Comparison of a Whole-Virus Enzyme Immunoassay (EIA) with a Peptide-Based EIA for Detecting Rubella Virus Immunoglobulin G Antibodies Following Rubella Vaccination

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Received 8 October 1992/Accepted 16 March 1993

A total of 250 human serum samples were tested for rubella virus immunoglobulin G antibodies by two enzyme immunoassays (EIAs), one using whole rubella virus antigen and the other based on the use of synthetic peptide antigen. The samples were taken from 125 volunteers before and after their immunization with the RA 27/3 rubella vaccine. This study indicates that a synthetic peptide-based EIA can favorably replace current viral lysate-based EIAs to detect rubella virus antibodies following immunization. Because the synthetic peptide used in this newly developed EIA represents a putative neutralization epitope of the rubella virus, it could also be instrumental in determining rubella immune status and in assessing vaccine program efficiency.

Rubella virus (RV) infection causes a mild illness with uncommon complications such as arthralgia, arthritis, or encephalitis (1). The major risk associated with rubella occurs during early pregnancy when severe damage to the fetus can result in deafness, cataract, cardiac abnormalities, microcephaly, and, sometimes, in fetal death (19). These congenital anomalies are referred to as congenital rubella syndrome (6). In order to prevent intraterine RV infection, different vaccination strategies are used. One of them is the systemic vaccination of infants with live attenuated measles, mumps, and rubella viruses at 15 months of age (5). Another approach involves schoolgirl vaccination. A third strategy is sometimes used and consists of postpartum immunization of seronegative women. It is well established that immunity acquired following natural RV infection remains longer than the vaccine-induced immunity (12). In both instances however, effective immunity is not always established, as numerous cases of RV reinfections have been documented (2, 7, 8, 11, 12, 17, 20).

Although 95% of vaccinees develop RV antibodies, nearly 10% fail to develop substantial protective immunity as demonstrated by reinfection frequencies (26). Currently, 10 to 20% of the normal adult population is seronegative for RV antibodies (1, 9, 14, 21). Such individuals are likely targets for primary RV infection or reinfection (23) and play a major role as carriers in the transmission of RV.

When present, the clinical manifestations of rubella are often nonspecific (4), particularly in adults, and the diagnosis of rubella is usually confirmed serologically. Neutralization assay (NT) determines the capacity of RV antibody to prevent RV infectivity. The hemagglutination inhibition (HI) assay is based on the ability of RV to hemagglutinate erythrocytes from specific animal sources. If specific antibodies are mixed with RV before the addition of erythrocytes, they will prevent hemagglutination. Because the NT and HI assay measure antibodies which inhibit an essential biologic function of the microorganism, the presence of such antibodies at a predetermined titer is generally thought to correlate well with immunity to disease, although they do not necessarily protect against reinfection. Therefore, in North America, the HI assay is still widely used despite the possibility of nonspecific reactions (3). Enzyme immunoassays (EIAs), which are faster and easier to perform and allow for isotype determination (24), have replaced the HI assay in many laboratories. A major problem with currently available EIAs, however, is the difficulty in obtaining RV antigens of reliable quality. RV is difficult to grow in cell culture and is also difficult to purify free of cellular debris. Different batches of RV antigens may contain different proportions of each of the viral proteins and also various contaminants. False-positive reactions of immunoglobulins with nonviral contaminants are often reported (22).

Synthetic peptides have demonstrated their ability to mimic efficiently epitopes on proteins. EIAs performed with synthetic peptide antigens improved sensitivity and specificity over EIAs based on the use of viral lysates or recombinant antigens (13).

In this paper, we compare the performance of a new synthetic peptide-based EIA (SP-EIA) with that of an RV lysate-based EIA (RV-EIA), in detecting RV antibodies.

MATERIALS AND METHODS

Serum samples. A total of 125 healthy female volunteers participated to this study. They were selected for RV immunization during the early postpartum period, on the basis of their RV seronegative status by the currently used RV-EIA (Enzygnost; Behringwerke, Marburg, Germany). In all cases, blood samples were drawn just before vaccination and approximately 6 weeks afterward. The serum from each blood sample was separated and kept frozen at −20°C until testing.

RV-EIA. The Enzygnost kit, which employs as antigen a viral lysate from RV grown on BHK-21 cells, was used. Each serum (diluted 1/204) was incubated for 1 h at 37°C in both a negative control well and an RV antigen well according to the manufacturer’s guidelines. Negative control wells were coated with uninfected cell lysates. Wells were washed, and alkaline phosphatase-conjugated anti-human

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immunoglobulin G (IgG) was added. After 1 h at 37°C, wells were washed and the para-nitrophenyl phosphate substrate solution was added. After 30 min at 37°C, color development was stopped with NaOH. The \( A_{405} \) was read. A positive reaction was indicated by a difference equal to or greater than 0.3 in the optical density (OD) of the RV antigen-coated well minus the OD of the control well (\( \Delta OD \)). A sample with a \( \Delta OD \) value between 0.2 and 0.3 was repeatedly tested. When the result was less than 0.2 on repetition, the sample was considered negative; when the result was still between 0.2 and 0.3, the sample was classified as doubtful; and when the result was greater than 0.3, the sample was considered positive.

**SP-EIA.** The DETECT-RUBELLA G kit (BioChem ImmunoSystems, Montréal, Canada) is based on the use of a synthetic-peptide antigen (referred to as BCH-178C), which corresponds to amino acids 213 to 239 of the E1 RV glycoprotein (Thérien strain). This region is conserved among all known strains of RV, including the RA 27/3 vaccine strain. It includes two cysteine residues which have been linked through a disulfide bridge. To perform SP-EIA, serum samples were diluted (1/51) with sample diluent, and 100 \( \mu l \) was added to microplate wells. After 1 h at room temperature, the wells were washed with washing buffer and 100 \( \mu l \) of peroxidase-conjugated F(ab')\(_2\) anti-human IgG was added. After 1 h at room temperature, the wells were washed and 100 \( \mu l \) of enzyme substrate (tetramethylbenzidine-H\(_2\)O\(_2\)) solution was added to each well. After 30 min at room temperature, color development was stopped by adding to each well 100 \( \mu l \) of 1 N H\(_2\)SO\(_4\). The \( A_{450} \) values were determined. A cutoff value is calculated as the mean value for three negative controls to which a value of 0.1 is added. A sample is considered positive when its \( A \) value is equal to or greater than the cutoff value and negative when it is lower than the cutoff value.

**NT.** When available, sera of interest were serially diluted and tested for their capacity to prevent 50% of the cytopathic effect compared with that of a reference. The NT was performed by an independent laboratory (Institute Armand Frappier, Laval, Québec, Canada) using the RK-13 host cell line with 100 PFU of RV strain M33 (10).

**RESULTS**

**Determination of relative EIA sensitivities.** The sensitivity of both EIAs was determined by using a reference serum (Behringwerke) calibrated in EIA units. Twofold serial dilutions of a 100-U standard were tested according to the manufacturer’s directions. Sensitivity of the Enzygnost was determined to be 0.063 U/ml of RV-specific IgG in this reference serum (Fig. 1). The sensitivity of SP-EIA was determined to be 0.016 U/ml of RV-specific IgG in the same serum (Fig. 1). However, different enzymatic probes and substrates used in these EIAs could explain such a difference in sensitivity and make the SP-EIA appear to be more sensitive than the RV-EIA.

**Comparative testing of RV-specific antibodies with RV- and SP-EIA.** In this study, 125 volunteers found to be seronegative by RV-EIA during early pregnancy testing were vaccinated immediately after delivery (RV vaccine; Rhône-Poulenc). Blood samples were drawn just before and at 6 weeks after vaccination. Four of these 125 women who were seronegative by RV-EIA were found to be positive by SP-EIA prior to their vaccination (Table 1). Primary response, as documented by the presence of RV IgM (determined by RV-EIA; data not shown), was demonstrated in one of these four cases. The three other female volunteers did not show such an IgM response.

After vaccination, the seroconversion rates were 96.8% (121 of 125) and 95.2% (119 of 125) as determined by RV-EIA and SP-EIA, respectively. Figure 2 shows the distribution of all serum reactivities after vaccination. High \( A \) values were generally reported when SP-EIA, with a mean of 2.31, was used compared with the RV-EIA, with a mean of 0.95.

**Follow-up studies of vaccine failures.** Six individuals failed to develop RV peptide antibody (SP-EIA) and four failed to develop whole-RV antibody (RV-EIA) after their vaccination. One (Table 2, patient B) of these women was negative by both EIAs. In order to verify whether the individuals with vaccine failure would subsequently seroconvert, follow-up studies were performed with five women from whom additional serum samples could be obtained. Table 2 shows both RV-EIA and SP-EIA results obtained for all subsequent serum samples. Patients A, B, and E failed to develop RV antibodies measurable by SP-EIA but became positive by RV-EIA; these responses appeared to be transient, however, as judged by patients A and E. Sera from patients A and E lost their reactivity and eventually were classified as doubtful (sample A3) or negative (sample E3). Patients B and C chose to receive a second dose of RV vaccine. Patient C, who had already been found to be positive on day 37 by SP-EIA, showed a measurable antibody titer by NT on day 224 and finally seroconverted according to RV-EIA results after a second immunization. In this patient, an earlier seroconversion was clearly revealed by SP-EIA. Patient D seroconverted late (sample D3) without a revaccination as determined by RV-EIA; her seroconversion had been detected earlier by SP-EIA and by NT (sample D2).

**DISCUSSION**

Antibody response to RV infection varies among individuals and depends on whether immunization is acquired
TABLE 1. RV antibodies in 125 serum samples from volunteers before and following RA 27/3 vaccination measured by SP-EIA and RV-EIA

<table>
<thead>
<tr>
<th>RV-EIA result</th>
<th>Prevaccination (No. of samples (%))</th>
<th>Postvaccination (No. of samples (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
<td>121 (96.8)</td>
<td>4 (3.2)</td>
</tr>
<tr>
<td>Doubtful</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>121 (96.8)</td>
<td>4 (3.2)</td>
</tr>
</tbody>
</table>

Following natural infection or vaccination. Various antibody specificities can be measured. Most immunoassays measure only the formation of immune complexes, whereas the NT and HI assay also allow detection of antibodies inhibiting a specific biologic function of the antigen. Therefore, in the context of immune status surveillance, the most relevant antibodies to monitor are those which have HI or neutralizing activity. However, the NT which measures the capacity of a given serum to block infection of cells in culture by the RV is difficult to perform, time consuming, and expensive. On another hand, RV serology based on the use of the RV-EIA does not reliably distinguish between protective and nonprotective antibodies. Several cases of congenital rubella after reinfection during pregnancy have been reported during the last decade (7, 8, 11, 20). In the course of this study, we have identified patients unable to raise an antibody response following RV vaccination. Cases of vaccine failure have also been reported by others (7, 8, 12, 16, 20, 25). In such cases, reinfection has been observed to occur with either no IgM response (12) or, rarely, with an IgM response accompanied by clinical symptoms (25). Our previous observations suggest that BCH-178C, the peptide used in the SP-EIA, represents a neutralizing epitope of RV (18, 25). As shown in Table 2, vaccination does not always induce neutralizing antibodies. The NT did not resolve all discrepant cases; it shows that in some cases earlier detection can be made by SP-EIA (samples C2 and D2). In other cases, RV antibodies are probably directed to a different neutralization site (sample B4). Immunization can be misinterpreted when serological diagnostic tests show a positive result with no correlation between the presence of antibodies and their neutralizing capacity. Some patients’ sera tested by RV-EIA showed high levels of nonspecific reactivity (control well A1 > 0.5 OD unit). As recommended by the manufacturer, these values were subtracted from the corresponding readings measured on wells coated with the RV antigens. Of the 125 prevaccinated women who were seronegative by RV-EIA, four were found to be positive by the SP-EIA. This difference possibly reflects the higher sensitivity usually observed with the SP-EIA (Fig. 1). Nevertheless, after

![FIG. 2. Scattergram distribution of 125 postvaccination serum samples. The female volunteers were all selected on the basis of their seronegative status by RV-EIA prior to vaccination. Arrows indicate assay cutoff values, and the stippled zone indicates doubtful results by the RV-EIA.]}

TABLE 2. Vaccine failure cases as shown by SP-EIA and RV-EIA

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Day of sample</th>
<th>A_{405} by RV-EIA</th>
<th>A_{405} by SP-EIA</th>
<th>NT titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0</td>
<td>0.01</td>
<td>0.02</td>
<td>ND</td>
</tr>
<tr>
<td>A2</td>
<td>47</td>
<td>1.30</td>
<td>0.03</td>
<td>ND</td>
</tr>
<tr>
<td>A3</td>
<td>792</td>
<td>0.23</td>
<td>0.02</td>
<td>ND</td>
</tr>
<tr>
<td>B1</td>
<td>0</td>
<td>0.02</td>
<td>0.03</td>
<td>ND</td>
</tr>
<tr>
<td>B2</td>
<td>45</td>
<td>0.13</td>
<td>0.04</td>
<td>ND</td>
</tr>
<tr>
<td>B3</td>
<td>112</td>
<td>0.22</td>
<td>0.04</td>
<td>ND</td>
</tr>
<tr>
<td>B4</td>
<td>191</td>
<td>0.35</td>
<td>0.04</td>
<td>12</td>
</tr>
<tr>
<td>C1</td>
<td>0</td>
<td>0.00</td>
<td>0.04</td>
<td>ND</td>
</tr>
<tr>
<td>C2</td>
<td>37</td>
<td>0.00</td>
<td>0.69</td>
<td>0</td>
</tr>
<tr>
<td>C3</td>
<td>224</td>
<td>0.19</td>
<td>1.09</td>
<td>16</td>
</tr>
<tr>
<td>C4</td>
<td>297</td>
<td>1.18</td>
<td>1.93</td>
<td>ND</td>
</tr>
<tr>
<td>D1</td>
<td>0</td>
<td>0.00</td>
<td>0.03</td>
<td>0</td>
</tr>
<tr>
<td>D2</td>
<td>52</td>
<td>0.12</td>
<td>1.28</td>
<td>24</td>
</tr>
<tr>
<td>D3</td>
<td>491</td>
<td>0.57</td>
<td>2.18</td>
<td>20</td>
</tr>
<tr>
<td>E1</td>
<td>0</td>
<td>0.00</td>
<td>0.05</td>
<td>ND</td>
</tr>
<tr>
<td>E2</td>
<td>80</td>
<td>0.35</td>
<td>0.07</td>
<td>ND</td>
</tr>
<tr>
<td>E3</td>
<td>630</td>
<td>0.09</td>
<td>0.06</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Letters A to E indicate five different volunteers from whom samples were taken three or four times. B3 and C3 were samples from volunteers who were revaccinated with day 0 vaccine.
* The number of days after vaccination on which a blood sample was drawn.
* Corrected A_{405} values. The cutoff in this test is 0.30. A result is considered doubtful when the A_{405} value falls between 0.2 and 0.3.
* The cutoff value is 0.19 absorbance unit for the SP-EIA.
* Neutralization titers determined by serial dilution. ND, not determined.
* Values in boldface were positive results.
receiving the RA 27/3 vaccine, six women did not have detectable RV antibodies by the SP-EIA, indicating a seroconversion rate of 95.2% (119 of 125). The seroconversion rate by RV-EIA was 96.8% (121 of 125). Interestingly, the two additional patients who tested positive by RV-EIA were volunteers A and E (Table 2), who had transient RV antibodies before they lost their immunity (samples A3 and E3).

A previous study has shown that a series of neutralizing monoclonal antibodies raised against the whole RV strongly recognize the BCH-178C synthetic peptide (18, 25). The same synthetic peptide has been used as an immunogen to develop monoclonal antibodies. These antibodies bind to RV and show in vitro RV-neutralizing activity at a titer of 1:20 (data not shown). Moreover, Wolinsky and associates (27) have recently published their work defining a neutralization domain on SP15 (E1 208–239), which is a synthetic peptide nearly identical to BCH-178C (E1 213–239). These investigators demonstrated the ability of a hyperimmune rabbit anti-SP15 sera to neutralize the infectivity of RV, confirming the capacity of this domain to induce antibodies reactive with the native virus. In the same work, Wolinsky and associates showed that SP27 (E1 221–239), a truncated form of SP15, maintains its immunological activity. This work further demonstrated the ability of BCH-178C to bind to RV-neutralizing antibodies. Collectively, these observations provide further evidence that the BCH-178C synthetic peptide described here represents a neutralizing epitope of RV.

In the last few years, an increase in rubella and congenital rubella syndrome has been reported in the United States (15). This raises the question of whether immune status to RV is reliably reported by the current tests. Testing based on reactivity with a neutralizing epitope would be a better marker of immune status. Although the epitope represented by BCH-178C is probably not the only neutralizing domain on RV, the use of a test based on a synthetic peptide representing such a neutralizing epitope as proposed here would improve the quality of RV diagnosis and contribute to the reduction in the number of congenital rubella syndrome cases. In addition, the BCH-178C synthetic peptide used in the SP-EIA is a potential candidate to be included in future RV vaccines.

REFERENCES