Detection of Rubella Virus-Specific Immunoglobulin G (IgG), IgM, and IgA Antibodies by Immunoblot Assays

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Immunoblot (IB) assays were developed for detection of rubella virus (RV)-specific immunoglobulin G (IgG), IgM, and IgA antibodies in human serum following natural infection or immunization. IB assays performed under nonreducing conditions were compared with those performed under reducing conditions and with immunoprecipitation assays. Significant loss of antigenicity (>90%) of RV E1 and E2 proteins was observed when IB assays were performed in the presence of 2-mercaptoethanol as compared with assays under nonreducing conditions. In contrast, the antigenicity of RV capsid protein was not influenced by reducing agents. Sensitivity of IB for RV-specific IgG antibodies was determined to be 0.01 IU/ml under nonreducing conditions. In the determination of RV-specific IgM and IgA antibodies by IB, pretreatment of serum with protein G to remove competing high-affinity RV-specific IgG or rheumatoid factor significantly improved assay sensitivity. IB assays were observed to be superior to immunoprecipitation assays in their ability to better define the specificities of RV-specific antibodies and to detect antibodies of all immunoglobulin classes. However, the conformational sensitivity of RV protein antigenicity should be an important consideration in the interpretation of RV-specific antibodies by IB assays.

Rubella virus (RV), the sole member of the genus Rubivirus of the family Togaviridae, is of clinical significance due to its capacity to induce congenital malformation and persistent infection in the human fetus when exposure occurs during the first trimester of pregnancy (44). RV infection or immunization occurring postnatally may also give rise to chronic manifestations such as arthralgia, arthritis, and neurologic sequelae (12, 30, 38) thought to arise from RV persistence in host tissues. Most RV infections produce lifelong immunity mediated by circulating antibodies and specific T lymphocytes (13, 27). However, immunity to RV is most frequently ascertained by detection of specific antibodies by enzyme-linked immunosorbent assay (ELISA) or other immunoassay methods which employ whole virus. RV contains three major structural proteins which are antigenic in both animals and humans: the envelope glycoproteins, E1 and E2, and the capsid (C) protein, which is associated with the viral RNA (8, 34, 42). Epitopes involved in hemagglutination and neutralization have been identified on E1 with the aid of RV-specific murine monoclonal antibodies (17, 19, 41), and E1 appears to be immunodominant in the normal serologic response to RV (19). An additional weak neutralization domain on E2 was also described (13). Although to date no other biological roles have been assigned to the E2 or C protein, there is increasing interest in these proteins as indicators of abnormal responses to RV (7). Katow and Sugiuira (19) demonstrated that although antibodies to E1 were predominant following most RV infections, anti-E2 antibodies were relatively more abundant following congenital infection. We have also observed abnormal distributions of anti-E1, -E2, and -C reactivities in RV hemagglutination inhibition- or ELISA-seronegative individuals developing arthritis in close temporal association with RV infection or immunization (26, 38), suggesting that previous RV exposure was associated with a failure to develop or sustain functional immunity. Similar dissociations between seroconversion and development of effective immunity to RV have been reported by others (15, 18, 36), indicating a need for a more sensitive and specific method of serodiagnosis.

The fine specificities of RV antibodies have been analyzed by immunoprecipitation (IP) followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26). However, this procedure is laborious, time-consuming, and hazardous in that it utilizes radiolabelled virus, and it is usually limited to detecting antibodies of the immunoglobulin G (IgG) class only. Immunoblot (IB) assays which are simpler to perform can be used to determine not only the specificities of antibodies generated in response to RV infection or immunization but also their class distribution. As detection of RV-specific IgM antibodies is critical to the diagnosis of maternal RV exposure and congenital rubella and may be used as a criterion for recommending therapeutic abortion, more-sensitive and -definitive methods for detecting this class of antibody are continually being sought. Here we report the development of sensitive and specific IB assays for detection and analysis of RV-specific IgG, IgM, and IgA antibodies and their comparison with IP methods for determining antibody response profiles following natural infection or immunization with RV. The results show that the use of IB circumvents problems associated with coprecipitation of antigens in IP and allows more sensitive and definitive determination of specific IgM and IgA antibodies than ELISA techniques. However, the sensitivity of determining E1- and E2-specific antibodies by IB was significantly influenced by the choice of nonreducing over reducing conditions during the initial electrophoretic separation of RV proteins. Hence, the relative conformational sensitivity of E1 and E2 antigenicity should be borne in mind when interpreting the results of antibody analysis by IB techniques.

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MATERIALS AND METHODS

Virus preparation. RV M33 strain (RV:ADCC, VR-315) was grown on Vero cell monolayers harvested and clarified by centrifuging at 1,000 × g for 15 min. Viral titers (in PFU) were determined by plaque formation after incubation of each RV preparation with RK-13 cells (11). Viral particles were concentrated by precipitation in 10% polyethylene glycol and then resuspended in phosphate-buffered saline (PBS) (60 mM Na2HPO4, 1.5 mM KH2PO4, 2 mM KCl, 150 mM NaCl, 0.02% NaN3) containing 0.5% Triton X-100.

Patient and reference sera. Sera were obtained from adult female vaccinees (who had received a single dose of RV [RA27/3] vaccine [Meruvax; Merck Sharpe & Dohme]) at 0 (preimunization), 1, 2, 3, 4, 5, and 6, and 24 weeks postvaccination. Other sera were collected from patients who had experienced wild RV infection during the 1985 epidemic in British Columbia, Canada. These sera were categorized by intervals following onset of a rubelliform rash into early acute-phase (0 to 6 days postonset), acute-phase (7 to 30 days postonset), and convalescent-phase (0.5 to 3 years postonset) sera. Reference serum pools were prepared from sera which had been identified as having high RV-specific IgG, IgA, or IgM reactivity in ELISAs employing detergent-solubilized whole RV (see below) and reactivity with RV E1, E2, and C proteins by IgG, IgA, or IgM IB, respectively. The IgG reference pool was standardized against the World Health Organization International Rubella Reference serum (46) and shown by ELISA to contain 943 IU of anti-RV IgG antibody per ml, of which approximately 50.1, 30.1, and 19.8% of the total antibody was directed to E1, E2, and C proteins, respectively, as determined by densitometry of IB assays. Reference pools for RV-specific IgA and IgM antibodies were assigned values of 100 and 1,000 arbitrary units per ml, respectively. All sera were stored at −70°C and thawed just before use.

Removal of serum IgG with protein G. For IgM and IgA antibodies determinations in IB assays, pretreatment of serum samples was required to reduce competition of RV-specific IgG antibodies for RV antigens. Briefly, protein G-Sepharose (Pharmacia, Uppsala, Sweden) was suspended to a concentration of 70% (vol/vol) in phosphate buffer (pH 7.2; 20 mM Na2HPO4, 20 mM KH2PO4) in a volume of 0.3 ml. The protein G-Sepharose suspension was pelleted by centrifugation at 14,000 × g for 15 s. The supernatant was decanted, and 0.5 ml of serum was added and incubated for 30 min at room temperature with gentle mixing. The supernatant (containing all antibody isotypes except IgG) was removed for determination of specific IgM and IgA antibodies by ELISA and IB as described below.

Whole-RV ELISAs. ELISAs for RV-specific IgG, IgA, and IgM antibodies were performed as previously described (38). Briefly, detergent-solubilized RV (M33) prepared as described above was diluted optimally in bicarbonate-carbonate coating buffer (pH 9.6) and coated onto polystyrene microplates overnight at 4°C. After blocking the virus-coated wells with PBS (pH 7.4) containing 0.5% (wt/vol) bovine serum albumin for 1 h at room temperature, patient serum (diluted 1:100 to 1:1,600) or reference serum (diluted to give concentrations ranging from 0.062 to 1.0 IU/ml) was added and the microplates were incubated for 1 h at 37°C. The plates were then washed four times in PBS containing 0.05% (vol/vol) Tween 20, and optimally diluted alkaline phosphatase-conjugated goat anti-human IgG, IgA, or IgM antisera (affinity purified γ, α, or μ chain specific; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) were added to the wells correspondingly. After a second 1-h incubation at 37°C, the plates were washed in PBS containing 0.05% Tween 20 as described above and enzyme substrate (p-nitrophenyl phosphate, 2 mg/ml in diethanolamine-Mg2+ buffer, pH 9.8) was added to the wells. The A405 was determined with a Bio-Rad (Richmond, Calif.) model 3550 microplate reader. Results were calculated by linear regression and interpolation from standard curves prepared with the reference sera described above.

SDS-PAGE and IB assay. SDS-PAGE was performed with 0.75-mm minigels (Mini-Protein; Bio-Rad) by using a discontinuous buffer system (as described by Laemmli [22]) and 10% polyacrylamide separating gels. Virus preparations were diluted 1:1 with sample buffer (60 mM Tris, 3% SDS, 10% glycerol, 0.02% bromphenol blue, pH 6.8) either with 1% 2-mercaptoethanol (reducing) or without mercaptoethanol (nonreducing) and heated at 100°C for 3 min. Prestained molecular markers (Sigma, St. Louis, Mo.) ranging in molecular weight from 26,000 to 180,000 were added in a separate lane. Electrophoresis was performed at 110 V for 100 min. After electrophoresis, the RV proteins were electrophoretically transferred onto nitrocellulose (NC) membranes (Bio-Rad; 0.45-μm pore size) at 300 mA of constant current for 30 min by established techniques (40). The blots were then washed with washing buffer (TBS [pH 7.4; 20 mM Tris, 150 mM NaCl] containing 0.5% Tween 20) for 10 min, blocked for 40 min at room temperature in blocking buffer (TBS, 4% milk powder), and air dried. Dried blots were stored at −20°C until required and cut into 0.5-cm-wide strips just before use. Serum samples diluted in blocking buffer (1:25 for IgG, IgA, and IgM) were incubated with blot strips at 4°C overnight and washed in washing buffer for 1 h. Optimal dilutions of alkaline phosphatase-conjugated affinity-purified goat anti-human IgG, IgM, and IgA (Kirkegaard & Perry Laboratories) were added, correspondingly, and the strips were incubated at room temperature for 2 h with gentle mixing. After washing as described above, the strips were incubated in 30 ml of substrate solution (pH 9.5; 100 mM Tris–5 mM MgCl2–100 mM NaCl containing 5 mg of bis-chloroindolyl phosphate and 10 mg of nitroblue tetrazolium [dissolved in 10% and 70% formamide, respectively]) to visualize RV protein bands. Densitometric tracings on the IB strips were made with a Video Densitometer 620 (Bio-Rad), and the relative density (optical density in millimeters [OD mm]) of each band was determined by integration of the area under each peak. Relative amounts (R%) of antibody binding to E1, E2, and C were expressed as a percentage of the absorbance area of each peak compared with the total area under all peaks and were calculated according to the following formula: R% = ([OD mm (peak X)/OD mm (peak E + E2 + C)] × 100.

Radioimmunoprecipitation. A modification of the conventional protein A-Sepharose technique (26) was used for radioimmunoprecipitation. Briefly, a protein A-Sepharose suspension (Pharmacia) was washed in TBS containing 1% Triton X-100 and resuspended in a minimal volume of the same buffer. Patient or control serum (30 μl) diluted in 200 μl of TBS–Triton X-100 was added to 50 μl of washed protein A-Sepharose suspension, and the mixture was allowed to adsorb to the protein A with gentle mixing for 4 h at 4°C. The beads were then washed well in TBS–Triton X-100, [35S]methionine-labelled detergent-solubilized RV (41) was added in a total volume of 200 μl, and the mixture was incubated for 4 h or overnight at 4°C. After washing the beads well by centrifugation through TBS–Triton X-100 followed by TBS, precipitated proteins were eluted from the protein A-Sephe-
arose by adding 60 μl of SDS-PAGE sample buffer and boiling for 5 min. Samples were clarified by centrifugation at 10,000 rpm in a Silencer H31 microcentrifuge (Western Scientific, Vancouver, Canada) before loading onto gels for SDS-PAGE, which was performed as described above.

RESULTS

Effect of reducing agents on the antigenicity of RV structural proteins. To determine whether disulfide bond-disrupting agents such as 2-mercaptoethanol influence the apparent antigenicity of RV E1, E2, and C in IB assays, RV proteins were separated by SDS-PAGE under reducing and nonreducing conditions with sample buffers with or without 2-mercaptoethanol (final concentration, 1%). After SDS-PAGE, the separated proteins were electrophoretically transferred to NC membranes. When the NC blots were developed with a pooled IgG reference serum (containing 943 IU of RV-specific IgG antibody per ml) diluted to 1:200, reactive monomeric bands were observed at approximately 58 kDa (E1), 42 to 47 kDa (E2), and 33 kDa (C) in bands performed after SDS-PAGE under reducing conditions; bands were observed at 58 kDa (E1), 42 to 47 kDa (E2), and 66 kDa (C dimer) in bands performed after SDS-PAGE in the absence of 2-mercaptoethanol (data not shown). Higher-molecular-mass bands thought to be E1 homodimers and E1-E2 heterodimers were also observed. When NC blots were developed with sera obtained from three subjects 6 months after immunization with RV RA 27/3 vaccine (Fig. 1), strong reactivities were observed with E1 and E2 proteins in bands performed under nonreducing conditions. In marked contrast, it was observed that the addition of 2-mercaptoethanol to the SDS-PAGE sample buffer destroyed much of the antigenicity of E1 and to a lesser extent that of E2, while C protein antigenicity was relatively unaffected.

In a separate experiment (Fig. 2), the relative sensitivities of RV IgG IBs performed under reducing (Fig. 2A) or nonreducing (Fig. 2B) SDS-PAGE conditions were compared by developing NC blot strips with dilutions of pooled anti-RV IgG reference serum or individual patient sera obtained during the acute and convalescent phases following natural infection. All sera were serially diluted to give equivalent concentrations of 10, 1, 0.1, and 0.01 IU of RV-specific IgG antibody per ml as determined previously by ELISA. It was observed that under nonreducing conditions, serum samples diluted to 0.01 IU/ml still produced detectable bands by IB. In sharp contrast, use of reducing conditions decreased the antigenicity of E1 and E2 by approximately 90% (as determined by densitometric comparison of relative areas under each protein peak). Hence, the use of 2-mercaptoethanol in SDS-PAGE effectively

FIG. 1. Comparison of RV IgG IBs performed after SDS-PAGE under reducing or nonreducing conditions. Detergent-solubilized RV (M33 strain) was separated by SDS-PAGE performed under reducing (+) or nonreducing (−) conditions. RV proteins were blotted electrophoretically onto NC. NC blot strips were reacted with sera obtained from RA 27/3 vaccinees at 6 months postimmunization. The relative positions of monomeric protein bands (E1, E2, and C), E1-E1 and E1-E2 dimers (D), and C dimer (Cd) are indicated.

FIG. 2. Evaluation of the effect of reducing agents on the sensitivity of RV IgG IBs. IgG reference standard serum (pooled) and two representative acute- and convalescent-phase postrubella serum specimens were diluted to give final RV antibody concentrations of 10, 1, 0.1, and 0.01 IU/ml based on previously determined ELISA values. (A) Reducing conditions; (B) nonreducing conditions. Lanes 1, 5, and 9, 10 IU/ml; lanes 2, 6, and 10, 1 IU/ml; lanes 3, 7, and 11, 0.1 IU/ml; lanes 4, 8, and 12, 0.01 IU/ml. E1, E2, and C indicate positions of monomeric RV protein bands; D indicates relative positions of presumed E1-E1 (upper band) and E1-E2 (lower band) dimers; Cd refers to the C dimer observed under nonreducing conditions. The relative positions of molecular weight markers (MW) are indicated.
reduced the sensitivity of the RV IgG IB assay 10-fold to approximately 0.1 IU/ml.

Effect of protein G treatment on the sensitivity of RV IgM and IgA IB assays. To investigate the potential interference of RV-specific IgG antibodies or rheumatoid factor on the sensitivity of detecting RV-specific IgM or IgA antibodies in IB assay, sera were pretreated by protein G-Sepharose adsorption to remove IgG antibodies prior to performing ELISAs or IB assays for RV-specific IgM or IgA antibodies. It was observed that in samples with high levels (>1,000 IU/ml) of RV-specific IgG antibody (as measured by ELISA) the binding of specific IgM and IgA antibodies was significantly inhibited in both ELISA and IB. Figure 3 and Table 1 depict representative results with a serum sample before and after protein G treatment showing that RV-specific IgM and IgA antibodies could be more effectively measured in both ELISA (Table 1) and IB (Fig. 3) after removal of serum IgG.

To determine the efficiency of protein G adsorption of serum IgG, patient sera and RV IgG, IgM, and IgA reference sera before and after protein G treatment were serially diluted, subjected to SDS-PAGE, and electrophoretically transferred to NC membranes. Isotype-specific bands were detected with enzyme-conjugated anti-human IgG, IgM, and IgA antisera. After densitometric scanning of the developed blots, the relative absorbances of isotype bands observed before and after protein G treatment of the serum showed that protein G effectively removed >90% of the IgG with a loss of <10% of serum IgM and IgA antibody (data not shown).

Comparison of IB and IP assays for determination of RV antibody specificity. IP techniques have been used extensively in the analysis of the RV antibody response following natural infection or immunization. In the present study IP was compared with IB for analysis of the fine specificities of antibodies in sera obtained from typical subjects after natural RV infection or immunization with RA 27/3 vaccine. Comparative antigen recognition profiles of six representative serum samples by IB and IP are shown in Fig. 4. In these serum samples obtained during the convalescent phase following natural infection or ≥6 months following vaccination, densitometric tracings of IB bands and IP autoradiographs revealed qualitative and quantitative differences between these two techniques (Table 2) in the interpretation of antibody specificities. In these sera E2 antibody reactivity was detected more frequently after IP than in IB assays. The relative proportions of anti-E2 antibodies as detected by IP were also greater than those observed by IB. For example, in serum 2 (Table 2) the relative proportion of anti-E2 antibody as determined by IP was 32%. In contrast, no E2-specific antibodies were observed when the same serum was tested by IB.

To evaluate the utility of IB analysis in defining the RV-specific isotype antibody specificities in patients follow-

![Image](https://jcm.asm.org/)

**TABLE 1. Effect of pretreatment of serum with protein G on the detection of RV-specific IgG, IgM, and IgA antibodies**

<table>
<thead>
<tr>
<th>Serum treatment</th>
<th>IgG by:</th>
<th>IgM by:</th>
<th>IgA by:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA (IU/ml)</td>
<td>IB</td>
<td>ELISA (AU°/ml)</td>
<td>IB</td>
</tr>
<tr>
<td>None</td>
<td>1,601.3</td>
<td>E1, E2, C</td>
<td>175.0</td>
</tr>
<tr>
<td>Protein G</td>
<td>0.05 E1</td>
<td>1,028.0 E1, E2</td>
<td>1,294.0</td>
</tr>
</tbody>
</table>

* AU, arbitrary units.

FIG. 3. Effect of pretreatment of serum with protein G on sensitivity of detection of RV-specific IgM and IgA antibodies by IB. Protein G-treated (+) and untreated (−) sera were reacted with RV proteins blotted onto NC under nonreducing conditions. Relative positions of E1 and E2 monomers, E1-E1 and E1-E2 dimers (D), and C dimer (Cd) are indicated.
TABLE 2. Comparison of IB and IP assays for determination of relative proportions of RV-specific IgG antibodies for each of the RV structural proteins

<table>
<thead>
<tr>
<th>RV structural proteins</th>
<th>% Distribution of RV-specific antibodya in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IB with serum no.</td>
</tr>
<tr>
<td></td>
<td>1 2 3 4 5 6</td>
</tr>
<tr>
<td>E1</td>
<td>0 92 61 58 49 37</td>
</tr>
<tr>
<td>E2</td>
<td>0 02 23 25 44 50</td>
</tr>
<tr>
<td>C</td>
<td>0 8 15 17 7 6</td>
</tr>
</tbody>
</table>

a Percentage of total cumulative area measured as optical density in millimeters (OD mm) under all peaks as determined bydensitometry. Serum numbers correspond to those shown in Fig. 4.

ing natural infection, sequential serum samples taken during the early acute, acute, and convalescent phases (see Materials and Methods) after onset of rash were subjected to IB. It was observed that in three serum samples (Fig. 5) anti-E1 IgG and IgA antibodies appeared during the acute phase (7 to 30 days postonset) and persisted throughout the follow-up period of 1 to 3 years after initial infection. Anti-C antibodies of all three immunoglobulin classes were detected in acute-phase sera but had disappeared by 1 to 3 years postinfection. Anti-E2 antibodies of the IgG and IgA classes were not detected until 1 month after onset, and these were observed to increase in the convalescent-phase samples. IgM reactivity with E1, E2, and C was observed only in acute-phase sera.

**DISCUSSION**

The majority of investigations which have analyzed the fine specificity of RV-directed antibodies have employed either IP (26, 31) or SDS-PAGE under reducing conditions followed by IB (3, 5, 6, 31, 43) to detect RV-specific IgG antibodies. Apart from being time-consuming, laborious, and hazardous because of the use of radioactively labelled virus, IP techniques have been limited by their capacity to detect RV-specific antibodies within the IgG class only. A further limitation of this methodology has been in its potential for false-positive interpretation of E2-reactive antibodies in serum due to coprecipitation of E2 as E1-E2 dimers reacting with E1-specific antibodies (16, 41, 42). Furthermore, the disproportional distribution of methionines in the RV structural proteins has to be corrected for during densitometric analysis, if [35S]methionine is used as a metabolic labelling agent. E1 contains only three methionines, whereas E2 and C contain seven and six, respectively (3), resulting in the underestimation of E1 in the immunoprecipitate. In the present study, notable differences were observed between the distribution of antibody specificities in a given serum sample as determined by IP or IB assay. In the present study, the relatively larger percentages of E2-specific antibodies detected by IP compared with those detected by IB suggest that coprecipitation of E2 as E1-E2 heterodimers may indeed lead to erroneous interpretations of E2 antibody specificity when the former method is used. Although epitopes on E2 may be masked by the association of this protein with E1 in the viral envelope in intact RV (42), during processing and presentation of RV antigens to the immune system such epitopes may give rise to detectable antibodies, as monoclonal antibody studies have shown (43). Apart from the identification of a weak neutralization domain on E2 with monoclonal antibodies (13), few other functions have been identified for this protein. However, the predominance of E2-specific antibodies in patients with altered immunity to RV (19) has sparked interest in the potential role of this protein in RV-associated pathology.

Detection of RV-specific IgM antibodies is an important criterion for diagnosis of acute and possibly persistent RV infection. RV-specific IgM determinations have been used in the early postnatal period to confirm congenital RV infection (33, 37). Detection of RV-specific IgA antibodies in serum has been of interest with respect to their associations either with prevention of reinfection (14) or as indicators of persistent RV infection (29). Unfortunately, assays for RV-specific IgM and IgA antibodies often lack sensitivity and specificity because of interference from high-affinity RV-specific IgG antibodies which may compete for antigen binding (2) or from IgM anti-IgG rheumatoid factors or heterophile antibodies which may give rise to false-positive results, particularly in ELISAs and other solid-phase assays (4, 23, 28, 33, 35). A further problem encountered in determination of RV-specific IgM is reported cross-reactivity of antibodies with other pathogens, such as parvovirus (21), which may be observed in acute-phase sera in particular. As seroconversion to RV-specific IgM is taken as an indicator of RV exposure in pregnant women and may be a criterion for recommending pregnancy termination, it is critical that these problems of sensitivity and specificity be overcome. These potential problems should also be borne in mind when

![FIG. 5. IB analysis of RV-specific IgG, IgM, and IgA antibodies at different intervals following wild RV infection. NC blots of RV proteins separated by nonreducing SDS-PAGE were reacted with representative sera obtained at the following intervals after the appearance of a rubelliform rash: 0 to 6 days (early acute phase) (lanes 1, 5, and 9), 7 to 30 days (acute phase) (lanes 2, 6, and 10), 1 to 11 months (convalescent phase) (lanes 3, 7, and 11), and 1 to 3 years (late convalescent phase) (lanes 4, 8, and 12). Relative positions of molecular weight markers (MW) are indicated. D, E1-E1 and E1-E2 dimers; Cd, C dimer.](http://jcm.asm.org/DownloadedFrom/602x792/10x2-602x792)
interpreting the results of IgM and IgA IB assays. Staphylococcal protein A has been used extensively to adsorb IgG from serum. However, its utility is limited by its failure to bind human IgG3 (25). Protein G purified from group G streptococci binds to all human IgG subclasses (1) and hence is more efficient than staphylococcal protein A adsorption or other methods, such as gel filtration or sucrose density gradient centrifugation (9, 10), in removing IgG from serum. The present study shows that preadsorption of serum with protein G is significantly improved the sensitivity of detecting RV-specific IgM and IgA antibodies by both ELISA and IB, particularly in the presence of high levels of RV-specific IgG antibody. Furthermore, the use of IB allows direct visualization of antibody reactivity with RV proteins, thereby reducing the probability of false-positive interpretation due to nonspecific adherence of antibodies.

Most IB studies reported to date have employed SDS-PAGE under reducing conditions (5, 6, 31, 43) for the analysis of interactions between RV proteins and specific antibodies. The consensus from these studies was that although RV-specific IgG antibodies reacted with all three RV proteins, IgM antibodies reacted preferentially with E1 while IgA antibodies were directed to C protein. By employing the conditions in IB assays, we have demonstrated that anti-E1, -E2, and -C antibodies may be found in the IgG, IgM, and IgA classes. This divergence from the observations of other studies is likely due to the failure to consider the potential impact of reducing agents on RV protein antigenicity, although altered profiles of monomeric versus dimeric E1 and E2 proteins have been observed by others (8, 41). The sensitivity of E1 and E2 to reducing conditions may be due to damage to discontinuous epitopes which are present on the native virus. Katow and Sugiuara (20) observed that 2-mercaptoethanol significantly reduced RV hemagglutinin activity by inducing conformational changes in RV envelope proteins. Both E1 and E2 are rich in cysteine residues (3) which are likely involved in maintaining tertiary structure through intrachain disulfide bonding. Hence, the disruption of disulfide bonds and loss of tertiary structure would be expected to destroy at least some of the antigenicity of E1 and E2, as was observed in the present study. In contrast, C protein contains only two cysteine residues, of which one is involved in intrachain bonding. Thus, it is likely to display mostly linear (continuous) epitopes and hence would be more resistant to reducing treatment, as was also observed in this study. Therefore, we conclude that nonreducing SDS-PAGE should be used routinely in RV IB assays to preserve RV protein antigenicity and to improve assay sensitivity.

It is becoming increasingly evident that more-sensitive and-specific methodology is required for serodiagnosis of recent RV infection and for determining seroconversion after RV vaccination. There are numerous reports in the literature which illustrate dissociation between the results of RV serologic tests and vaccine failure as determined by RV reinfection or associated pathology. For example, it has been shown that certain individuals deemed seronegative by RV hemagglutination inhibition and neutralizing antibody assays had antibodies reactive with electrophoretically purified E2 and C proteins in ELISA with little or no reactivity with E1 protein (26, 39), suggesting previous exposure to RV with failure to develop functional immunity. Other studies (24, 32) have documented reinfecion in rubella hemagglutination inhibition-seropositive individuals who contracted wild RV infection. Also, recent observations of molecular mimicry resulting in RV-induced autoimmunity (45) have increased interest in the fine specificities of the RV immune response. The enhanced sensitivity and specificity afforded by the RV IB assays described herein will be useful not only for detection of recent RV infections and differentiation of borderline-positive seroconversion in the management of congenital RV infection but also for dissecting the fine specificity of the anti-RV antibody response and its relationship to effective immunity.

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