Humoral Immune Response to Primary Rubella Virus Infection

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Received 14 November 2005/Returned for modification 22 December 2005/Accepted 14 January 2006

Rubella virus is a positive-sense, single-stranded RNA virus belonging to the Togaviridae family in the genus Rubivirus (17). Infection with rubella virus usually results in a mild disease that only rarely produces significant sequelae. However, primary rubella virus infection during the first trimester of pregnancy may result in the transmission of virus through the placenta and infection of the fetus. This may in turn result in congenital rubella syndrome (CRS), the most common manifestations of which are blindness, mental retardation, and deafness (15).

The risk of the fetus developing CRS is 40 to 60% if infection occurs during first two months of pregnancy, 35 to 40% if it occurs in the third month of pregnancy, and 10% if it occurs in the fourth month of pregnancy (19).

Rubella virus infection occurs worldwide, with a seasonal peak of infections in spring in temperate climates. Globally, only 57% of countries have rubella vaccination programs, and it is estimated that more than 100,000 cases of CRS occur each year in developing countries (27).

The humoral immune response to infection commences with the production of low-affinity immunoglobulin M (IgM) molecules. Class switch recombination then results in the generation of antibody isotypes with the appropriate effector function to eliminate the invading organism. Concurrently, somatic hypermutation of the complementarity-determining regions results in the generation of antibodies of progressively increasing affinity (11).

Classically, serological diagnosis of primary rubella virus infection has relied on the detection of rubella virus-specific IgM (1) or the demonstration of a fourfold increase in the IgG titer to rubella virus antigens in sera that are taken sequentially and assayed in pairs (5). These diagnostic methods are subject to false-positive results due to nonspecific IgM reactivity (1, 5, 7, 23, 25, 28) and false-negative results when the initial sample is obtained too late to demonstrate an increasing IgG titer (5, 9).

Consequently, there has been increasing interest in the use of avidity assays to distinguish recent rubella virus infection from immune responses occurring later in the course of infection (4, 10, 18, 22).

The avidity of an antibody is a combination of the number of available binding sites and the strength (affinity) with which each individual binding site can bind the antigen. Avidity assays are generally based on commercially available enzyme-linked immunosorbent assays (ELISAs) modified by the addition of a chaotropic agent (e.g., 8 M urea [18] or 100 mM diethylamine [22]) following sample application. It is assumed that this treatment will cause the dissociation of low-affinity antibody-antigen interactions, while high-affinity antibodies remain bound. The immunological interactions upon which avidity assays are based have not been thoroughly investigated, and little effort has been directed towards standardizing the results generated by the different methods. Despite the availability of commercial avidity assays for the detection of rubella virus-specific antibodies (Enzynost; Dade-Behring), many laboratories simply modify commercial ELISAs, thus developing in-house assays. Difficulties in interpreting the results can arise if careful attention is not paid to all of the variables inherent in each assay, and contradictory results have been observed with avidity assays for other viral infections, depending upon which chaotrope is used and upon which antigens the assays are based (2, 3, 24). Factors affecting the results include variations in antibody specificity, as well as changes in titer, affinity, and antibody isotype distribution at different time points during the immune response to infection.
In this study, maturation of the humoral immune response to rubella virus was followed for recently infected individuals by use of antibody isotype-specific viral lysate-based ELISAs and avidity assays. In addition, antibody isotype and avidity data generated using the putative immunodominant peptide from the E1 glycoprotein have been directly correlated with the relative affinity constants calculated from kinetic analysis on a surface plasmon resonance-based BIAcore 2000 biosensor (Biaco Ab & Uppsala, Sweden).

MATERIALS AND METHODS

Plasma samples. Four seroconversion panels consisting of sequential samples obtained from individuals recently infected with rubella virus were used in this study. Panels 1 and 2 consisted of nine specimens taken between days 19 and 107 and between days 16 and 78, respectively, following symptom onset. These panels were collected by the National Serology Reference Laboratory, Australia. Panel 3 was a commercial seroconversion panel (PTR901, Boston Biomedica Inc., Massachusetts) which consisted of nine specimens taken between 15 days before and 31 days after symptom onset. Panel 4 consisted of four specimens taken between day 1 and day 70 after symptom onset. This panel was collected by Queensland Medical Laboratories, QLD, Australia. Anti-rubella virus antibody-positive specimens and negative control specimens were obtained from the Australian Red Cross Blood Service.

All the specimens in the present investigation were characterized by use of Vidas IgG and Vidas IgM assays (bioMerieux, Marcy-l'ETOile, France), AxSYM IgG and AxSYM IgM assays (Abbott Laboratories, Diagnostics Division, Abbott Park, IL), and an in-house hemagglutination inhibition assay (8).

Viral lysate. KIS rubella viral lysate (PanBio, QLD, Australia) was sonicated three times for 10 seconds on ice, aliquoted, and stored frozen at −70°C.

Western blot. KIS rubella virus antigen (10 μl) was diluted in nonreducing sample buffer, and the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V (14). The proteins were transferred to nitrocellulose (0.45 µm pore size) for 2 h at 0.22 A. The nitrocellulose was incubated with strips at room temperature for 4.5 h. Strips were washed extensively with TBS-T. The bands were visualized by use of a 5-bromo-4-chloro-3-indolyl-5-diluted 1 in 1,000, anti-human IgA was diluted 1 in 10,000, and anti-human IgM incubated with strips at room temperature for 4.5 h after antibody addition. Strips were washed extensively with TBS-T. Plates were washed twice in PBS-T, and then 100 μl of 8 M urea in PBS-T was added per well, and the plate was incubated at 37°C for 1 h and then washed three times in PBS-T. Sheep polyclonal horseradish peroxidase (HRP)-conjugated anti-human IgG (Chemicon Australia Pty. Ltd., Victoria, Australia), mouse monoclonal HRP-conjugated anti-human IgG clone JDC-1 (Southern Biotechnology Associates Inc., Birmingham, AL), and mouse monoclonal HRP-conjugated anti-human IgG clone SA-DA4 (Southern Biotechnology Associates Inc., Birmingham, AL) were all used at a dilution of 1 in 1,000. Mouse monoclonal HRP-conjugated anti-human IgM clone SA-DA4 (Southern Biotechnology Associates Inc., Birmingham, AL) was used at a dilution of 1 in 2,000, and HRP-conjugated goat anti-human IgA (Biosource International, Camarillo, CA) was used at a dilution of 1 in 5,000. The appropriate HRP-conjugated secondary antibody was diluted in BLOTTO, 100 μl was added per well, and the plate was incubated at 37°C for 1 h and then washed three times in PBS-T.

Two μM ABTS [2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] in 25 mM sodium citrate buffer, pH 4.5, containing 0.3% hydrogen peroxide was used as a substrate for the HRP by adding 100 μlwell and incubating in the dark at room temperature for 20 min. The reaction was stopped by the addition of 50 μl of 5% oxalic acid per well, and the absorbance of the wells was read at 405 nm.

The avidity assays were run in parallel with the standard ELISA using the same specimens, plasma dilutions, and secondary isotyping antibodies. The mean absorbance of the negative controls for each plate was subtracted from the optical density at 405 nm, and the absorbance of the wells was read at 405 nm. The avidity assay was used as a cutoff for recent infection, with an AI of 40% considered as highly avid and indicative of past exposure (4, 10).

BIAcore analysis. The binding kinetics of the specimens to the E1 peptide were analyzed using a 1,000 instrument (Biacore AB, Uppsala, Sweden). The E1 peptide was synthesized with an N-terminal amine group for coupling to a BIAcore CM5 sensor chip (Biaco Ab, Uppsala, Sweden). The E1 peptide was synthesized on an Applied Biosystems 430A automated synthesizer by the solid-phase synthesis procedure of Merrifield (16) by use of 2-chlorotertiobutyloxycarbonyl chemistry. E1 208–239 was recon-}

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Estimated Day Post-Symptom Onset

FIG. 1. Antibody isotype-specific Western blots were performed on a rubella virus seroconversion panel. This seroconversion panel (panel 1) consisted of nine specimens with estimated times after symptom onset of 19, 37, 43, 50, 57, 71, 80, 88, and 107 days. Plasma specimens were diluted 1 in 100 and incubated with K1S rubella viral lysate-based Western blot strips. Biotinylated anti-human isotyping secondary antibodies were used to probe the strips for total IgG (A), IgG1 (B), IgG3 (C), IgA (D), and IgM (E); this was followed by incubation with an avidin-alkaline phosphatase conjugate. The bands were visualized by use of a BCIP–nitroblue tetrazolium phosphatase substrate system. The apparent molecular masses of three structural proteins of the rubella virus are 58 kDa for E1, 42 to 47 kDa for E2, 33 to 38 kDa for the capsid, and 70 kDa for the capsid homodimer (Cd) (28).

RESULTS

Seroconversion panel alignment. Because the exact date of infection was unknown and for the purpose of comparison, the four seroconversion panels were aligned using the maximum peak of IgM reactivity. This maximum peak of reactivity was identified by using a Vidas IgM enzyme immunoassay (EIA) and was designated as day 7 after symptom onset because, in the course of a typical rubella virus infection, the IgM antibody reaches its maximum concentration at approximately 7 days after symptom onset (6). We acknowledge that using an antibody marker to set a time frame for an investigation comparing antibody responses is not ideal. However, no other method of comparing the panels was available. Following infection with rubella virus, viremia disappears prior to symptom onset, and consequently, samples are rarely collected early enough to permit the detection of nucleic acid or antigen.

Western blot analysis. Western blots were used to confirm that all three rubella virus structural proteins were present in the K1S rubella viral lysate. The reported, apparent molecular masses of the rubella virus proteins are 58 kDa for E1, 42 to 47 kDa for E2, 33 to 38 kDa for the capsid, and 70 kDa for the capsid homodimer (28), which corresponded well with the bands we observed on our Western blot probed for total IgG (Fig. 1A). Antibody isotype-specific Western blots demonstrated E1 to be the immunodominant protein, and the four antibody isotypes investigated were all found to react with this protein. IgA and IgM reacted early during the course of infection (Fig. 1D and E, respectively) while the IgG1 and IgG3 responses gradually intensified with time (Fig. 1B and C, respectively).

Rubella viral lysate ELISA. Specimens from the four seroconversion panels were analyzed by use of an antibody isotype-specific ELISA with rubella viral lysate as the antigen. The total IgG response was seen to increase gradually with time in all four seroconversion panels (Fig. 2A). The IgG1 reactivity followed this same pattern (Fig. 2B), indicating that IgG1 is the major antibody isotype contributing to this total IgG reactivity. The IgG3 (Fig. 2C), IgA (Fig. 2D), and IgM (Fig. 2E) responses all demonstrated the presence of early transient peaks of reactivity which declined with time.

K1S rubella viral lysate avidity assay. The viral lysate-based avidity assay for total IgG demonstrated rapidly increasing AI in two of the panels but a consistently high-avidity response for all samples collected over the other two panels (Fig. 3A). This response was mirrored by the IgG1-specific avidity assay (Fig. 3B), indicating that the high-avidity response observed in the total IgG assay was primarily due to a high-avidity IgG1 response. The IgG3-specific (Fig. 3C) and IgA-specific (Fig. 3D) avidity assays showed increasing avidity with time. However, the transition to a high-avidity response (>40% AI) did not occur until well after the initial peak of high-titer antibody (evident in the conventional ELISA) had disappeared. Consequently, by the time the avidity of these antibody isotypes had increased, the corresponding titer had decreased to levels where this reactivity could not significantly contribute to the overall AI observed. The IgM-specific avidity assay (Fig. 3E) demonstrated a very low avidity index over the course of infection examined, despite the presence of an initial high-titer peak of reactivity observed with the standard ELISA.

Reactivity to E1208–239. The major humoral immune response to the immunodominant peptide E1208–239 was an IgG1 response which increased with time for all four seroconversion panels (Fig. 4A). This binding pattern was mirrored by the total binding response curves obtained with the BIAcore instrument (Fig. 4C). Application of the chaotropic agent in the avidity assay demonstrated that all samples taken following infection for each panel were of high avidity (Fig. 4B), which indicates that the immune response to this particular epitope matures in affinity very rapidly. This was confirmed by the extremely low $K_D$ values obtained with the BIAcore instrument (Fig. 4D), which indicates that the relative affinity was high for all the seroconversion samples very soon after infection.

DISCUSSION

We have demonstrated that during the primary immune response to infection by rubella virus, there is an initial peak of IgM reactivity to the viral proteins, which is closely followed by transient peaks of both IgG3 and IgA reactivity. During the
course of infection, an IgG1 response develops and gradually increases in both titer and affinity until it dominates the immune response. While previous studies have reported the detection of IgM soon after rubella virus infection and prior to the appearance of significant amounts of IgG (20) and the observation of the presence of both IgG1 and IgG3, the times at which they appear following infection has been found to vary (21). By using seroconversion panels rather than single samples obtained from individuals, we have been able to demonstrate the relative times following infection that the various antibody isotypes appear.

Our data indicate that treatment with the chaotropic agent 8 M urea appears to eliminate the detection of the early low-affinity IgM, IgA, and IgG3 peaks of reactivity while apparently leaving the developing IgG1 response unaffected, explaining the principle underlying these avidity assays. IgM antibodies do not undergo affinity maturation (11, 13), which was reflected in the results we obtained with the avidity assay. Although a high-titer peak of IgM was identified in the viral lysate-based ELISA, this reactivity was completely absent in all samples from all panels in the avidity assay. Despite the presence of intense peaks of IgG3 and IgA reactivity soon after infection, the AI was not found to increase until after these peaks of reactivity had decreased significantly when the avidity assay was conducted for these antibody isotypes. Consequently, neither of these antibody isotypes could have made any significant contribution to the overall AI observed. Previous studies have also reported avidity assays to be helpful and reliable in the diagnosis of recent primary rubella virus infection (10), where IgG avidity has been found to increase with time after primary infection but not following reinfection (4, 12, 21). Analyzing seroconversion panels in an avidity assay probed with antibody
isotype-specific secondary antibodies, we have been able to determine the interactions which underlie these findings.

To obtain good-quality kinetic data with the BIAcore instrument, highly purified proteins are required for immobilization. Rubella viral lysate could not be used for immobilization, since the possibility that some antigens could preferentially bind to the chip could not be discounted. For this reason, we probed viral lysate-based Western blots with antibody isotype-specific secondary antibodies to identify the antigen that would provide the most comprehensive analysis of the interactions we had observed. Not only was E1 found to be immunodominant, but it also was the only antigen recognized by all the antibody isotypes we investigated. Consequently, we used a peptide analogue of the putative immunodominant epitope (E<sup>208–239</sup>) for immobilization to the biosensor chip and subsequent kinetic analysis.

The total binding to the E<sup>208–239</sup>-coated biosensor chip was analogous to that observed in the peptide-based ELISA. The peptide-based avidity assay demonstrated that a high-avidity response to this peptide is generated rapidly following infection, and this was confirmed by the extremely low dissociation rates calculated from the sensograms. The AI measured in the avidity assay and the relative affinity measured with the BIA-core instrument indicate that the immune response to the E1 immunodominant peptide matured too rapidly to provide a means of identifying recent infection, since a high-affinity IgG1

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**FIG. 3.** An antibody isotype-specific, rubella viral lysate-based avidity assay was performed. Plates coated with K1S rubella viral lysate were used to analyze specimens from four seroconversion panels, panels 1 (●), 2 (▼), 3 (■), and 4 (▲), for the presence of different antibody isotypes. The AI was calculated by dividing the absorbance at 405 nm following treatment with 8 M urea by the absorbance at 450 nm without treatment and multiplying by 100. The dashed line at 40% AI represents the arbitrary cutoff for recent infection. Panels were probed with a polyclonal anti-human total IgG antibody (A), with an anti-human IgG1 secondary antibody (B), with an anti-human IgG3 antibody (C), with an anti-human IgA antibody (D), and with an anti-human IgM secondary antibody (E). The AI is plotted against the estimated day postinfection.
response was already present in the samples collected immediately after infection, with only very low and quite variable reactivity observed for the other antibody isotypes.

A reliable assay to distinguish between the immune response generated by recent exposure and the immune response existing as a result of past exposure or immunization is an essential facet of the management of the health of a pregnant woman when a rubelliform rash develops or when there is suspected exposure to rubella virus. We have investigated the immunological basis of the avidity assays which are currently being used for this purpose and found that these assays do appear to discriminate between the initial peaks of low-affinity IgM, IgA, and IgG3 reactivities and the high-affinity IgG1 response indicative of past exposure.

In the absence of a more specific marker of recent infection, assays relying on the ability of chaotropic agents to dissociate low-avidity interactions provide valuable information for confirming the diagnosis of recent rubella virus infection. However, it is essential to ensure that an appropriate antigen is employed, because the immune response to each specific antigen matures at a different rate. For this reason, it is also necessary to evaluate fully each assay with large numbers of well-characterized samples.

ACKNOWLEDGMENTS

We thank Frofa Katsis from St. Vincent’s Institute, Melbourne, for performing peptide synthesis and Bruce Kemp from St. Vincent’s Institute, Melbourne, for providing access to the BIACore 2000 biosensor. We also thank Robin Wood at Queensland Medical Laboratories for providing seroconversion panel 4 and Wayne Dimech and Lena Panagiotopoulos from the National Serology Reference Laboratory, Australia, for providing the Vidas and AxSYM EIA results.
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