Large-Scale Production of Rubella Precipitinogens and Their Use in the Diagnostic Laboratory

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A method for producing large quantities of rubella theta and iota precipitating antigens in Vero cells is described. Using this reagent, the detection of anti-theta antibodies was found to be as sensitive as the hemagglutination inhibition test for both the determination of immune status and the diagnosis of acute rubella infection. The detection of anti-iota antibodies may permit diagnosis of rubella infection, when the collection of the first serum has been delayed and the early rise of hemagglutinin-inhibiting and anti-theta antibodies has been missed.

Soluble rubella antigens detectable by immunodiffusion have been described by several investigators (5, 14, 15). This study is concerned with the two antigens designated theta and iota by Le Bouvier (5). In patients with rubella, anti-theta antibody rises promptly and can be detected for years, whereas anti-iota is made more slowly and declines sooner. Precipitin responses have proved to be useful parameters for evaluation of vaccine-induced immunity (3, 8).

Despite their diagnostic potential and simplicity of execution, tests for precipitating antibodies have not been adopted for routine use because of difficulties in obtaining suitable reagents. This report describes a method for concentrating the antigens from rubella virus-infected cell culture fluids. Concentrated theta or iota antigens were used in conjunction with immune sera for serological surveys and for testing patients' sera following natural infection. Immunodiffusion titers are compared with titers of rubella hemagglutinin-inhibiting (HAI), complement-fixing (CF), and, in some cases, neutralizing antibodies.

MATERIALS AND METHODS

Virus. Wild-type rubella virus, strain Therien, was used. The virus had been isolated in Vero monkey kidney cells from the serum of an adult woman with clinical rubella and had been passaged at least 10 times in this cell line (10).

Cells. In preliminary experiments virus and antigen production were compared in several cell systems: primary rabbit kidney cells, continuous lines of BHK-21 hamster, pig kidney, and Vero monkey kidney cells. Vero cells were found to give the highest titers of virus, hemagglutinin, and precipitinogens. Yields with decreasing potency were obtained in BHK-21, primary rabbit kidney, and pig kidney cells, in that order.

Virus was grown as previously described (6, 11). Roller bottles covered with a confluent monolayer of Vero cells (2 × 10⁴) were infected with a virus input of 1 to 10 mean tissue culture infective doses per cell, and the virus was allowed to adsorb for 4 h at 37 C. The medium was then replaced with 100 ml of Eagle minimal essential medium containing 5% kaolin-treated newborn calf serum and 100 U of penicillin, 100 µg of streptomycin, and 10 U of nystatin per ml.

Beginning on the fourth day after inoculation, the medium was harvested daily for 6 days and replaced with fresh medium. The harvested medium was centrifuged at low speed to remove cellular debris and stored at -70 C.

Antigen concentration and purification. Initial steps were according to the method of Liebhaber and Gross for purifying virus (10). Harvested tissue culture fluid (3,600 ml) was gradually precipitated with 50% (wt/vol) ammonium sulfate at 4 C, maintaining the pH at 7.4 with 1 N NaOH. This suspension was shaken for 1 h and centrifuged at 3,000 rpm for 20 min, and the precipitate was dissolved in 250 ml of TS buffer [0.01 M tris(hydroxymethyl)aminomethane, pH 7.4, 0.1 M NaCl]. The antigen was precipitated again with 30% ammonium sulfate; after centrifugation, the precipitate was dissolved in 35 ml of TS buffer. This suspension was then layered on a discontinuous sucrose gradient prepared in a cellulose nitrate tube for the Beckman SW25.2 rotor. The gradient was prepared by overlaying from bottom to top, 2 ml of 60% (wt/wt), 4 ml of 50%, 5 ml of 40%, and 10 ml of 30% sucrose in TS. After a 24-h centrifugation at 22,000 rpm at 4 C, 1-ml fractions were collected by piercing the bottom of the tube. Each fraction was examined for infectivity, hemagglutination, and theta and iota antigens. The identity of the immunoprecipitin lines was confirmed by comparison with standards kindly provided by George Le Bouvier.
fractions containing the viral particles were pooled and used for preparation of nucleocapsid antigen as described below. The fractions containing the precipitating antigens were also pooled, dialyzed 48 h against TS at 4°C, and concentrated 5- to 10-fold by forced dialysis against Aquacide. The pooled concentrate (0.5 ml) was then placed on a 4.5-ml, 20 to 60% (wt/wt) continuous sucrose gradient. The tubes were centrifuged at 35,000 rpm for 1 h at 4°C in a Beckman SW50.1 rotor, and 0.3-ml fractions were collected, dialyzed against TS buffer for 24 h, and then tested for hemagglutinins and precipitating antigens.

**Theta- and iota-specific immune sera.** A CF-negative serum from a patient who experienced rubella 2 years previously (HAI, 64; anti-theta, 8 U/μl; anti-iota, 0 U/μl) gave a sharp theta immune precipitate's leading antigen pool and was selected as the standard for anti-theta antibody. The anti-iota serum (HAI, 8; anti-theta, 0 U/μl; anti-iota, 2 U/μl) was prepared in a rabbit hyperimmunized with the nucleocapsid fraction of Nonidet P-40-treated purified rubella virus, using a modification of the method of Liebhaber described in a recent paper (1). Instead of disrupting the virus prior to separation of its components by density gradient centrifugation, we sedimented the virus through a zone of detergent layered over the gradient. Tubes for the SW50.1 rotor were prepared as follows. A 3-ml continuous sucrose gradient (20 to 60%, wt/wt) in TS-buffered D2O containing 0.01 M MgCl₂ was prepared. One milliliter of 10% Nonidet-P40 in TS buffer containing 0.01 M MgCl₂ was layered over the gradient. Dialed purified virion fractions were then placed on top, and the gradients were centrifuged at 45,000 rpm for 4 h at 4°C. The visible nucleocapsid band, which formed at a density of about 1.25 g/ml, was collected through the side of the tube by means of a needle and syringe.

**Immunodiffusion.** Tests were done on glass slides (75 by 25 mm) covered with 2.5 ml of gel consisting of 0.4% agarose in 0.01 M TS buffer and 0.1% sodium azide. Aliquots (20 μl) of reactants were placed in wells 5 mm in diameter and 3 mm apart. The slides were incubated in moist chambers at room temperature, and the results were read after 24 h.

**Quantitation in units.** This was done according to the technique described by Le Bouvier (4). Serial dilutions of standard antigen and antibody prepared as described above were reacted against each other to determine the equivalence zone for each system (theta or iota). The change in relative position of the precipitate's leading edge corresponding to a given change in concentration of one or other reactant was noted. The highest dilution of the standard antibody reactant which still gave a distinct band of precipitate in the equivalence zone with any dilution of the homologous standard antigen reactant was taken as containing 1 U of the antibody per μl. That dilution of the antigen reactant with which this band of precipitate was obtained was defined as containing 1 U of antigen per μl. Because the standard units are defined as concentrations giving a line in the equivalence zone, lower concentrations of reactants can be detected, and these are reported as fractions of a unit.

In a given test, unknown antisera were compared with one or two dilutions of the standard antibody reactant with respect to the position of the precipitate that formed with 2 U of standard antigen reactant per μl. On the basis of this comparison, the titer of a particular serum (units per microliter) was assigned.

**Titration of hemagglutinin and HAI antibody.** Tests were done by the method developed by Liebhaber, using dextran-sulfate and calcium chloride to remove nonspecific inhibitors from sera (9).

**Titration of CF antibody.** Levels of CF antibody were determined by the standard microtitration technique, employing 4 U of rubella soluble antigen. The antigen was prepared as described above for theta and iota, except that the second gradient separation was omitted.

** Infectivity and neutralizing antibody assays.** These were done by the interference test described previously (13).

**Serum specimens.** A total of 658 sera were used to compare the immunodiffusion tests with the HAI test for determining rubella immune status. These sera came from several sources, but the majority were collected during field trials of rubella vaccines conducted in Danbury, Connecticut (2, 12). Among these are prevaccination sera and specimens obtained over a 5-year period from 55 naturally immune children, identified in the study mentioned above. The balance comprises clinical specimens submitted to our laboratory for determination of rubella antibody levels.

To follow the appearance of anti-theta and anti-iota antibodies after clinical rubella, serial serum specimens were collected from 25 patients during the 4-week period post-onset.

**RESULTS**

Table 1 summarizes the steps in the concentration and purification procedure. A 3,600-ml amount of tissue culture fluid with no detectable precipitating antigen activity was concentrated 2,000-fold to yield a preparation with titers of 40 and 36 U/μl for theta and iota, respectively. Figure 1 shows that after the first centrifugation there was a considerable overlap between the hemagglutinin and the precipitating antigen activities, but after the second high-speed centrifugation (Fig. 2) good separation was obtained. Using this procedure, the theta and iota antigens were always found in the same fractions, as would be expected from their previously determined sedimentation characteristics (6). When antigens so prepared were tested by immunodiffusion against rubella early convalescent sera, only two lines of reaction, specific for theta and iota, were observed. Since in the test procedure for the determination of the precipitating antibodies only 20 μl of the antigen reactant (2 U/μl) is needed to test four sera for each specificity, the amount prepared from 3,600 ml provided enough to test about 2,000 sera for both anti-theta and anti-iota.
antibodies. Virus harvests varied in potency, but those with hemagglutinin titers of 512 or more regularly were good sources of precipitins.

Antibodies to theta and iota as a measure of immune status. Among the 658 sera tested for HAI antibodies, 304 had titers lower than 8. This high percentage of seronegative subjects is explained by the fact that 30% of the sera were from children 3 to 8 years of age. Sera from these 304 subjects were also found to be negative for rubella CF antibodies, but 12 (4%) were found to have low titers of anti-theta antibodies (geometric mean titer [GMT], 1.2 U/μl). These 12 sera contained low levels of neutralizing antibodies (GMT, 7) as well.

Table 2 shows the precipitin results for the 354 subjects who had HAI titers higher than 8. A fairly good correlation could be observed between the HAI and the anti-theta antibodies. In this group only two had no detectable anti-theta antibody, but both of these sera were positive by neutralization test as well as by HAI. These results indicate that detection of anti-theta antibody in the evaluation of rubella immunity is as sensitive as the HAI test. In contrast, anti-iota antibodies were found in only 48%, but increased in frequency in persons with higher HAI titers.

Anti-theta antibodies seem to persist for a long period without any significant change in titer. In 55 children from whom serial blood specimens were collected over a 5-year period, no important changes in titer could be detected. The GMT was 4.6 U/μl at the first bleeding and 4.3 and 4.1 U/μl after 3 and 5 years, respectively.

Precipitating antibodies after acute natural rubella. The results for 25 patients studied are shown in Fig. 3. It is clear that anti-theta and HAI antibodies follow a similar time course.

Table 1. Procedure for the concentration and purification of rubella precipitating antigens

<table>
<thead>
<tr>
<th>Steps</th>
<th>Vol (ml)</th>
<th>Theta (U/μl)</th>
<th>Iota (U/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude medium</td>
<td>3,600</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>First precipitation</td>
<td>250</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>Second precipitation</td>
<td>36</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Overnight dialysis against TS buffer</td>
<td>36</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Centrifugation on a discontinuous sucrose gradient</td>
<td>20</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Overnight dialysis against TS buffer and forced dialysis with Aquacide</td>
<td>2</td>
<td>48.0</td>
<td>36.0</td>
</tr>
<tr>
<td>Centrifugation on linear sucrose gradient</td>
<td>1.8</td>
<td>48.0</td>
<td>36.0</td>
</tr>
</tbody>
</table>

Fig. 1. Distribution of rubella hemagglutinin (HA; •) and theta antigen (O) after preliminary centrifugation on the discontinuous sucrose gradient.

Fig. 2. Distribution of rubella hemagglutinin (HA; •) and theta antigen (O) after final separation on linear sucrose gradient.
of development, as shown previously by Le Bouvier (7). At the time of appearance of the rash, 36% of the subjects had demonstrable HAI and 27% had anti-theta antibodies. Both tests showed that all had converted by the second day post-rash.

The development of the anti-iota response clearly was delayed by comparison. In 63% of the cases the antibodies became demonstrable 2 weeks after onset, with a GMT of 1.8 U/µl, and 100% conversion was reached only after 4 weeks. Maximal titerS were attained 6 to 8 weeks after onset, with a GMT of 3 U/µl.

**DISCUSSION**

Rubella precipitating theta and iota antigens can be prepared and purified from infected Vero cells. Titers are sufficiently high so that adequate amounts of diagnostic reagents can easily be obtained. These antigens also can be produced in pig kidney cells and in BHK-21 cells, but results with the latter were not reproducible. The use of an antigen preparation containing both theta and iota reactivities did not present a problem, since monospecific antisera were employed in the tests. For simplicity, however, it would be desirable to develop preparative methods for separation of the two antigens.

Our data show that the detection of theta antibodies can be useful in the evaluation of immune status as well as in the diagnosis of rubella infections. The detection of these antibodies was sometimes more sensitive than the HAI test, since 4% of the samples which were negative by CF and HAI were found to contain neutralizing and anti-theta antibodies.

The diagnosis or exclusion of recent rubella infection is a frequent problem, especially during pregnancy. This problem cannot always be solved with the serological methods usually utilized. This is particularly true if the collection of the first specimen has been delayed, since in this case the rapid rise of HAI and anti-theta antibodies may be missed. For additional diagnostic aid in these cases, the demonstration of a rise in CF antibodies can be used, because these antibodies develop more slowly than the HAI. However, it is important to use soluble rubella CF antigen for such tests, as the CF antibody response to virion or large-particle antigen mimics the HAI response in time course. The slow development of anti-iota antibodies makes them equally useful as an aid to diagnosis when serum specimens are obtained more than 2 weeks post-onset (3, 5, 7). Of course, demonstration of rubella-specific immunoglobulin M by any of these methods provides definitive confirmation of recent infection.

We conclude that the detection of rubella precipitating antibodies can be applied routinely in the diagnostic laboratory. The tests are ideally suited for this purpose, because they are easy to perform, require little time, and are inexpensive and reproducible.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

phase protein found in association with rubella virus infection. J. Exp. Med. 139:497–511.