

Rubella Antibodies Detected by Several Commercial Immunoassays in Hemagglutination Inhibition-Negative Sera

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Although a very good correlation was found between the level of rubella antibodies measured by a standard hemagglutination inhibition (HI) test and by an enzyme-linked immunosorbent assay (ELISA) procedure (Cordia R), an appreciable proportion (31%) of ELISA-positive specimens were encountered among HI-negative sera. The reverse was rarely seen. Many of the HI-negative, ELISA-positive sera were also found to be positive for rubella antibodies by one or more other assay methods, including an immunofluorescence assay (IFA) procedure (FIAX), passive hemagglutination (PHA) (Rubacell and PHAST), latex agglutination (Rubascan), and a second ELISA procedure (Rubelisa). The specificity of all of the ELISA-positive HI-negative specimens was substantiated by absorption experiments. In these tests, the ELISA reactivities were blocked by rubella antigens, but not by a variety of tissue culture control antigens or by influenza virus grown on the same cell line. The findings indicate that many of the newer methods available for rubella antibody detection are more sensitive than HI for detecting low levels of rubella antibodies. Until more clinical information is available concerning the protective nature of these low levels of antibody, caution should be exercised in assessing the significance of these results.

Although rubella (German measles) is usually a benign infection of children or young adults, its occurrence in women during early pregnancy causes a high frequency of congenital abnormalities of the newborn (13, 15). The current strategy for rubella control is based on routine vaccination of all children ≥ 12 months of age, vaccination of all school children not immunized in infancy, and vaccination of susceptible adults, particularly females and hospital personnel (5). The determination of immune status by the detection of specific antibody is often used to detect susceptible adults and can, therefore, be of considerable importance. The hemagglutination inhibition (HI) test (14) has been considered the standard assay against which a variety of new commercial tests have been compared.

Commercial serological tests used in the studies reported in this paper include Cordia R (Cordis Laboratories, Miami, Fla.), Rubacell (Abbott Laboratories, North Chicago, Ill.), Rubella FIAX (International Diagnostic Technology, Santa Clara, Calif.), Rubascan (Hynson, Westcott and Dunning, Baltimore, Md.), Rubelisa (MA Bioproducts, Walkersville, Md.), and the PHAST test (Calbiochem-Behring, La Jolla, Calif.).

In this report, the performances of these assays are compared with a standard HI test and with each other. Particular emphasis was placed on sera which were HI negative ($<1:8$) or weakly positive ($1:8$ or $1:16$). In both this work and in reports by others (2, 6, 9-11, 16-18), a number of HI-negative sera ($<1:8$) have been found to be positive by many of the newer assays. In the present study, the specificity of these reactions for rubella antibodies was evaluated by comparing a number of different tests on the same sera, as well as by absorption or blocking experiments.

MATERIALS AND METHODS

Specimens. Five different serum panels were obtained and assayed for antibodies to rubella virus. Panel 1 consisted of 511 consecutive serum specimens which had been submitted to the Michigan Department of Public Health State Laboratory for routine serological testing (these sera were assayed by HI at Michigan and then coded and sent to Cordis Laboratories for Cordia R ELISA testing). Panel 2 contained 188 selected specimens which had been submitted to the North Carolina State Laboratory of Public Health for serological testing and included 87 HI-negative ($<1:8$, $\pm 1:8$) and 101 weakly HI-positive ($1:8$, $1:16$) sera. Panel 3 was also obtained from North Carolina and consisted of 130 selected sera, including 75 HI-negative ($<1:8$) and 55 weakly HI-positive ($1:8$, $1:16$) specimens. Panel 4 was made up of 26 HI-negative

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(<1:8) sera selected from Panel 3 (17 of these specimens were determined to be weakly positive for rubella antibody in previous ELISA tests). Panel 5 consisted of 90 specimens obtained from North Carolina and selected to include 45 HI-negative (<1:8) and 45 weakly HI-positive (1:8) sera.

Antibody tests. Non-kit HI tests were performed according to the U.S. Public Health Service publication *A Procedural Guide to the Performance of the Standardized Rubella Hemagglutination-Inhibition Test* (4), using the heparin-MnCl₂ treatment for removal of nonspecific inhibitors and chick erythrocytes as indicator cells. Where indicated, human O cells were substituted for chick erythrocytes. All other antibody tests were performed according to the instructions of the manufacturers.

Blocking experiments. Rubella virus used in the blocking assays (Gilchrist strain) was grown in baby hamster kidney cells (BHK-21) by the procedure of Halonen et al. (7), as adapted by the Centers for Disease Control, Atlanta, Ga. (K. Herrmann, personal communication). The cells and debris were removed by centrifugation at 14,000 × g for 15 min, after which the viral particles were harvested from the clarified supernatant fluid by ultracentrifugation at 113,000 × g for 1 h. The viral pellets were washed once with 0.1 M Tris buffer at pH 7.2 containing 0.1 mM EDTA and suspended in the same buffer at 1 to 2 mg of protein per ml, as determined by absorbance measurements at 280 and 260 nm (8). The concentrated viral suspensions were inactivated by exposure to Tween 80 and ethyl ether by the procedure of Norrby (12) and stored frozen at -20°C for later use.

Four preparations served as control antigens. Two of these were obtained from uninfected BHK-21 cultures. In the first, the spent culture medium was centrifuged at low speed (14,000 × g, 15 min), and the supernatant was used. In the second, these cell-free supernatants were subjected to ultracentrifugation (113,000 × g, 1 h) to obtain traces of a pellet which was used. This pellet was not obtainable in sufficient quantities for use in the absorption experiments. It was possible, however, to prepare disks coated with this material to serve as tissue culture antigen controls. The third control antigen was prepared by using rubella virus-infected culture supernatant fluid after the bulk of the virus had been removed by ultracentrifugation at 113,000 × g for 1 h. A fourth control antigen consisted of influenza virus (A/Victoria strain) grown on BHK-21 cells and processed as above for viral particles.

The absorption experiments were performed by preincubating the appropriately diluted serum samples for 45 min at 37°C with rubella antigen, tissue culture control materials, antigens prepared from influenza virus, or no antigen. After this, the normal Cordia R procedure was performed.

RESULTS

The results of parallel blind coded ELISA (Cordia R) and HI (human O cells) tests to determine the immune status of 511 individuals (panel 1) covering a wide range of titers are shown in Fig. 1. The correlation of the results between the two procedures was good ($r =$

0.905, $P < 0.001$). However, 10 of 54 (19%) HI-negative sera (<1:8) were positive by the ELISA procedure.

Similar parallel testing with the ELISA (Cordia R) assay was done on another panel of 188 specimens (panel 2), using primarily negative (<1:8 or ±1:8, sera with incomplete inhibition) and weakly positive sera (1:8 or 1:16) as determined by HI (chick erythrocytes) (Fig. 2). Once again, the overall correlation was good ($r = 0.832$, $P < 0.001$). However, of the 87 HI-negative sera, 34 (39%) ELISA-positive sera were encountered. In all of these discrepant cases, indicated by triangles in Fig. 2, the ELISA reactions were weakly positive. Among the 25 sera which showed a partial inhibition of hemagglutination in the HI test at the 1:8 dilution, 15 (60%) were positive by ELISA. On the other hand, of the 62 sera which were clearly negative by HI at 1:8 dilution, only 19 (31%) were ELISA positive. The probability that these differences were due to chance was 0.01. In addition, the median value of the ELISA-positive reactions in the sera which were clearly negative by HI was appreciably lower than the median of the ELISA-positive sera in the group

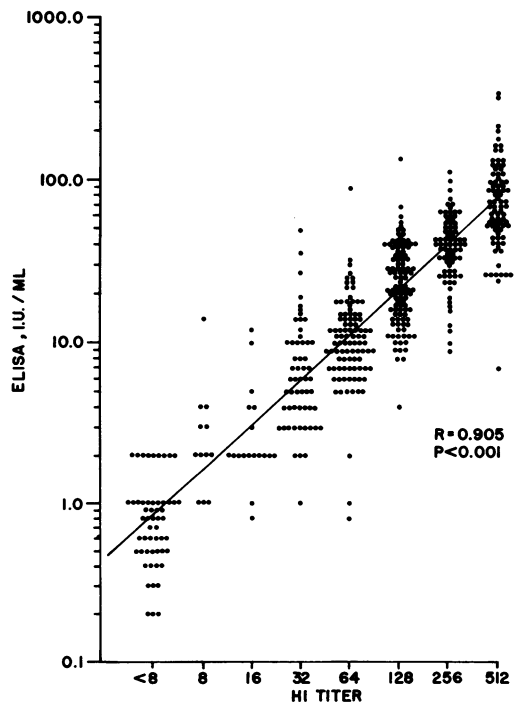


FIG. 1. Rubella antibodies (IU/ml) as determined by ELISA (Cordia R) and HI titrations (human O cells) in a blind study with serum specimens from 511 individuals. In this more sensitive version of the ELISA procedure, values ≥ 1 were generally indicative of the presence of rubella antibodies.

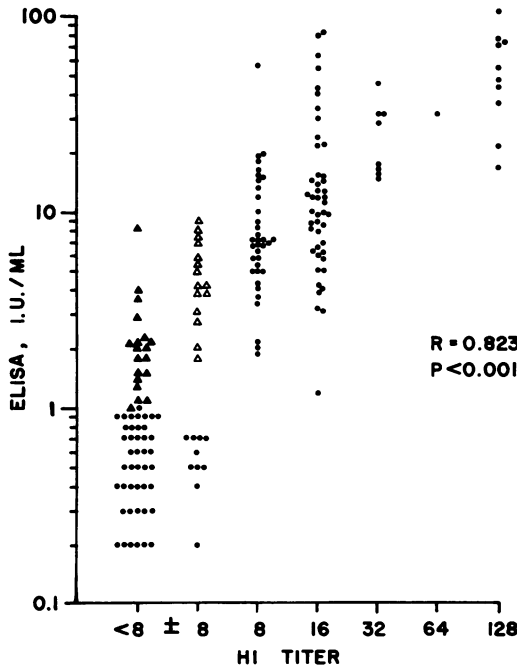


FIG. 2. Rubella antibodies (IU/ml) as determined by ELISA (Cordia R) and HI titration (chick erythrocytes) on 188 serum specimens which were negative or weakly positive by HI. Symbols: ▲, ELISA positive, HI negative (<1:8); △, ELISA positive, HI negative, with partial inhibition (\pm 1:8). In this more sensitive version of the ELISA procedure, values \geq 1 were generally indicative of the presence of rubella antibodies.

showing partial inhibition by HI at 1:8. In contrast to these results, specimens which were HI positive but ELISA negative were not encountered in this group of specimens.

To substantiate the specificity of the HI-negative, ELISA-positive tests, we retested specimens after absorption with either rubella antigen prepared from baby hamster kidney cell culture supernatant fluid or the control antigen prepared from uninfected cell culture supernatant fluid. The results of a typical absorption experiment are shown in Fig. 3. It can be seen that the reactivity of each positive specimen was blocked by exposure to rubella antigen. In contrast, absorption with control antigen prepared from uninfected spent tissue culture supernatant fluid had no appreciable effect on the majority of these specimens. Two specimens having weakly positive ELISA absorbance values (580 and 560) had values just below the cutoff value (470 and 460, respectively) after absorption with control antigen. As expected, exposure to rubella virus had no effect on the reactivity of the three negative specimens tested.

Even more significantly, similar results were obtained when the control antigen was rubella-infected tissue culture supernatant fluid from which the bulk of the rubella virus had been removed by ultracentrifugation. Once again, the reactivity of positive specimens was blocked by preexposure to rubella antigen but was not significantly affected by the presence of an equivalent amount of the ultracentrifuged control antigen (Fig. 4). Again, two borderline ELISA-positive specimens, which had absorbance values of 520 and 560, gave values just below the cutoff value (460 and 480, respectively), after absorption with control antigen. The drop in the latter absorbance values and the similar results presented for two weak-positive specimens in Fig. 3 are within the range of experimental error.

Finally, no blocking was observed when an influenza virus antigen was used for absorption. The influenza virus had been grown in the same line of cells used to prepare the rubella antigen. The influenza virus concentrates failed to inhibit the antirubella activity in the five ELISA-positive specimens tested. Two of these specimens

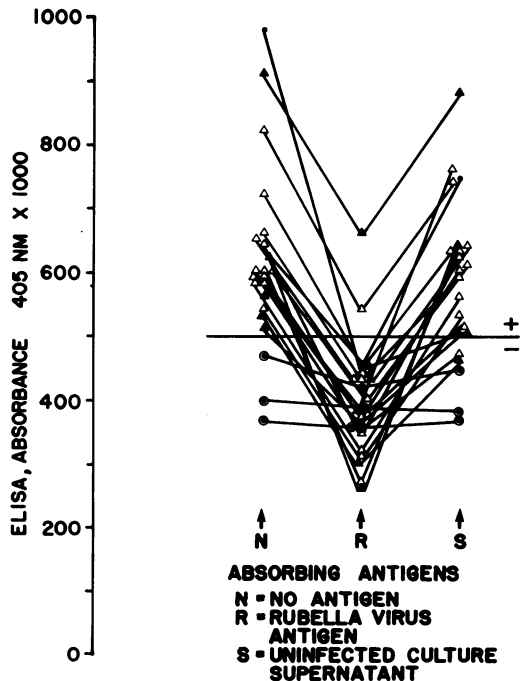


FIG. 3. Blocking of rubella antibodies in serum specimens positive by ELISA and HI (●), positive by ELISA and negative by HI with partial inhibition (△), positive by ELISA and negative by HI (▲), and negative by ELISA and HI (○). The specimens were preincubated before testing by ELISA with rubella antigen (R), uninfected BHK-21 culture supernatant (S), or no antigen (N).

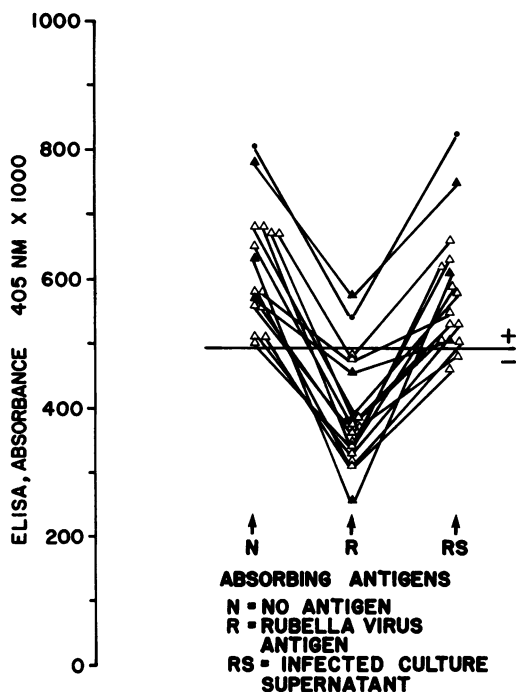


FIG. 4. Blocking of rubella antibodies in serum specimens positive by ELISA and HI (●), positive by ELISA and negative by HI with partial inhibition (△), and positive by ELISA and negative by HI (▲). The specimens were preincubated before testing by ELISA with rubella antigen (R), infected BHK-21 culture supernatant from which the cells and the bulk of the rubella virus had been removed by centrifugation (RS), or no antigen (N).

were strongly positive by HI and ELISA, and three were weakly positive by ELISA but HI negative. The ELISA activity in all five specimens was significantly inhibited by absorption with rubella antigen.

Conversely, six sera were tested by the normal Cordia ELISA assay, using BHK-21-derived influenza virus as antigen on the solid phase. As might be expected, these sera reacted variably, and their reactivity was unrelated to the rubella virus antibody titer. Absorption with influenza virus strongly blocked these reactions, whereas cross-absorption with rubella virus grown on the same cell line failed to effect the influenza virus antibody reactivity.

Three control antigen preparations were substituted on the solid phase disks in place of rubella antigen in the normal rubella assay. These preparations consisted of: uninfected tissue culture supernatant; traces of pellet obtained by high-speed centrifugation of uninfected tissue culture supernatant; and infected tissue culture supernatant after removal of rubella virus by

high-speed centrifugation. One hundred and eighteen sera, including the specimens used in the absorption experiments (Fig. 3 and 4), were assayed with the three types of tissue culture antigen control disks. All of these specimens gave ELISA absorbance values below the qualitative cut-off value in assays against all three types of antigen control disks. The qualitative cut-off value was ca. 500 in this series of experiments. In addition, no changes in the qualitative test results were observed when the absorbance values obtained with antigen control disks were subtracted from the absorbance values obtained with the viral antigen disks.

Another set of negative and weakly positive sera (panel 3), including 55 weakly HI-positive (1:8 or 1:16) and 75 HI-negative (<1:8) sera were tested by PHA (Rubacell) and ELISA (Cordia R). All 55 sera which were low positive by HI were also positive by both ELISA and PHA (Table 1). However, only 50 of the 75 sera which were negative by HI were found to be negative by both ELISA and PHA. Of the 25 remaining HI-negative sera, 20 were positive by both ELISA and PHA. Of the other five HI-negative sera, two were positive by PHA only, and three were positive by ELISA only. The latter five sera were tested by IFA (FIAX), and all five were found to be positive for rubella antibody.

Twenty-six HI-negative (<1:8) sera (panel 4) were selected from panel 3 and independently tested in two additional laboratories by several methods. All 26 specimens were assayed by HI, using human O cells or chicken erythrocytes, as well as by ELISA (Cordia R), IFA, and PHA. When chicken erythrocytes were used as indicator, all sera were HI negative for rubella antibodies in two independent laboratories, but seven were positive by HI with human O cells. In addition, 13 were positive by ELISA; two laboratories reporting PHA results found 12 and 14 positive samples, respectively, and two laboratories performing IFA reported 17 and 12 positive results, respectively. Thus, approximately

TABLE 1. Results of ELISA (Cordia R) and PHA tests on 130 selected serum specimens weakly positive or negative for rubella antibodies by HI^a

| No. of samples | Result ^b | | |
|----------------|-------------------------|-------|-----|
| | HI (chick erythrocytes) | ELISA | PHA |
| 55 | + | + | + |
| 50 | - | - | - |
| 20 | - | + | + |
| 2 | - | - | + |
| 3 | - | + | - |

^a Of the 130 specimens, 55 were weakly positive by HI and 75 were negative.

^b Total positive specimens: HI, 55; ELISA, 78; PHA, 77.

TABLE 2. Comparison of five rubella antibody tests with 45 HI-negative sera

| No. of specimens | Result ^a | | | | |
|------------------|-------------------------|--------------------------------|-------------------------------|------------------|----------------|
| | HI (chick erythrocytes) | Latex agglutination (Rubascan) | ELISA (Cordia R) ^b | ELISA (Rubelisa) | PHA (PHAST) |
| 28 | - | - | - | - | - |
| 4 | - | - | E ^c | - | - |
| 3 | - | + | + | + | + |
| 2 | - | + | + | - | + |
| 2 | - | - | - | - | + ^d |
| 1 | - | - | E | - | + |
| 1 | - | - | E | E | - |
| 1 | - | + | + | E | + |
| 1 | - | - | - | + | - |
| 1 | - | + | + | - | - |
| 1 | - | + | - | - | + |

^a Test results which were not in agreement with the consensus (agreement in at least three of the five methods used) were considered discrepant. Total discrepancies: HI, 6 (13%); latex agglutination, 2 (4%); ELISA (Cordia R), 1 (2%); ELISA (Rubelisa), 3 (7%); PHA, 3 (7%).

^b Modified version with decreased sensitivity (higher serum dilution).

^c E, Equivocal, as per limits defined by the manufacturer.

^d One serum hemolyzed.

half of the 26 HI-negative sera in panel 4 were positive by ELISA, PHA, or IFA. Not all the same sera were reactive in all tests, but six sera were positive by all tests except the chick erythrocyte method. Although the total PHA-positive samples (in each of two labs) differed by only two, the same sera were not always positive, so that in six cases PHA reactions were negative in one laboratory but positive in the other. This type of discrepancy occurred in seven instances

with the IFA. These results were not unexpected in view of the low levels of rubella antibody being detected.

Most recently, a set of 45 HI-negative (<1:8) and 45 weakly HI-positive (1:8) sera (panel 5) were tested by ELISA (Cordia R and Rubelisa), latex (Rubascan), and PHA (PHAST) procedures. In Table 2 it can be seen that 12 (27%) of the 45 HI-negative sera (<1:8) were positive by one or more of the other techniques. Eight of these 12 were positive by at least two of the other methods. Of the 45 sera which were minimally positive by HI (1:8), all were also positive by two or more of the other methods (Table 3), whereas 22 were positive by all assays.

DISCUSSION

The results of parallel assays presented here and in other reports indicate that HI and ELISA are essentially equivalent in tests of HI-positive sera (9, 10, 16). However, several groups of investigators have suggested that the newer more sensitive assay methods are capable of detecting low levels of rubella antibodies which escape detection by standard HI procedures. The methods employed include ELISA (2, 11, 16-18), IFA (6, 18), latex agglutination (10), and enhanced HI (1, 11). Their conclusions are supported by the findings presented in this report.

An appreciable proportion (31%) of ELISA-positive rubella antibody specimens were encountered among HI-negative samples. The percentage was higher when chick erythrocytes were used than when human O cells were used. Our absorption results support the conclusion that the positive ELISA reactions observed with HI-negative sera were due to the presence of low levels of specific rubella antibody. When ELISA-positive, HI-negative sera were preab-

TABLE 3. Comparison of five rubella antibody tests with 45 weakly HI-positive sera

| No. of specimens | Result ^a | | | | |
|------------------|-------------------------|--------------------------------|-------------------------------|------------------|----------------|
| | HI (chick erythrocytes) | Latex agglutination (Rubascan) | ELISA (Cordia R) ^b | ELISA (Rubelisa) | PHA (PHAST) |
| 22 | + | + | + | + | + |
| 6 | + | + | + | - | + |
| 6 | + | + | E ^c | + | + |
| 4 | + | + | E | - | + |
| 2 | + | + | - | - | + |
| 2 | + | + | - | + | + ^d |
| 1 | + | - | + | + | + |
| 1 | + | + | E | E | + |
| 1 | + | + | + | E | + |

^a Test results which were not in agreement with the consensus (agreement in at least three of the five methods used) were considered discrepant. Total discrepancies: HI, 0; latex agglutination, 1 (2%); ELISA (Cordia R), 4 (9%); ELISA (Rubelisa), 12 (26%); PHA, 0.

^b Modified version with decreased sensitivity (higher serum dilution).

^c E, Equivocal, as per limits defined by the manufacturer.

^d One serum was lipemic.

sorbed with rubella antigen, influenza antigen, or various tissue culture control antigens (all prepared from BHK cells), only the rubella antigen was able to significantly block the rubella ELISA reactions. In addition, such sera were unreactive in the ELISA procedure when various tissue culture control antigens were substituted for rubella antigen on the solid phase.

Other investigators have confirmed specific antirubella activity in ELISA-positive, HI-negative sera. Morgan-Capner et al. (11), using a more sensitive HI procedure, detected rubella antibody in 46 sera which were positive by ELISA and single radial hemolysis, but which were negative by a standard HI procedure. Buimovici-Klein et al. (2) and Vejtorp (16) have demonstrated antirubella HI activity in isolated immunoglobulin G (IgG) fractions obtained from HI-negative, ELISA-positive sera. Similar fractions from HI-negative ELISA-negative sera did not reveal HI antibody. In addition, Buimovici-Klein et al. (2) have shown that lymphocytes obtained from five ELISA-positive, HI-seronegative individuals underwent blast transformation upon exposure to rubella virus. The latter finding indicates previous exposure to rubella virus and probable immunity in individuals whose sera lacked detectable HI antibody. Lymphocytes from individuals whose sera were negative by both HI and ELISA did not respond to stimulation with rubella antigen.

The accumulating body of evidence thus indicates that low levels of rubella antibody may be detected in HI-negative sera by more sensitive procedures, e.g., ELISA. However, little is known about the clinical significance of these low levels of rubella antibody. Until clinical observations have confirmed that these low levels of rubella antibody provide protective immunity for mother and fetus, the sensitivity of all of the newer assay methods should probably not exceed that of the standard HI test. Therefore, the sensitivity of the commercial version of the ELISA procedure (Cordia R) described here has been adjusted by increasing the test sample dilution to yield results which correlate more closely with standard HI test results. Ideally, an epidemiological study on the susceptibility to rubella infection of HI-negative, ELISA-positive individuals versus HI-negative, ELISA-negative individuals would resolve the clinical immunity question. Obviously this study would be difficult to conduct. Two investigations have been reported which are relevant to this question. Balfour et al. (1) demonstrated evidence of residual immunity in 11 previously vaccinated schoolgirls who were seronegative by HI. When these girls were revaccinated, they had accelerated immune responses, little or no rubella-specific IgM, no vaccine-induced reactions, and,

most significantly, no viremia. More sensitive HI tests revealed that many of the girls studied did in fact have rubella antibody before revaccination. In contrast, four of five seronegative women who were being vaccinated for the first time showed a more delayed immune response, and three of the four women tested had viremia. Two had vaccine-induced reactions. Butler et al. (3) investigated a group of adolescents with documented histories of rubella vaccination. Those who were seronegative by a standard HI test were revaccinated. Eleven of these individuals were examined for the development of rubella-specific IgG and IgM antibody by HI. Rubella-specific IgG developed in all 11, and none developed a specific IgM response. However, rubella-specific IgM was detected in two seronegative vaccinees who had no previous history of rubella vaccination. The authors concluded that although the HI antibody in the vaccinees had waned, residual immunity remained, resulting in a secondary immune response upon revaccination. In agreement with the findings cited above, we also observed that rubella antibody may sometimes be undetectable by the chick cell HI test in rubella vaccinees (personal observation). Negative HI results thus raise questions regarding the need to revaccinate these individuals. More sensitive serological tests for detecting rubella antibodies may resolve these questions.

Extensive challenge studies should be done to determine whether the low levels of rubella antibody which are detected by the more sensitive tests do in fact confer immunity. This can be accomplished by extending the investigations of Balfour et al. (1), comparing larger groups of individuals who are HI seronegative and either positive or negative by more sensitive assays methods, e.g., ELISA. The occurrence or absence of viremia in these individuals upon challenge with live rubella vaccine could be used as an indicator of immunity and related to the serological determinations. On the other hand, accelerated immune responses or the occurrence of rubella-specific IgM antibody or both may not be completely reliable indicators of immune status due to the difficulties encountered in quantitating these responses.

Epidemiological or other studies may convincingly demonstrate the protective role of the low concentrations of rubella antibody discussed here. Should this prove to be true, the HI test, which is now the basis of reference for rubella immunity, should be replaced.

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