG should probably be checked before the method is used routinely. If radial diffusion and immunoelectrophoresis are not available for this, one could employ paper or cellulose acetate electrophoresis, as done in clinical laboratories, to estimate dilutions and purity.

The DEAE extraction technique has some practical advantages over the other methods now in use for similar purposes. After being set up, the extraction requires only a few minutes of actual working time per specimen, and a large number of sera can be processed simultaneously. Its use in place of the multiple techniques presently necessary for removing the nonspecific inhibitors directed to different viruses would simplify diagnostic HI serology. Finally, whether the DEAE extraction will eliminate the necessity of absorbing test sera with the heterologous red cells used to identify HI end points remains to be seen. Although it did so for the goose hemagglutinins of 19 sera tested for arbovirus antibodies, "naturally" occurring IgG antibodies specific for
other heterologous cells have been demonstrated in human sera (11, 23). If such hemagglutinins persist in DEAE-extracted IgG fractions, a serum control would easily identify those specimens requiring further absorption with red cells.

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