Variables of the Rubella Hemagglutination-Inhibition Test System and Their Effect on Antigen and Antibody Titers

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A systematic study was made of certain variables of the rubella hemagglutination-inhibition (HI) test system and their effect on antigen and antibody titers. Erythrocytes from pigeons and 1-day-old chicks gave similar antigen and antibody titers, but goose erythrocytes gave lower titers. Indicator erythrocytes could be stored in Alsever's solution at 4°C for as long as 2 weeks without losing sensitivity in hemagglutination (HA) and HI tests. Antigen titers varied by eightfold or more in different diluent systems; titers were generally higher at pH 6.2 than at pH 7.2. A diluent without Ca²⁺ gave antigen titers as high as those obtained in diluents with added Ca²⁺ ions. Antibody titers also varied in different diluent systems. HEPES diluents at pH 6.2 gave higher antibody titers than those obtained in other diluents, but occasional "false-positive" inhibition reactions were seen. Kaolin suspended in borate saline at pH 9.0 effectively removed inhibitor from sera without absorbing specific antibody, but at pH 7.3 it removed various amounts of specific antibody. Antibody titers of sera treated with kaolin at pH 9.0 were similar to those of sera treated with heparin-MnCl₂; treatment with dextran sulfate-CaCl₂ gave lower antibody titers. Antigens varied widely in sensitivity for detecting HI antibody and in the ability to detect diagnostically significant increases in antibody. Sensitivity in detecting antibody was not related to the HA titer of the antigens. Tween-ether-treated antigens gave lower antibody titers but were more reliable than corresponding untreated antigens for serological diagnosis of infection.

Hemagglutination-inhibition (HI) tests for assay of rubella antibody have been shown to be as reliable as the more cumbersome and time-consuming neutralization and indirect fluorescent-antibody tests for diagnosis of infection and for determination of immunity status (4, 8, 12, 14, 19). With the release of live, attenuated rubella virus vaccines, there is increased interest on the part of diagnostic laboratories in performing rubella HI tests as a guide to the administration of vaccine. In light of the important actions which may be taken by physicians on the basis of HI test results, it is essential to use a well-standardized procedure of maximum sensitivity and reliability.

Since the introduction of rubella HI tests late in 1966 (10, 20), a number of modifications in the test procedures have been described (1, 3, 5–7, 16, 19). The sensitivity of viral HI tests is markedly influenced by each of the variables in the test system; these include the indicator erythrocytes, the composition of the diluents, the type and amount of antigen, the method employed for removal of nonspecific inhibitors from sera, the method used for absorption of natural agglutinins from sera, and the conditions of incubation.

In an effort to determine which of the several procedures described to date gives the most reliable antibody assays, we have made a systematic study of certain variables of the rubella HI test system and have determined the effect of these variables on both antigen and antibody titers.

MATERIALS AND METHODS

HI test procedures and reagents. The rubella HI test procedures or reagents studied and compared were those developed by Halonen et al. (10) at the National Communicable Disease Center (NCDC), by Stewart et al. (20) at the National Institutes of Health (NIH), by Auletta et al. (1) at Microbiological Associates, Inc., and the National Institutes of Health (MBAIH), by Dold and Northrop (5, 6) at the University of Illinois, by Plotkin et al. (16) at the Wistar Institute.
by Cooper et al. (3) at New York University, and by H. Liebhaber (personal communication) at Yale University. All tests were conducted by the microtiter method. Serum-antigen mixtures were incubated at 4 C for 1 hr before addition of the indicator erythrocytes. Tests were then incubated at 4 C for 90 min or longer before results were read.

**Hemagglutinating (HA) antigens.** Rubella HA antigens were prepared in this laboratory by using the RV strain (obtained from John L. Sever and G. M. Schiff, National Institutes of Health, Bethesda, Md.) of virus propagated in BHK-21 cells. The cells were infected in suspension as previously described (18) and then planted either in prescription bottles or in roller bottles in growth medium consisting of 10% fetal bovine serum and 90% Eagle’s minimal essential medium (MEM). After 3 days of incubation, when confluent monolayers of cells had formed, the cultures were washed and maintenance medium consisting of 2 or 5% kaolin-treated fetal bovine serum in Eagle’s MEM was added. Cultures were harvested 3 to 5 days later, and rubella HA antigens were prepared from both the fluid and cellular phases of the cultures. Unconcentrated, infected tissue culture fluids (TC fluids) were used without treatment and also after treatment with Tween 80 and ether (T-E-treated).

Concentrated hemagglutinin preparations were prepared from the TC fluids by centrifuging at 35,000 X g for 3 hr and resuspending the pellets in 0.4% bovine albumin-borate saline (BABS) to 0.1 or 0.01 of the original volume of culture fluid; these concentrates were used untreated and after T-E treatment. Antigens were also prepared by extraction of the cellular phase of the cultures with 0.1 M glycine buffer at pH 9.5 for 6 hr at 37 C (17); these alkaline extracts were used as antigens both untreated and after T-E treatment. In addition, several rubella HA antigens were purchased from Flow Laboratories and from Courtland Laboratories.

**Neutralizing antibody assays.** Sera were examined for rubella neutralizing antibodies by the interference neutralization test conducted in the BS-C-1 line of grivet monkey kidney cells; echovirus type 11 was used as the challenge virus (14).

**Complement-fixing antibody assays.** Complement fixation (CF) tests for rubella were conducted by our standard microtiter procedure (13) by using antigens prepared by alkaline buffer extraction of infected BHK-21 cells (17).

**RESULTS**

**Effect of indicator erythrocytes on rubella antigen and antibody titers.** In assays for viral HI antibody, it is essential to use erythrocytes which possess maximum sensitivity to agglutination by the virus. If erythrocytes of low sensitivity are employed, more antigen is required, and this excess of antigen in the test system may result in low antibody titers.

Table 1 compares the sensitivity of chick (1-day-old chicks), pigeon, and goose erythrocytes to agglutination by four different rubella antigen

| Antigen                              | HA titer of antigen vs. RBC<sup>a</sup> |
|--------------------------------------|----------------------------------------|
|                                      | Chicken (1-day-old chick) | Pigeon | Goose |
| Flow C961231                         | 51<sup>2</sup> | 1,024 | 64 |
| Courtland K690519                    | 128 | 256 | 64 |
| T-E-treated fluid lot 497<sup>b</sup> | 128 | 128 | 32 |
| Alkaline buffer extract lot 518<sup>c</sup> | 128 | 128 | 32 |

<sup>a</sup> Tests performed by the method of Halonen et al. (10) by using 0.25% suspensions of indicator erythrocytes. RBC, red blood cells.

<sup>b</sup> Reciprocal of antigen titer.

<sup>c</sup> Antigen prepared by Tween 80 and ether treatment of unconcentrated, infected BHK-21 culture fluid.

<sup>d</sup> Antigen prepared by extraction of infected BHK-21 cells with glycine buffer, pH 9.5; not treated with Tween-ether.

**Table 2. Effect of species of erythrocytes on rubella hemagglutination-inhibition (HI) antibody titers: comparison of chick, pigeon, and goose erythrocytes**

| Serum                              | HI titer in tests<sup>a</sup> with erythrocytes from |
|------------------------------------|------------------------------------------------------|
|                                    | Chick (antigen 1:16) | Pigeon (antigen 1:24) | Goose (antigen 1:4) |
| 1–6                                | <8<sup>b</sup> | <8 | <8 |
| 7                                 | 32 | 32 | 8 |
| 8                                 | 32 | 32 | 8 |
| 9                                 | 32 | 16 | 8 |
| 10                                | 32 | 32 | 16 |
| 11                                | 64 | 64 | 16 |
| 12                                | 64 | 64 | 16 |
| 13                                | 64 | 64 | 16 |
| 14                                | 64 | 128 | 16 |
| 15                                | 128 | 64 | 16 |
| 16                                | 128 | 64 | 32 |
| 17                                | 512 | 512 | 128 |
| 18                                | 512 | 512 | 128 |
| 19                                | 512 | 512 | 256 |
| 20                                | 2,048 | 4,196 | 512 |

<sup>a</sup> Tests conducted by the procedure of Halonen et al. (10) by using 0.25% suspensions of indicator erythrocytes and Courtland antigen lot K69027.

<sup>b</sup> Reciprocal of antibody titer.

preparations. Pigeon erythrocytes gave antigen titers either the same or twofold higher than those obtained with cells from 1-day-old chicks, whereas goose erythrocytes give antigen titers fourfold...
lower than those seen with the other two species or erythrocytes. Thus, four times as much antigen was required for HI tests with goose erythrocytes as for tests with chick or pigeon erythrocytes. Results shown in Table 1 were obtained by using the NCDC (Halonen et al.) test procedure, but comparable results were also obtained by using diluents from a Courtland kit and the diluent of the Wistar procedure (16).

The effect of the species of indicator erythrocytes on rubella HI antibody titers is illustrated in Table 2. Sera were titrated in parallel by using chick, pigeon, and goose erythrocytes with the same antigen, diluted to contain 4 HA units for each species of erythrocyte. All sera were treated with kaolin at pH 9.0 to remove nonspecific inhibitors, and tests were conducted by the procedure of Halonen et al. Antibody titers obtained with goose erythrocytes were two- to fourfold lower than those obtained with chick or pigeon erythrocytes. In the Courtland and Wistar diluents, goose erythrocytes also gave lower HI antibody titers than did chick or pigeon erythrocytes.

Considerable variation was noted in the sensitivity of different lots of goose erythrocytes to hemagglutination by rubella virus.

Erythrocytes stored for prolonged periods of time tend to become less sensitive to agglutination by the virus and also to hemolyze and to agglutinate nonspecifically. A study was made to ascertain the maximum length of time that indicator erythrocytes could be stored without a loss of sensitivity and reliability for use in rubella HI tests. Chick, pigeon, or goose erythrocytes were held at 4°C in Alsever’s solution as an approximate 20% suspension, and at intervals samples were removed, washed, and prepared as a 0.25% suspension for use in antigen titrations. Tests were conducted by the procedure of Halonen et al. against three different antigen preparations. In Table 3, it is seen that chick and pigeon cells retained their initial sensitivity to hemagglutination for 13 to 19 days, and goose cells showed an initial loss of sensitivity during the first week and then showed no change for as long as 24 days. After 2 weeks of storage, none of the species of erythrocytes showed hemolysis or spontaneous agglutination. On the basis of these results, it is considered that erythrocytes stored in Alsever’s solution are satisfactory for rubella HI tests for as long as 2 weeks.

Effect of composition and pH of diluents on rubella antigen and antibody titers. Various diluents have been described for rubella HI test systems. Some of these (5, 6, 10; H. Liebhaber, personal communication) give a final pH of 6.2 in the test, which has been reported to be the optimal pH for rubella hemagglutination, whereas other diluents (1, 3, 9, 16, 20) have a pH of 7.1 to 7.3. For the procedure of Halonen et al. (10), serum and antigen were diluted in 0.4% BABS (pH 9.0), and the erythrocytes were diluted in a phosphate-buffered saline (PBS) adjusting diluent which gave a final pH in the test of 6.2 when the erythrocyte suspension was mixed with an equal volume of the serum-antigen mixtures in BABS, pH 9.0. For the procedure of Stewart et al. (20), erythrocytes, serum, and antigen were all diluted in dextrose-gelatin-Veronal (DGV) buffer (2) containing 0.2% bovine serum albumin; this diluent had a pH of 7.3. Certain other procedures used DGV at pH 7.2 or 7.3 without bovine albumin (3, 7, 19). Auletta et al. (reference 1; MBA-NIH method) employed a diluent for all test components consisting of 0.9% NaCl, 0.1% CaCl₂, and 0.1% MgSO₄·7H₂O, having a pH of 7.1. Dold and Northrop (5, 6) employed a PBS at pH 6.2 containing 0.03% bovine albumin, 0.5% dextrose, 0.03% gelatin, 0.1% calcium chloride, and 0.01% magnesium chloride (ADGP buffer) as a diluent for all reagents. Plotkin et al. (16; Wistar method) employed a

### Table 3. Effect of age of indicator erythrocytes on titers of rubella hemagglutinating (HA) antigens

| Species of RBC* | Age of RBC | HA titers of antigenb | Flow Laboratories C961231 | Alkaline extract lot 518b | Tween-ether lot 487b |
|-----------------|------------|-----------------------|---------------------------|--------------------------|---------------------|
| Chicken         | days       |                       |                           |                           |                     |
| 5               | 128        | 128                   | 32                        |                          |
| 12              | 128        | 128                   | 32                        |                          |
| 15              | 128        | 128                   | 32                        |                          |
| 19              | 128        | 128                   | 32                        |                          |
| 26              | 32         | 64                    | 16                        |                          |
| Pigeon          |            |                       |                           |                           |                     |
| 3               | 256        | 256                   | 64                        |                          |
| 10              | 256        | 256                   | 64                        |                          |
| 13              | 256        | 256                   | 64                        |                          |
| 17              | 128        | 128                   | 64                        |                          |
| 24              | 128        | 128                   | 64                        |                          |
| Goose           |            |                       |                           |                           |                     |
| 3               | 64         | 64                    | 32                        |                          |
| 10              | 32         | 64                    | 16                        |                          |
| 13              | 32         | 64                    | 16                        |                          |
| 17              | 32         | 32                    | 16                        |                          |
| 24              | 32         | 32                    | 16                        |                          |

* Red blood cells.

b Tests conducted by the procedure of Halonen et al. (10) by using 0.25% suspensions of indicator erythrocytes.

* Antigen prepared by extraction of infected BHK-21 cells with glycine buffer, pH 9.5.

* Antigen prepared by Tween 80 and ether treatment of unconcentrated, infected BHK-21 culture fluids.

* Reciprocal of antigen titer.
Table 4. Effect of diluents on titers of rubella hemagglutinating (HA) antigens

| Antigen                      | HA titer of antigen in diluent systema |
|------------------------------|---------------------------------------|
|                              | NCDC (Halonen et al.) | NIH (Stewart et al.) | MBA-NIH (Auletta et al.) | Wistar (Plotkin et al.) | Yale HSAG (Lieberhaber) | HEPES pH 6.2, 0.1% FBS | ADGP (Dold and Northrop) | Antigen titer range |
| Infected BHK-21 fluids lot 528, unconc., untreated                                  | 32 | 4b | 4 | 4 | 8 | 4 | 8 | 4-32 |
| T-E treated                                | 32 | 8b | 16 | 16 | 32 | 128 | 32 | 32-128 |
| Infected BHK-21 fluids lot 526, concd. 10X⁺, untreated                                  | 32 | 32b | 32 | 128 | 64 | 128 | 32 | 32-128 |
| T-E-treated                                | 64 | 64b | 128 | 128 | 128b | 256 | 128b | 64-256 |
| Infected BHK-21 fluids lot 523, concd. 100X⁺, untreated                                | 256 | 4b | 8 | 4 | 64a | 64 | 32 | 4-256 |
| Alkaline buffer extract of infected BHK-21 cells lot 506-515, untreated              | 256 | 64b | 64 | 128 | 16b | 32 | 16 | 16-256 |
| Alkaline buffer extract of infected BHK-21 cells lot 327, T-E-treated                 | 128 | 32b | 128 | 128 | 256 | 1,024 | 128 | 32-1024 |
| Flow Laboratories C961231                                                             | 256 | 128b | 256 | 256 | 32b | 256 | 32b | 32-256 |
| Courtland Laboratories K690519                                                        | 128 | 64b | 512 | 256 | 64b | 1,024 | 64b | 64-1024 |

a Suspensions (0.25%) of chick erythrocytes used for all tests.
b Agglutination pattern = rough instead of smooth shields.
c Hemagglutinins concentrated by high-speed centrifugation.

0.01 M HEPES (N-2-hydroxyethyl-piperazine-N'2-ethanesulfonic acid) buffer containing 0.15 m NaCl, 0.001 m CaCl₂, and 0.1% fetal bovine serum (FBS; treated with heparin-MnCl₂ to remove nonspecific inhibitors) and having a pH of 7.25. R. Liebhaber (personal communication) employed a 0.025 M HEPES buffer containing 0.14 M NaCl, 0.001 M CaCl₂, 1.0% bovine serum albumin, and 0.00025% gelatin and having a pH of 6.2 (HSAG buffer).

Table 4 compares the titers obtained for nine different rubella antigens assayed in parallel in each of the diluent systems described above. These antigens represented unconcentrated, infected tissue culture fluids, hemagglutinin preparations which had been concentrated by high-speed centrifugation, and alkaline buffer extracts of infected cells. Both untreated and T-E-treated antigens were tested. In addition to the HSAG buffer, a modification of this buffer was tested in which 0.1% FBS (treated with heparin-MnCl₂ to remove nonspecific inhibitors) was substituted for the bovine albumin and gelatin (HEPES, pH 6.2, 0.1% FBS). Chick erythrocytes (1-day-old chicks) were used as a 0.25% suspension in all test systems.

Each antigen showed marked variation in titer in the different diluents, and the buffers at pH 6.2 did not regularly give higher antigen titers than those at pH 7.1 to 7.3. With the HEPES diluents at pH 6.2, the titers of certain T-E-treated antigens tended to be higher than those obtained in other buffer systems. It has been reported that calcium ions are essential to, or greatly enhance, hemagglutination by rubella virus (1, 6, 9). However, in these comparisons, antigen titers were as high, and in some cases higher, in the PBS-BABS diluent (10) lacking Ca²⁺ as in certain other diluents containing Ca²⁺ at concentrations of 10⁻³ to 10⁻⁴ M. In three of the diluents, which contained bovine albumin and gelatin (the NIH diluent, HSAG, and ADGP), the agglutinated erythrocytes formed rough patterns rather than smooth shields, making it difficult to read end points.

To study further the effect of pH on hemagglutination by rubella virus, seven antigens were titrated in parallel at pH values ranging from 5.8 to 7.6 in three different diluent systems. These were (i) the PBS-BABS system (2) on which the Halonen procedure is based, (ii) DGV with 0.2% bovine albumin, and (iii) 0.025 M HEPES containing 0.1% FBS rather than bovine albumin and gelatin. Table 5 shows that the titers of all of the antigens (with the exception of the alkaline buffer extract in the HEPES system) were higher in the pH range of 6.2 than in the range of 7.2. Again, the effect of the composition, as well as pH, of the diluents was seen on the antigen titers. T-E-treated antigens 524 and 487 had markedly higher titers in the HEPES system than in the PBS-BABS or DGV systems. The 100-fold concentrate of hemagglutinins from an infected culture fluid had a titer of 1:256 at the optimal
Table 5. Effect of pH on titers of rubella hemagglutinating (HA) antigens: a comparison of three buffering systems

| Buffering system | pH | Infected fluid 324, untreated | Infected fluid 324, T-E-treated | Infected fluid 487, T-E-treated | Infected fluid 323, concd 100X | Alkaline extract 506-515 | Flow C901231 | Courtland K900519 |
|------------------|----|------------------------------|-------------------------------|-------------------------------|-----------------------------|---------------------|----------------|-------------------|
| PBS-BABS         | 5.8| <4                           | 8                             | 8                             | 256                         | 256                 | 128            | 256               |
|                  | 6.0| 8                            | 16                            | 8                             | 256                         | 256                 | 128            | 64                |
|                  | 6.2| 16                           | 16                            | 8                             | 256                         | 256                 | 128            | 64                |
|                  | 6.4| 8                            | <4                            | 128                           | 128                         | 64                  | 128            | 16                |
|                  | 6.6| 8                            | <4                            | 128                           | 128                         | 64                  | 128            | 16                |
|                  | 6.8| 8                            | <4                            | 128                           | 128                         | 64                  | 128            | 16                |
|                  | 7.0| 8                            | <4                            | 64                            | 32                          | 16                  | 64             | 8                 |
|                  | 7.2| 4                            | <4                            | 64                            | 32                          | 16                  | 64             | 8                 |
|                  | 7.4| 4                            | <4                            | 64                            | 32                          | 16                  | 64             | 8                 |
|                  | 7.6| 4                            | <4                            | 32                            | 16                          | 64                  | 8              |                   |
| DGV, 0.2% bovine albumin | 5.8| 8                            | 16                            | 16                            | 8b                          | 128b                | 32             | 32                |
|                  | 6.0| 8                            | 32                            | 32                            | 128b                        | 512b                | 128            |                   |
|                  | 6.2| 8                            | 16                            | 16                            | 8b                          | 128b                | 256b           | 128b              |
|                  | 6.4| 8                            | 8                             | 16                            | 8b                          | 128b                | 128b           | 64b               |
|                  | 6.6| 8                            | 8                             | 16b                           | 8b                          | 64b                 | 128b           | 64b               |
|                  | 6.8| 8                            | 8                             | 16b                           | 8b                          | 64b                 | 128b           | 64b               |
|                  | 7.0| 8                            | 8                             | 16b                           | 8b                          | 128b                | 64b            | 64b               |
|                  | 7.2| 4                            | 8                             | 8                             | 4b                          | 128b                | 64b            | 64b               |
|                  | 7.4| 4                            | 8                             | 8                             | 4b                          | 128b                | 64b            | 64b               |
|                  | 7.6| 4                            | 8                             | 8                             | 4b                          | 128b                | 64b            | 64b               |
| 0.025 M HEPES, 0.1% FBS | 5.8| 4                            | 128                           | 64                            | 32                          | 16                  | 512            | 256               |
|                  | 6.0| 8                            | 128                           | 64                            | 32                          | 16                  | 512            | 256               |
|                  | 6.2| 8                            | 64                            | 64                            | 32                          | 16                  | 512            | 512               |
|                  | 6.4| 8                            | 64                            | 32                            | 16                          | 32                  | 256            | 256               |
|                  | 6.6| 8                            | 64                            | 32                            | 8                            | 64                  | 256            | 256               |
|                  | 6.8| 8                            | 32                            | 32                            | 8                            | 32                  | 256            | 128               |
|                  | 7.0| 4                            | 16                            | 16                            | 4                            | 32                  | 256            | 128               |
|                  | 7.2| 4                            | 8                             | 8                             | 4                            | 32                  | 256            | 128               |
|                  | 7.4| 4                            | 8                             | 8                             | 4                            | 32                  | 256            | 128               |
|                  | 7.6| 4                            | 8                             | 8                             | 4                            | 32                  | 256            | 128               |

* Suspension (0.25%) of chick erythrocytes used for all tests.

b Agglutination patterns = rough instead of smooth shields.

pH in the PBS-BABS system but only 1:32 in the other two systems. The titer of the alkaline buffer extract antigen (not T-E-treated) was greatly enhanced at low pH levels in the PBS-BABS system but not in the other two systems.

Tables 6 through 8 show the effect of the composition of diluents on rubella HI antibody titers. All sera were treated with kaolin at pH 9.0 to remove nonspecific inhibitors. It is seen in all three tables that some sera lacking neutralizing (and fluorescent) antibody to rubella virus had low inhibitory titers of 1:8 in the MBA-NIH and HEPES buffering systems; these can probably be considered "false-positive" reactions. Table 6 shows that antibody titers of individual sera varied widely in the five different buffering systems. Titers in the NCDC, NIH, and ADGP systems were similar, whereas higher titers were obtained in the MBA-NIH and the HEPES systems. The possibility was considered that differences in antibody titer might be attributable to differences in the amount of antigen employed rather than to the diluent per se, since higher antibody titers were obtained in the diluent systems in which the antigen was more dilute. However, in Table 7, which shows titers of sera tested in parallel in the PBS-BABS system (10) and the HEPES system against an alkaline buffer extract antigen used at nearly the same dilution in both systems, and in Table 8, which compares titers of sera tested in the ADGP diluent and the HEPES diluent by using another antigen at nearly the same dilution in both systems, it is seen that higher antibody titers were obtained in the
HEPES system. Although the HEPES diluent gave higher antibody titers, it is noteworthy that a number of apparently false-positive reactions were obtained in this diluent system.

Table 6. Effect of diluents on rubella hemagglutination-inhibition (HI) antibody titers: a comparison of five diluent systems

| Serum no. | Neutralization titer | HI antibody titer in diluent system<sup>a</sup> | HI antibody titer, in diluent system<sup>a</sup> | HI titer range |
|-----------|----------------------|-----------------------------------------------|-----------------------------------------------|----------------|
|           | NCDC (antigen<sup>b</sup> 1:12) | NIH (antigen<sup>b</sup> 1:6) | ADGP (antigen<sup>b</sup> 1:12) | MBA-NIH (antigen 1:24) | HEPES, pH 6.2, 0.1% FBS (antigen 1:48) | |
| 2166      | <4                   | <8    | <8    | <8    | <8    | <8    | |
| 2510      | <4                   | <8    | <8    | <8    | <8    | <8    | <8-8 |
| 2627      | <4                   | <8    | <8    | <8    | <8    | <8    | <8-8 |
| 2041      | <4                   | <8    | <8    | <8    | <8    | <8    | <8-8 |
| 2545      | <4                   | <8    | <8    | <8    | <8    | <8    | <8-8 |
| 3868      | 4                    | 16    | 16    | 32    | 128   | 128   | 16-128 |
| 3921      | 8                    | 16    | 32    | 32    | 64    | 64    | 16-64 |
| 1410      | 8                    | 64    | 64    | 32    | 64    | 64    | 32-64 |
| 0513      | 16                   | 128   | 64    | 64    | 128   | 128   | 64-256 |
| 0849      | 32                   | 64    | 64    | 128   | 256   | 512   | 64-256 |
| 2202      | 32                   | 64    | 64    | 64    | 256   | 256   | 64-256 |
| 0855      | 64                   | 64    | 64    | 64    | 256   | 256   | 64-256 |
| 0577      | 128                  | 256   | 256   | 256   | 256   | 1024  | 256-1024 |

<sup>a</sup> Suspension (0.25%) of chick erythrocytes used for all tests.
<sup>b</sup> Antigen was a hemagglutinin preparation concentrated 10-fold by high-speed centrifugation and treated with Tween 80 and ether.
<sup>c</sup> Italicized numbers represent probable false-positive reactions.

Table 7. Effect of diluents on rubella hemagglutination-inhibition (HI) antibody titers<sup>a</sup>

| Serum no. | Neutralization titer | HI antibody titer in diluent system<sup>a</sup> | HI antibody titer in diluent system<sup>a</sup> | NCDC (antigen 1:12) | HEPES, pH 6.2, 0.1% FBS (antigen 1:16) | |
|-----------|----------------------|-----------------------------------------------|-----------------------------------------------|--------------------------------------------------------------------------------------|----------------|
| 2811      | <4                   | <8    | 8c    | 8c    |<8    | |
| 2510      | <4                   | <8    | 8c    |<8    |<8    | |
| 2911      | <4                   | <8    |<8    |<8    |<8    | |
| 2045      | <4                   |<8    |<8    |<8    |<8    | |
| 3046      | 4                    | 8     | 128   | 128   |<8    | |
| 1024      | 8                    | 16    | 128   | 128   |<8    | |
| 0977      | 16                   | 32    | 128   | 128   |<8    | |
| 0717      | 32                   | 64    | 256   | 256   |<8    | |
| 0855      | 64                   | 128   | 256   | 256   |<8    | |
| 0573      | 128                  | 256   | 512   | 512   |<8    | |

<sup>a</sup> Tests conducted with an antigen prepared by alkaline buffer extraction of infected BHK-21 cells.
<sup>b</sup> Suspension (0.25%) of chick erythrocytes used for tests.
<sup>c</sup> Italicized numbers represent probable false-positive reactions.

Table 8. Effect of diluents on rubella hemagglutination-inhibition (HI) antibody titers<sup>a</sup>

| Serum no. | Neutralization titer | HI antibody titer, in diluent system<sup>a</sup> | ADGP (antigen 1:12) | HEPES, pH 6.2, 0.1% FBS (antigen 1:16) | |
|-----------|----------------------|-----------------------------------------------|--------------------------------------------------------------------------------------|----------------|
| 2166      | <4                   |<8    | 8c    |<8    | |
| 2910      | <4                   |<8    | 8c    |<8    | |
| 2041      | <4                   |<8    |<8    |<8    | |
| 3812      | <4                   |<8    |<8    |<8    | |
| 3046      | 4                    | 8     | 32    |<8    | |
| 2711      | 8                    | 16    | 64    | 64    | |
| 0472      | 16                   | 32    | 128   | 128   | |
| 0717      | 32                   | 32    | 128   | 128   | |
| 0849      | 32                   | 32    | 256   | 256   | |
| 0577      | 128                  | 64    | 1,024 | 1,024 | |

<sup>a</sup> Tests were conducted with an antigen prepared from unconcentrated, infected BHK-21 culture fluids treated with Tween 80 and ether.
<sup>b</sup> Suspension (0.25%) of chick erythrocytes used for tests.
<sup>c</sup> Italicized numbers represent probable false-positive reactions.

Effect on HI antibody titers of method employed for removal of nonspecific inhibitors from sera. Absorption of sera with kaolin was the method first described for the removal of nonspecific inhibitor of rubella hemagglutinins from test sera (10, 20). It is recognized that kaolin may remove various amounts of specific antibody from sera (1, 15), particularly immunoglobulin (Ig)M (15). In this laboratory, satisfactory removal of rubella inhibitor has been achieved by using kaolin in borate saline at pH 9.0, as described for arboviruses HI tests (2) and for the rubella HI test of Halonen et al. (10). Removal of inhibitor appeared to be complete, as sera lacking neutralizing and fluorescent-antibody for rubella virus did not show HI activity after treatment, and further there was no evidence of appreciable loss of rubella antibody, since sera with neutralizing and fluorescent antibody for rubella also showed HI antibody (14). However, other laboratories utilizing kaolin suspended in DGV at pH 7.3 have reported that kaolin used at a ratio of four parts of a 25% suspension to one part of serum frequently failed to remove inhibitor (3, 19), and that increasing the amount of kaolin to a ratio of six parts of kaolin suspension to one of serum gave better absorption of inhibitor but tended to remove specific antibody (19). Table 9 shows the effect of the pH of the kaolin suspension and the amount used for absorption on rubella inhibitors and specific antibody. At pH 9.0 and 7.3 and at both concentrations tested, kaolin removed inhibitor from 10 sera lacking neutralizing or fluorescent antibody for rubella. The 17 sera
TABLE 9. Effect of pH and amount of kaolin on rubella hemagglutination-inhibition (HI) antibody

| Serum Neutralization titer | HI antibody titer of serum treated with kaolin<sup>a</sup> |
|---------------------------|--------------------------------------------------|
|                           | Borate saline (pH 9.0) | DGV (pH 7.3) |
|                           | 4 K:1 serum<sup>b</sup> | 6 K:1 serum<sup>b</sup> | 4 K:1 serum<sup>b</sup> | 6 K:1 serum<sup>b</sup> |
| 1–10                      | <4                  | <8                  | <8                  | <8                  |
| 11                        | 8                   | 8                   | 8                   | <8                  |
| 12                        | 8                   | 8                   | 8                   | <8                  |
| 13                        | 8                   | 16                  | 16                  | <8                  |
| 14                        | 16                  | 16                  | 16                  | <8                  |
| 15                        | 16                  | 16                  | 16                  | <8                  |
| 16                        | 16                  | 32                  | 32                  | 16                  |
| 17                        | 16                  | 32                  | 32                  | 16                  |
| 18                        | 16                  | 64                  | 64                  | 32                  |
| 19                        | 16                  | 64                  | 64                  | 32                  |
| 20                        | 32                  | 64                  | 64                  | 32                  |
| 21                        | 32                  | 64                  | 64                  | 32                  |
| 22                        | 32                  | 128                 | 128                 | 64                  |
| 23                        | 32                  | 128                 | 128                 | 64                  |
| 24                        | 64                  | 128                 | 128                 | 64                  |
| 25                        | 64                  | 256                 | 256                 | 256                 |
| 26                        | 64                  | 128                 | 128                 | 128                 |
| 27                        | 128                 | 1,024               | 1,024               | 1,024               |

<sup>a</sup> Tests conducted by the method of Halonen et al. (10) by using chick erythrocytes.
<sup>b</sup> Sera treated at a ratio of four parts of 25% kaolin to one part of serum.
<sup>c</sup> Sera treated at a ratio of six parts of 25% kaolin to one part of serum.
<sup>d</sup> Italicized numbers represent false-negative reactions.

containing various levels of rubella neutralizing antibody showed the same HI titers after treatment with each concentration of kaolin at pH 9.0. On the other hand, treatment of these 17 sera with kaolin at pH 7.3 reduced the HI antibody in some of the low-titered sera to undetectable levels; reduction of antibody titers was particularly marked in sera treated at pH 7.3 at a ratio of six parts of kaolin suspension to one of serum. These results illustrate the importance of using kaolin at pH 9.0 to minimize absorption of specific rubella antibody.

More recently, heparin and manganese chloride (3, 5, 7, 16) and dextran sulfate and calcium chloride (W. D. Sedwick et al., Bacteriol. Proc. 1968, p. 180; H. Liehhaber, personal communication) have been employed to remove rubella HA inhibitor from sera. Both of these methods selectively precipitate the beta-lipoproteins containing inhibitory activity. To compare the effectiveness of various methods for removal of nonspecific inhibitors and also to study their effect on specific antibody, sera were treated in parallel by four different methods and then tested for rubella HI antibody. Tests were conducted in DGV diluent by using a 0.25% suspension of chick erythrocytes. The four methods used for removal of inhibitor were (i) absorption with kaolin at pH 9.0 at a ratio of four parts of 25% kaolin suspension to one part of serum, (ii) treatment with heparin-MnCl₂ at the concentrations described by Mann et al. (15), (iii) treatment with heparin-MnCl₂ at 10 times these concentrations (3), and (iv) treatment with dextran sulfate-CaCl₂ (H. Liehhaber, personal communication).

In Table 10, it is seen that all four methods effectively removed inhibitory activity from sera lacking neutralizing antibody to rubella. Titers of sera treated with kaolin at pH 9.0 were similar to those of sera treated with heparin-MnCl₂, but in some instances kaolin-treated sera had twofold higher titers. Titers of sera treated with dextran sulfate-CaCl₂ tended to be lower than those of sera treated by the other two methods, and one showed a false-negative reaction.

TABLE 10. Effect of various methods used for removal of nonspecific inhibitors on rubella hemagglutination-inhibition (HI) antibody titers

| Serum Neutralization titer | HI titer<sup>a</sup> after treatment with |
|---------------------------|----------------------------------|
|                           | Kaolin, (pH 9.0)<sup>b</sup> | Heparin-MnCl₂<sup>c</sup> | 10X Heparin-MnCl₂<sup>a</sup> | Dextran sulfate-CaCl₂<sup>d</sup> |
| 1–8                       | <4                  | <8                  | <8                  | <8                  |
| 9                         | 4                   | 32                  | 16                  | 16                  |
| 10                        | 4                   | 32                  | 32                  | 32                  |
| 11                        | 8                   | 32                  | 32                  | 16                  |
| 12                        | 8                   | 64                  | 64                  | 32                  |
| 13                        | 8                   | 64                  | 64                  | 32                  |
| 14                        | 8                   | 64                  | 64                  | 32                  |
| 15                        | 16                  | 32                  | 32                  | 16                  |
| 16                        | 16                  | 128                 | 128                 | 64                  |
| 17                        | 16                  | 128                 | 128                 | 64                  |
| 18                        | 16                  | 128                 | 128                 | 128                 |
| 19                        | 16                  | 128                 | 128                 | 128                 |
| 20                        | 32                  | 256                 | 256                 | 64                  |
| 21                        | 32                  | 256                 | 256                 | 128                 |
| 22                        | 32                  | 256                 | 256                 | 128                 |
| 23                        | 32                  | 128                 | 128                 | 64                  |
| 24                        | 64                  | 512                 | 256                 | 256                 |
| 25                        | 64                  | 512                 | 256                 | 128                 |
| 26                        | 128                 | 1,024               | 512                 | 256                 |
| 27                        | 128                 | 1,024               | 1,024               | 1,024               |

<sup>a</sup> All tests conducted with a 0.25% suspension of chick erythrocytes.
<sup>b</sup> Used at a ratio of four parts of 25% kaolin suspension to 1 part of serum.
<sup>c</sup> Method described by Dold and Northrop (5).
<sup>d</sup> Method described by Cooper et al. (3).
<sup>e</sup> Serum (0.2 ml) in 0.4 ml of diluent treated with 0.05 ml of 5% dextran sulfate solution and 0.1 ml of 1 M CaCl₂ solution at 4°C for 2 hr.
TABLE 11. Lack of relationship between titer of rubella hemagglutinating (HA) antigens and sensitivity in detecting hemagglutination-inhibition (HI) antibody: a comparison in three different diluent systems

| Diluent system       | Antigen                                      | Dilution of antigen used to contain 4 HA units | HI antibody titers of various sera³ |
|----------------------|----------------------------------------------|-----------------------------------------------|-----------------------------------|
|                      |                                              |                                               | 3921  0472  9849  0855  0577       |
| PBS-BABS (Halonen et al.) |                                        |                                               |                                   |
|                      | Unconcl. TC fluid, T-E-treated, lot 497     | 1:6                                           | <8    16    8    32    32             |
|                      | Unconcl. TC fluid, T-E-treated, lot 528     | 1:2                                           | 16    32    32    64    128           |
|                      | Conc. 10X by centrifugation, untreated, lot 526 | 1:4                                          | 32    64   128   512   512            |
|                      | Conc. 100X by centrifugation, untreated, lot 533 | 1:24                                         | 8     16    16    64    128           |
| ADGP (Dold and Northrop) |                                        |                                               |                                   |
|                      | Unconcl. TC fluid, T-E-treated, lot 497     | 1:12                                          | 8     32    32    32    32             |
|                      | Unconcl. TC fluid T-E-treated, lot 528      | 1:1                                           | 16    32    32    64    64             |
|                      | Conc. 10X by centrifugation, untreated, lot 526 | 1:4                                          | 32    64   256  128   512            |
|                      | Conc. 100X by centrifugation, untreated, lot 533 | 1:32                                         | 64    64   512  128  1,024          |
| HEPES, pH 6.2, 0.1% FBS |                                         |                                               |                                   |
|                      | Unconcl. TC fluid, T-E-treated, lot 497     | 1:16                                          | 64    64   256  64   1,024          |
|                      | Unconcl. TC fluid T-E-treated, lot 528      | 1:12                                          | 64    128  1,024 256  1,024         |
|                      | Conc. 10X by centrifugation, untreated, lot 526 | 1:12                                         | 256   256  1,024 128  1,024        |
|                      | Conc. 100X by centrifugation, untreated, lot 533 | 1:64                                         | 128   128  512  128  1,024        |
| Rubella neutralization titers of sera |                                |                                               | 8     16    32    64    128          |

* Sera absorbed with kaolin at pH 9.0 to remove nonspecific inhibitors. All tests were conducted with a 0.25% suspension of chick erythrocytes.

**Effect of titer and type of antigen on rubella HI antibody titers.** Antigen preparations may differ in sensitivity for demonstrating rubella HI antibody, but the reasons for these differences are not known. Halonen et al. (11) noted that antibody titers varied as much as eightfold against different antigen preparations and that higher-titered antigens gave higher antibody titers. It was suggested that low-titered antigens may contain nonhemagglutinating viral particles which compete with hemagglutinins for antibody but that in high-titered antigens such nonhemagglutinating particles can be diluted beyond the reactive range (11).

The results of several comparative studies in this laboratory suggested that the HA titer of an antigen is not directly related to its sensitivity in detecting rubella HI antibody. Table 11 compares the sensitivity of high- and low-titered rubella HA antigens prepared from unconcentrated, T-E-treated culture fluids and of high- and low-titered antigens concentrated by high-speed centrifugation (not T-E-treated). Despite the fact that antigen 497 had a higher HA titer and could be used more dilute than antigen 528, it gave lower antibody titers in all three diluent systems. Antigen 533 (concentrated 100 X) had a much higher titer than antigen 526 (concentrated 10 X), but it gave markedly lower antibody titers than antigen 526 in the diluent system of Halonen et al. and gave similar titers in the ADGP and HEPES systems.

The lack of correlation between the HA titer of an antigen and its sensitivity in detecting rubella HI antibody is further illustrated in Table 12. Thirty-six sera were tested in parallel by the method of Halonen et al. against three different antigen preparations, each diluted to contain 4 HA units. Antigen 525, which had a titer of 1:320, was a hemagglutinin preparation concentrated 10-fold by high-speed centrifugation and treated with T-E. Antigen C961242 was a commercial
TABLE 12. Comparison of hemagglutination-inhibition (HI) antibody titers obtained in parallel tests against three different antigen preparations

| Antigen                  | HI titer | HI titer vs. antigen 525 (diluted 1:80 to contain 4 HA units) |
|--------------------------|----------|---------------------------------------------------------------|
|                          |          | <8  | 8       | 16      | 32      | 64      | 128     | 256     | 512     | ≥1,024 |
| Antigen C961242 (diluted 1:40 to contain 4 HA units) | <8  | 6³  | -      | e       | -       | -       | -       | -       | -       | -      |
|                          | 8       | 1    | -      | -       | -       | -       | -       | -       | -       | -      |
|                          | 16      | -    | -      | -       | -       | -       | -       | -       | -       | -      |
|                          | 32      | -    | -      | -       | -       | -       | -       | -       | -       | -      |
|                          | 64      | -    | -      | -       | -       | -       | -       | -       | -       | -      |
|                          | 128     | 1    | 1      | 1       | 1       | -       | -       | -       | -       | -      |
|                          | 256     | 1    | 1      | 1       | 2       | 3       | -       | -       | -       | -      |
|                          | 512     | 1    | 1      | 1       | 4       | 1       | 3       | -       | -       | -      |
|                          | ≥1,024  | 1    | 1      | 1       | 4       | 1       | 3       | -       | -       | -      |

³ Tests conducted by the method of Halonen et al. (10) by using 0.25% suspension of chick erythrocytes.

³ Number of sera.

³ Reference marks for titers which would show complete correlation.

preparation (Flow Laboratories) with a titer of 1:160. Antigen 322 was prepared by T-E treatment of unconcentrated, infected BHK-21 culture fluids; this antigen initially had a titer of 1:32, but upon prolonged storage the titer had dropped to 1:8. In Table 12, it is seen that the highest-titered antigen (525) gave the lowest antibody titers as compared to the commercial antigen with a slightly lower titer, and even compared to the very low-titered antigen which had lost HA activity on storage, and thus might be expected to contain nonhemagglutinating particles which could compete for antibody.

To further compare the sensitivity of different rubella HA antigens for detecting HI antibody, several different types of antigen preparations were examined in parallel HI tests by using three different diluent systems. The antigens compared were unconcentrated, infected TC fluid, untreated and T-E-treated; a hemagglutinin preparation concentrated 10-fold by high-speed centrifugation, untreated and T-E-treated; a 100-fold concentrate, untreated; and an alkaline buffer extract, untreated. Results are shown in Table 13.

In the diluent system of Halonen et al., the highest antibody titers were obtained with the 10-fold concentrate, untreated. Both unconcentrated and concentrated T-E-treated antigens gave lower antibody titers than did corresponding untreated antigens. The antigen concentrated 100-fold gave lower antibody titers than did the 10-fold concentrate.

In the ADGP diluent, the 10-fold and 100-fold concentrated, untreated antigens gave comparable HI antibody titers, and the T-E-treated antigens gave HI titers nearly comparable to those of untreated antigens. The alkaline buffer extract gave unsatisfactory results in the ADGP system.

In the HEPES diluent, apparent false-positive reactions were again seen with some sera which lacked rubella-neutralizing antibody and gave negative reactions in other HI test systems. Antibody titers demonstrated with all of the antigens tended to be higher in the HEPES diluent than in the other two systems. There was less correlation between neutralizing antibody levels and HI antibody levels in the HEPES system than in the other two diluent systems.

In addition to reliability in demonstrating the presence or absence of antibody, another important quality of a rubella HA antigen is its sensitivity in detecting significant increases in HI antibody for diagnosis of rubella infections. Table 14 shows that rubella HA antigens may vary greatly in their ability to demonstrate significant HI titer rises in rubella infections. The antigens employed for this comparison were the three
TABLE 13. Effect of type of antigen on rubella (HI) antibody titers

| Diluent system                     | Serum no. | Neut - xation titers | HI antibody titer vs. antigen | Range of HI titers |
|-----------------------------------|-----------|-----------------------|------------------------------|-------------------|
|                                   |           | Uncon - treated       | T-E treated                  |                   |
|                                   |           | Uncon - treated       | T-E treated                  |                   |
|                                   |           | Uncon - treated       | T-E treated                  |                   |
|                                   |           | Uncon - treated       | T-E treated                  |                   |
| PBS-BABS (Halonen et al.)         | 2166      | <4                    | <8                           | <8                |
|                                   | 2510      | <4                    | <8                           | <8                |
|                                   | 2911      | <4                    | <8                           | <8                |
|                                   | 3812      | <4                    | <8                           | <8                |
|                                   | 3868      | 4                     | 16                           | 8                 |
|                                   | 2711      | 8                     | 16                           | 16                |
|                                   | 0472      | 16                    | 32                           | 16                |
|                                   | 0717      | 32                    | 64                           | 16                |
|                                   | 0855      | 64                    | 64                           | 16                |
|                                   | 0573      | 128                   | 128                          | 128               |
| ADGP (Dold and Northrop)          | 2166      | <4                    | <8                           | <8                |
|                                   | 2510      | <4                    | <8                           | <8                |
|                                   | 2911      | <4                    | <8                           | <8                |
|                                   | 3812      | <4                    | <8                           | <8                |
|                                   | 3868      | 4                     | 16                           | 16                |
|                                   | 2711      | 8                     | 16                           | 32                |
|                                   | 0472      | 16                    | 32                           | 64                |
|                                   | 0717      | 32                    | 64                           | 128               |
|                                   | 0855      | 64                    | 64                           | 128               |
|                                   | 0573      | 128                   | 128                          | 256               |
| HEPES, pH 6.2, 0.1% FBS           | 2166      | <4                    | 8                            | 8                 |
|                                   |           |                       | 8                            | 8                 |
|                                   | 2510      | <4                    | <8                           | <8                |
|                                   |           |                       | <8                           | <8                |
|                                   | 2911      | <4                    | <8                           | <8                |
|                                   |           |                       | <8                           | <8                |
|                                   | 3812      | <4                    | <8                           | <8                |
|                                   |           |                       | <8                           | <8                |
|                                   | 3868      | 4                     | 128                          | 128               |
|                                   | 2711      | 8                     | 256                          | 128               |
|                                   | 0472      | 16                    | 256                          | 64                |
|                                   | 0717      | 32                    | 256                          | 128               |
|                                   | 0855      | 64                    | 256                          | 128               |
|                                   | 0573      | 128                   | 512                          | 512               |

a Sera absorbed with kaolin at pH 9.0 to remove nonspecific inhibitors. Tests conducted with a 0.25% suspension of chick erythrocytes.

b Hemagglutinins concentrated from infected TC fluid by high-speed centrifugation.

c I t icalized numbers represent probable false-positive reactions.

d Antigens C961242 and 322 gave relatively high titers with the acute-phase sera, whereas antigen 525 gave low acute-phase titers; this lower sensitivity for demonstrating early antibody enhanced the diagnostic value of this particular antigen.

The relative diagnostic value of different types of antigens was further studied by using various types of antigens all prepared from the same lot of infected BHK-21 cell cultures. These were unconcentrated fluids, untreated and T-E-treated; 10-fold concentrated antigens, untreated and T-E-treated; and an alkaline buffer extract of the infected cells, untreated and T-E-treated. Each antigen, at a dilution containing 4 HA units, was
TABLE 14. Comparison of the reliability of three different hemagglutinating (HA) antigens for diagnosis of rubella infection

| No. of patients showing ≥ 4X CF rise | No. of patients showing ≥ 4X HI titera with HA antigens | Antigen C961242 | Antigen 322 | Antigen 525 |
|--------------------------------------|--------------------------------------------------------|-----------------|-------------|-------------|
|                                      | Antigen C961242 | Antigen 322 | Antigen 525 |
|                                      | 4             | 6            | 9            |
|                                      | 13            |              |              |

a Tests were conducted on paired sera from 13 patients showing fourfold or greater increases in rubella CF antibody.

b Tests performed by the method of Halonen et al. (10) by using a 0.25% suspension of chick erythrocytes.

c All antigens were used at dilutions containing 4 HA units.

TABLE 15. Examples of differences in hemagglutination-inhibition (HI) antibody titers detected with three different antigena

| Patient | Serum, days after onset | CF titer | HI antibody titer vs. units antigen |
|---------|-------------------------|----------|-----------------------------------|
|         |                         |          | Lot C961242 | Lot 322 | Lot 525 |
| Str.    | 5                       | <4       | 256         | 64     | 16     |
|         | 27                      | 16       | 512         | 128    | 64     |
| Hir.    | 3                       | <4       | 1,024       | 512    | 128    |
|         | 27                      | 8        | 1,024       | 1,024  | 512    |
| Pis.    | 7                       | 4        | 256         | 128    | 32     |
|         | 20                      | 16       | 512         | 512    | 128    |
| Cas.    | 5                       | <4       | 512         | 256    | 16     |
|         | 16                      | 8        | 1,024       | 512    | 128    |
| Mat.    | 3                       | <4       | 64          | 32     | 8      |
|         | 18                      | 16       | ≥1,024      | 1,024  | 512    |

a Tests, conducted by the method of Halonen et al. (10) by using chick erythrocytes, on paired sera from five patients showing ≥ fourfold increases in rubella CF antibody titer.

tested against paired sera from 11 patients with clinical rubella who showed fourfold or greater increases in rubella CF antibody.

Table 16 shows that concentrated antigens and alkaline extracts gave slightly higher titers than those obtained with unconcentrated antigens, and that untreated antigens generally gave higher antibody titers than did corresponding T-E-treated antigens. However, T-E-treated antigens detected a greater number of significant antibody titers. The antigen concentrated 10-fold by high-speed centrifugation and treated with T-E was the most sensitive for serological diagnosis of infection. This antigen was prepared in the same manner as antigen 525, which was also found to be highly sensitive for serological diagnosis of infection.

The increased diagnostic value of the T-E-treated antigens appeared to be related to a decreased sensitivity of the antigens for early antibody; differences in titers with untreated and T-E-treated antigens were not so marked for convalescent-phase sera.

DISCUSSION

Although pigeon erythrocytes sometimes gave twofold higher antigen titers than those obtained with chicken erythrocytes (1-day-old chicks), this slight difference in sensitivity was not reflected in higher antibody titers in HI tests with pigeon erythrocytes. On the other hand, goose erythrocytes gave antigen titers at least fourfold lower than those obtained with pigeon and chick erythrocytes, and the need to use larger amounts of antigen with goose erythrocytes resulted in lower HI antibody titers. Cell counts on 0.25% suspensions of chick and goose erythrocytes showed that they contained approximately equal numbers of cells. Thus, lower antigen titers obtained with goose erythrocytes would appear to be due to lower sensitivity to agglutination rather than to differences in the number of cells in the indicator suspensions. It has been suggested that goose erythrocytes may be used at a lower concentration of 0.08% to give more sensitive tests (H. Liebhaber, personal communication). Considerable variation was noted in the suitability of erythrocytes from different geese for use in rubella HA and HI tests, and another drawback to the use of goose erythrocytes was their tendency to give rough agglutination patterns.

It has been noted in this laboratory that human sera contain higher levels of natural agglutinins for pigeon erythrocytes than for chick erythrocytes, and these agglutinins are often difficult to absorb from test sera, even with large volumes of erythrocytes and prolonged incubation periods. For this reason erythrocytes from 1-day-old chicks, rather than pigeon erythrocytes, are used routinely. Also, erythrocytes from different pigeons may vary in sensitivity to agglutination by the virus.

The composition of the diluents was found to have a marked effect on HA antigen titers and to a lesser extent upon antibody titers. In all buffer systems compared, antigen titers were higher at pH 6.2 than at 7.2, but other differences in composition of the diluents also affected antigen titers. Although hemagglutination by rubella virus has been reported to be dependent upon the
### Table 16. Comparison of the diagnostic value of different types of antigen preparations

| Patient | Serum, days after onset | CF titer | HI antibody titer vs. antigen* | Range of HI titers |
|---------|-------------------------|----------|--------------------------------|-------------------|
|         |                         |          | Unconcentrated fluid | Concentrated 10X | Alkaline extract of cells |
|         |                         |          | Untreated | T-E | Untreated | T-E | Untreated | T-E |
| Str.    | 5                       | <4       | 32       | 16 | 64       | 16 | 128       | 32 | 16-128 |
|         | 27                      | 16       | 128      | 32 | 128      | 64 | 128       | 128 | 32-128 |
| Hir.    | 3                       | <4       | 128      | 64 | 256      | 64 | 256       | 128 | 64-256 |
|         | 27                      | 8        | 256      | 128 | 512      | 256 | 256       | 128 | 256-512 |
| Cas.    | 5                       | <4       | 64       | 8  | 64       | 16 | 64        | 32  | 8-64   |
|         | 16                      | 8        | 128      | 64 | 128      | 128 | 256       | 128 | 64-256 |
| Val.    | 7                       | <4       | 64       | 32 | 128      | 32 | 128       | 64  | 32-128 |
|         | 20                      | 16       | 128      | 64 | 256      | 128 | 256       | 128 | 64-256 |
| Sig.    | 4                       | <4       | 64       | 8  | 64       | 8  | 32        | 8   | 8-64   |
|         | 21                      | 32       | 256      | 128 | 512      | 256 | 512       | 256 | 128-512 |
| Mat.    | 3                       | <4       | 32       | 8  | 32       | 8  | 32        | 8   | 8-32   |
|         | 18                      | 16       | 256      | 64 | 256      | 128 | 256       | 256 | 64-256 |
| Kea.    | 7                       | <4       | 32       | 8  | 32       | 8  | 32        | 8   | 8-32   |
|         | 23                      | 32       | 64       | 32 | 64       | 32 | 64        | 32  | 32-64  |
| Wit.    | 2                       | <4       | 16       | <8 | 16       | <8 | 16        | <8  | <8-16  |
|         | 15                      | 32       | 128      | 128 | 256      | 256 | 512       | 256 | 128-512 |
| O'Le.   | 2                       | <4       | 8        | <8 | 8        | <8 | 8         | <8  | <8-8   |
|         | 18                      | 16       | 128      | 64 | 128      | 128 | 128       | 128 | 64-128 |
| Red.    | 3                       | <4       | 8        | <8 | 8        | <8 | 8         | <8  | <8-8   |
|         | 20                      | 16       | 128      | 64 | 128      | 128 | 128       | 128 | 64-128 |
| Tri.    | 4                       | <4       | 128      | 32 | 128      | 32 | 128       | 32  | 32-128 |
|         | 12                      | 16       | 128      | 32 | 256      | 64 | 128       | 64  | 32-256 |

No. of patients showing ≥ fourfold increase in HI antibody titers

| Serum, days after onset | CF titer | HI antibody titer vs. antigen* | Range of HI titers |
|-------------------------|----------|--------------------------------|-------------------|
|                         |          | Unconcentrated fluid | Concentrated 10X | Alkaline extract of cells |
|                         |          | Untreated | T-E | Untreated | T-E | Untreated | T-E |

*Tests were conducted by the method of Halonen et al. (10) by using a 0.25% suspension of chick erythrocytes.

*All antigens were prepared from the same lot of infected BHK-21 cell cultures.

*Hemagglutinins were concentrated from infected TC fluid by high-speed centrifugation.

Presence of Ca²⁺ ions (1, 6, 9), diluents containing 10⁻² to 10⁻³ M Ca²⁺ did not consistently give higher antigen titers than those obtained in the PBS-BABS diluent lacking Ca²⁺. The effect of the composition of diluents on FA titers varied with different types of antigen preparations.

In some instances, different diluents also gave widely varying antibody titers for the same serum specimens. Sera had similar titers in the PBS-BABS, DGV, and ADGP diluent systems, despite differences in the concentration of antigen required for each system. In the MBA-NIH diluent, and particularly in HEPES diluent at pH 6.2, antibody titers were higher than those obtained in other diluents, and in some instances false-positive inhibition was seen. Even in comparisons for which antigen could be used at similar dilutions in each system, antibody titers were consistently higher in the HEPES diluent. Further, sera with low and with high neutralizing antibody titers for rubella virus frequently showed similar, high HI titers in the HEPES diluent, whereas in other diluents the same sera showed HI titers which correlated more closely with neutral-
izing-antibody titers. Thus, certain types of diluents may give overly sensitive HI antibody assays, resulting in false-positive reactions and in inappropriately high antibody titers. In selecting a diluent system for rubella HI tests, reliability in detecting the presence or absence of antibody should be a more important consideration than ability to demonstrate high antibody titers.

It is noteworthy that indicator erythrocytes formed rough agglutination patterns in certain diluents containing bovine albumin and gelatin, making it extremely difficult to read antigen and antibody end points.

Treatment of sera with reagents which selectively precipitate the inhibitory beta-lipoprotein fraction (heparin-MnCl₂ or dextran sulfate-CaCl₂) would appear to be a more rational approach to removal of inhibitor than absorption with kaolin, which is known to remove certain classes of immune globulins. At present, however, methods for treatment with these reagents are not well standardized and certain problems have been encountered. Treatment with too low a concentration of the reagents may fail to effectively remove inhibitor, whereas treatment with higher concentrations may reduce specific antibody levels. Further, the reagents may produce precipitates with phosphate buffers used as diluents for serum, antigen, or erythrocytes. In the present studies, kaolin used at pH 9.0 effectively removed inhibitor from sera, and HI antibody titers of sera treated with kaolin at this pH were as high or higher than those of the same sera treated with heparin-MnCl₂ or dextran sulfate-CaCl₂. In other studies (3, 5, 7, 16), comparing the effect of kaolin and heparin-MnCl₂ on rubella antibody, the kaolin was used at a lower pH, and this may account in part for the lower antibody titers seen in some of the kaolin-treated sera.

Although kaolin may remove rubella IgM antibody, this is rarely a problem, except perhaps in demonstrating antibody in certain congenital infections. The removal of IgM antibody from acute-phase sera in postnatal rubella infections may actually enhance the diagnostic value of the test, as it may permit the demonstration of a diagnostically significant titer increase between the acute-phase serum (containing IgM antibody) and the convalescent-phase serum containing relatively greater amounts of IgG antibody. Antibody elicited by past rubella infections is IgG in nature, which is little affected by kaolin absorption at pH 9.0, and therefore kaolin treatment would appear to be satisfactory for use in HI tests to determine immunity status to rubella. Continuing efforts should be made to standardize a method for selective removal of rubella inhibitor, permitting assay of IgM antibody for certain purposes. Until such a method is available, however, absorption with kaolin at pH 9.0 appears to be a satisfactory method for removal of inhibitor if the limitations and pitfalls in the use of kaolin are recognized. Only acid-washed kaolin should be employed, and, since different batches may vary in effectiveness for removal of inhibitors, pretested kaolin should be used.

Although reasons for differences in sensitivity of rubella HA antigens were not fully elucidated, certain points were clarified. The sensitivity of an antigen for assay of antibody was found not to be related to its HA titer, but appeared to be related instead to the manner in which the antigen was prepared. The sensitivity of antigens for assay of HI antibody also varied in different diluent systems. In general, untreated antigens gave higher antibody titers than did corresponding T-E-treated preparations. Also, antigens concentrated 10-fold by high-speed centrifugation and antigens prepared by alkaline buffer extraction of infected cells tended to give higher antibody titers than did antigens derived from unconcentrated tissue culture fluids.

It was shown that the sensitivity of an antigen for demonstrating high antibody titers was not related to its diagnostic value in detecting significant HI antibody increases in rubella infections. In fact, certain antigens which gave high antibody titers were the least useful for serological diagnosis. T-E-treated antigens were shown to be more reliable than corresponding untreated antigens for demonstrating significant increases in antibody, and this appeared to be related to a lower sensitivity of the T-E-treated antigens for detecting early antibody in the acute-phase sera. The sera used for studies on comparative sensitivity of different antigens were all treated with kaolin, which removes most of the IgM, so it seems unlikely that differences in sensitivity of untreated and T-E-treated antigens for detecting early antibody reflect differences in avidity for IgM immunoglobulins. Further, it appears that T-E treatment is not the only factor which determines the diagnostic value of rubella antigen, since all three antigens used for the studies summarized in Tables 14 and 15 were T-E-treated, and two of them were relatively insensitive in demonstrating significant antibody titer rises.

Although T-E-treated hemagglutinin preparations concentrated 10-fold from infected culture fluids may give lower antibody titers than certain other antigens, extensive experience in our diagnostic laboratory has shown that they are equally reliable for detecting the presence or absence of antibody (determination of immunity status) and they may be more reliable than certain other types
of antigens for serological diagnosis of rubella infections.

The widely varying antigen and antibody titers obtained with different test systems emphasize the pressing need for standardization of the rubella HI test.

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