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Received 29 November 1993/Accepted 21 March 1994

Rubella virus (RV), a member of the Togavirus family (20), is the etiological agent of German measles in humans, the only known host. RV infection during pregnancy may result in spontaneous abortion and congenital defects (1). Vaccination with live, attenuated virus has been successful in reducing the incidence of congenital rubella syndrome (29). However, rubella-associated arthritis and the consequence of viral persistence in vaccinees resulting from RV vaccination remain major medical concerns (3). A new approach using RV-like particles (VLPs) is an alternative to conventional attenuated RV vaccines, since these particles contain all of the RV structural proteins and are nonreplicating. RV contains three major structural proteins, capsid protein (C, 33 kDa) and the E2 (42 to 47 kDa) and E1 (57 kDa) glycoproteins (22), that are proteolytically cleaved from a polyprotein precursor translated from a 24S subgenomic RNA in RV-infected cells (23). E2 and E1 are membrane glycoproteins located on the virion exterior (22, 38), and the capsid protein is associated with the genomic RNA, forming the nucleocapsid (22). Glycoprotein E1 is the dominant surface molecule of RV (5, 40). Virus-neutralizing (VN) and hemagglutination (HA) epitopes have been allocated to E1 by using murine monoclonal antibodies (MABS) (4, 11, 35, 39, 40).

Studies of the immune response to RV infection in humans have indicated that E1 is the major target antigen as compared with E2 and C (5, 25, 42). Genetically engineered RV structural proteins have been produced in Escherichia coli (4, 36, 41), insect cells (33), and mammalian cells (2, 13, 30, 32). However, the antigenicity of expressed proteins is either lost or greatly reduced after purification (17, 36). Furthermore, the capacity of expressed E1 protein to elicit antibody responses in animals is dependent on the native conformation of the E1 protein (30, 36).

Although it is known that RV-immune individuals have T cells that proliferate in vitro in the presence of RV, only recently have the specific determinants of this response come under study. Cell-mediated immunodominant domains have been identified within all three structural proteins (5, 6, 18, 19, 24–26). These domains stimulate major histocompatibility complex class II-restricted CD4+ helper T cells (24, 26, 27) or class I-restricted CD8+ cytotoxic T cells (18) in human populations. It appears that a practical approach to produce an efficacious rubella vaccine is to prepare genetically engineered VLPs that contain both linear and conformation-dependent epitopes of RV structural proteins (C, E2, and E1).

It has been proposed that immunogenicity can be achieved by presenting antigens on a polyvalent particle structure. This concept led to the development of chimeric virus (7, 15, 21), VLPs (12), or immunostimulating complexes (34), in which multiple copies of antigen are integrated in a particulate form. These particles have been found to induce both humoral and cell-mediated immune responses, including VN antibodies (12, 15, 21), T helper cells, or cytotoxic T lymphocytes (12, 15, 34) in animals. In this study, we report high-level expression of RV structural proteins in stable, transformed BHK cell lines. Pseudovirions have been found to be assembled and released from cells containing cDNA prepared from 24S subgenomic RNA. Virological and immunological properties of these VLPs have been characterized.

Isolation of BHK cell lines expressing RV structural proteins. Three RV cDNAs were constructed (Fig. 1) and used in the isolation of stable transformed BHK cell lines. These cDNAs encode the capsid protein (C), E2E1 polyprotein precursor (E2E1), or polyprotein precursor for all three structural proteins of RV (24S) (8, 13). The cDNAs were subcloned into the SmaI site of transfer vector pNUT (28), under the control of the metallothionein 1 promoter (Fig. 1). The resultant recombinant plasmids were transfected into BHK cells by the calcium phosphate method (10). Twenty-four hours after transfection, methotrexate (2.5 mM) was added to the culture medium and cells were incubated with this selection medium for 10 days. Methotrexate-resistant colonies were picked and screened for the integration of RV cDNAs into their chromosomes by PCR (31) and for the expression of RV structural proteins by Western immunoblot analysis (37). Isolated cell lines were stable under normal growth conditions, as they retained the capacity to express RV structural proteins after 4 months of continuous culturing. Cell lines were named accord-
FIG. 1. Schematic diagram of the modified pNUT vector and RV cDNAs. (Top) Plasmids used for construction of stable transformed BHK cells. RV cDNAs were cloned into the pNUT vector by using the Smal site which is flanked by the mouse metallothionein promoter (mMT-1) and the 3' polyadenylation sequence of the human growth hormone (hGH 3'). The 3' sequence of hepatitis B virus (HBV 3') allows the integration of plasmid DNA into chromosomes of transfected cells, and the presence of the dihydrofolate reductase (DHFR) cDNA permits the selection of transformed BHK cells with high concentrations of methotrexate. SV 40, simian virus 40 early promoter. (Bottom) Diagrammatic representation of RV cDNAs used in the construction of recombinant plasmids. The translation initiation site (ATG) from RV capsid protein was used in all constructs. The putative signal peptides and membrane anchor domains of E2 and E1 are indicated □ and ■, respectively.

Expression of RV structural proteins and assembly of VLPs in stable transformed cells. The expression of RV structural proteins from stable transformed cell lines was analyzed by immunoblotting (37). Monolayers of stable transformed cells were incubated with medium containing 30 μM zinc sulfate for 12 h to induce the expression of RV structural proteins from the promoter. Samples from medium and cell lysates were collected and directly subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to nitrocellulose membranes and probed with human anti-RV serum. In BHK-C cells, an intracellular protein species with molecular size of 34 kDa was observed (Fig. 2A, lanes C). This protein may represent the capsid protein of RV. In BHK-E2E1 cells, protein species corresponding to the endoplasmic reticulum and Golgi forms of RV E2 (13) and E1 glycoproteins were found in the cell lysate but not in the medium (Fig. 2A, lanes E2E1), indicating that the E2E1 polyprotein precursor was synthesized and proteolytically processed to give rise to E2 and E1 proteins. In BHK-24S cells, protein species corresponding to the C, E2, and E1 proteins of RV were present in the cell lysate as well as in the medium (Fig. 2A, lanes 24S), suggesting that the integrated cDNA of 24S RNA was active in directing the synthesis of RV structural proteins and these structural proteins were released from the cells. The secretion of RV structural proteins from BHK-24S increased with time and was linear over a period of 24 h under ZnSO4 induction (Fig. 2C).

The secretion of RV structural proteins into the medium was found to be dependent on the coexpression of C, E2, and E1, suggesting that these proteins are assembled into subviral particles prior to their release from the cells. To examine this possibility, medium from BHK-24S and RV-infected cells was subjected to ultracentrifugation (350,000 × g for 20 min) in the presence or absence of 1% of nonionic detergent Nonidet P-40. Resuspended pellets were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with human anti-RV serum. In the absence of Nonidet P-40, C, E2, and E1 were detected in the pellets from BHK-24S and
RV-infected cells (Fig. 2B, lanes 2 and 3). In the presence of Nonidet P-40, E1 and E2 glycoproteins remained in the supernatant after ultracentrifugation (not shown), although trace amounts of C were found in the pellet (Fig. 2B, lane 1). Thus, the assembled viral proteins are secreted as particles that sediment in a gravitational field. To confirm that proteins E1, E2, and C assembled into VLPs, samples from pelleted VLPs were centrifuged for 16 h at 90,000 x g through a density gradient of 20 to 50% sucrose. VLPs were recovered in fractions with densities of 1.17 to 1.19 g/ml, similar to that of native RV virion (1.175 to 1.20 g/ml) (14).

The morphology of the VLPs was analyzed by employing conventional electron microscopic techniques with routine Epon embedding of fixed BHK-24S cells. The VLPs found in BHK-24S cells were comparable in size to RV particles (60 nm) (Fig. 3A) and indistinguishable in appearance, with an electron-dense core surrounded by an envelope (Fig. 3B, C, and D). These particles were located predominantly within the vacuoles in the juxtanuclear region (Fig. 3D) or cytoplasm (Fig. 3C), which may represent the Golgi structure. Some particles were distributed in the cytoplasm (Fig. 3B), not associated with any membrane structure. Such particles were not observed in BHK-E2E1 or BHK-C cells (data not shown). Taken together, it is evident that VLPs were indeed assembled intracellularly prior to their release from the cells.

Antigenicity of the VLPs. HA activity of the VLPs was examined and compared with that of RV particles. Media were collected from BHK-24S cells at 12, 36, and 60 h after induction. RV virions were harvested from culture medium of RV-infected cells at an interval of 24 h over a period of 72 h starting 48 h postinfection. Equal amounts of medium from BHK-24S or RV-infected cells were subjected to high-speed centrifugation, and the pelleted VLPs or RVs were suspended in phosphate-buffered saline. HA assay was performed using a heparin-manganese chloride procedure (16), and the HA titer

FIG. 2. (A) Immunoblot analysis of proteins expressed in transformed BHK cells. Monolayers of BHK-C, BHK-E2E1, or BHK-24S cells were incubated with serum-free medium in the presence of 30 μM zinc sulfate for 12 h. Culture media were collected, and cell monolayers were lysed with RIPA buffer (50 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% Na deoxycholate). Samples were directly subjected to SDS-PAGE and immunoblotting. (B) Immunoblot analysis of proteins sedimented by ultracentrifugation. Samples from medium of induced BHK-24S (lanes 1 and 2) or RV-infected BHK cells (lane 3) were centrifuged at 350,000 x g for 20 min in the absence (lanes 2 and 3) or presence (lane 1) of 1% nonionic detergent Nonidet P-40. The pellets were resuspended in RIPA buffer and analyzed by SDS-PAGE and immunoblotting. The positions of RV structural proteins are indicated. The molecular weight markers (in thousands) are included for reference. (C) Time course of VLPs secretion from BHK-24S cells. Expression of RV structural proteins was induced by the addition of ZnSO₄ (30 μM in the culture medium). Culture medium was collected, and cells were lysed at the indicated times (hours postinduction). Samples from medium were subjected to centrifugation at 350,000 x g for 20 min and resuspended in RIPA buffer. The resuspended pellets and the cell lysates were analyzed by SDS-PAGE and immunoblotting. The positions of RV structural proteins are indicated.

FIG. 3. Electron microscopic analysis of the VLPs in BHK-24S cells. RV-infected BHK cells (A) or induced BHK-24S cells (B, C, and D) were fixed with formaldehyde-glutaraldehyde, postfixed with osmium tetroxide, ethanol dehydrated, and Epon embedded. Thin sections were analyzed by electron microscopy after staining. Arrows indicate VLPs or RV particles. Nu, nucleus.
was expressed as the end point of serial dilutions of sera at which erythrocyte aggregation was observed. The VLPs from BHK-24S cells displayed HA activity of 64, while RV particles retained HA activity when diluted to 1/32. This difference is due to the higher yield of VLPs from induced BHK-24S cells compared with that of RV from infected cells.

To evaluate the antigenicity of VLPs compared with that of RV, equal amounts of RV or VLPs (with respect to HA units) were used in each assay. Table 1 shows the antibody binding activities of VLPs and RV in immunoblot and enzyme-linked immunosorbent assay (ELISA) analysis using 12 MAb s against RV E1, E2, or C. Two of the E2 MAb s showed differences between the VLPs and RV in Western blotting (Table 1), and VLPs displayed a higher ELISA titer with MAB against C protein than did RV (Table 1). VLPs were also used in a solid-phase immunoassay to measure the immunoglobulin G response in humans. With 200 human serum samples, it was found that the correlation coefficient between the VLPs and whole RV antigens was 0.96 by a nonparametric regression analysis method (data not shown). This indicated that the immunogenic determinants on the VLPs resemble those of authentic RV.

**Immunogenicity of the VLPs.** To evaluate the immunogenic properties of the VLPs, we immunized mice (BALB/c, four in each group) with RV, RV, or soluble E1 protein expressed in transfected BHK cells. To quantify the amount of immunogens used in immunization, purified particles or soluble E1 protein was measured by ELISA using E1-specific MAb s. The same amounts of antigens (equivalent to 250 HA units) were emulsified in Freund’s complete adjuvant and used to immunize mice. Mice received three additional injections of antigens in Freund’s incomplete adjuvant at 3-week intervals. Mice were bled, and sera were collected for analysis. The presence of anti-RV antibodies was determined by radioimmunoprecipitation. As shown in Fig. 4, mice immunized with the VLPs produced antibodies against all three structural proteins of RV (Fig. 4, lanes 3), as did mice immunized with RV (Fig. 4, lane 4). Mice immunized with E1 protein also developed some anti-E1 antibody response (Fig. 4, lane 2).

ELISA was used to quantify the antibody titers against each of the RV structural proteins, by using individual purified RV structural proteins expressed in SF9 cells infected with baculovirus recombinants (unpublished data) as antigens. In the sera from mice immunized with VLPs, a significantly higher anti-C antibody titer was found, whereas anti-E1 and E2 antibody titers were slightly lower (Table 2). The biological functions of these antibodies were analyzed. Sera from VLP-immunized mice displayed VN activities (Table 2) as determined by plaque reduction assays (9). HA-inhibiting activities were also present in the sera from mice immunized with the VLPs, as well as in the sera from mice immunized with RV (Table 2). These results suggested that although VLPs were less active in inducing overall anti-E1 and E2 antibodies compared with RV, they induced the production of both VN and HA-inhibiting antibodies.

We have also determined cell-mediated immune responses against RV in VLP-immunized mice in a lymphocyte proliferation assay (5, 26). Lymphocyte proliferative responses of mice were determined in vitro by direct stimulation of lymphocytes with UV-inactivated RV or individual RV structural proteins (C, E2, and E1) purified from recombinant baculovirus-infected insect cells. Lymphocytes from VLP-immunized mice responded strongly to UV-inactivated RV as well as to the individual RV structural proteins in a dose-dependent manner (Fig. 5).

The assembly of RV virions involves at least two major steps: encapsidation and envelopment of nucleocapsids. In RV,

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**Table 1. Immunoreactivity of VLPs with RV-specific MAb s**

<table>
<thead>
<tr>
<th>MAb</th>
<th>Reactivity in Western blot(a) with:</th>
<th>ELISA titer(b) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>VLP</td>
</tr>
<tr>
<td>H15C22(C)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H32C43(E1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21B9H(E1)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3D5D(E1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14B1F(E1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3D9F(E1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16A10E(E1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E2-2(E2)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E2-4(E2)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E2-3(E2)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E2-6(E2)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H46C64(E2)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(a\) MAb s were used at a dilution of 1:100 for ascitic fluid or 1:5 for tissue culture supernatant. +, positive reactivity; -, negative reactivity.

\(b\) Highest dilution of antibodies yielding an optical density at 405 nm two times higher than background.

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**Figure 4.** Radioimmunoprecipitation of RV structural proteins expressed in COS cells. COS cells were transfected with pCMV5-24S (13), labelled with \(^{35}\)S methionine, and lysed. RV structural proteins were recovered from cell lysates with mouse anti-RV antibodies prebound to Sepharose 4B-protein A beads as previously described (13) and separated by SDS-PAGE. Sera were from mice immunized with E1 protein (lane 2), VLPs (lane 3), or RV (lane 4) or from preimmune mice (lane 1). The positions of RV structural proteins are indicated.

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**Table 2. Comparison of antibody titers of sera from mice immunized with different RV antigens**

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>ELISA titer to RV protein(a)</th>
<th>VN titer(b)</th>
<th>HA-inhibitory titer(c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>E2</td>
<td>E1</td>
</tr>
<tr>
<td>E1</td>
<td>&lt;10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>RV</td>
<td>40</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>VLP</td>
<td>320</td>
<td>40</td>
<td>80</td>
</tr>
</tbody>
</table>

\(a\) Individual RV structural proteins (C, E2, and E1) were purified from SF9 cells infected with baculovirus recombinants expressing each RV structural protein (unpublished data).

\(b\) Reciprocal of the highest antibody dilution that showed 50% reduction in plaque formation.

\(c\) Reciprocal of the highest antibody dilution that inhibited HA.
Encapsulation occurs in the cytoplasm as newly synthesized capsid protein interacts with genomic RNA to form icosahedral nucleocapsids. The packaging of genomic RNA into the nucleocapsid is believed to be a specific event, as the 40S genomic RNA but not the 24S subgenomic RNA is packaged into the RV virion (23). Recently, a stretch of 31 nucleotides on the 5' end of the RV genome has been identified as responsible for the binding of the genomic RNA to the capsid protein in vitro (unpublished data). Employing reverse transcription combined with PCR (31), we failed to detect any RV-specific RNA in the pseudovirion secreted from stable BHK-24S cells (data not shown), suggesting that capsid proteins can interact with each other and form a nucleocapsid-like structure. The VLPs were found to have a higher ELISA titer with a C-specific MAb (Table 1) and to elicit a stronger anti-C antibody response in mice than those from RV (Table 2), implying that the VLPs contain more C protein than RV because of either its relative amount or conformational exposure in the particles. Although the pseudovirions do not contain RV-specific RNA, we cannot rule out the possibility that they package some cellular RNAs or even DNAs into the nucleocapsid.

Incorporation of nucleocapsid into the membrane envelope to form virus particles is a poorly understood event in virus assembly. We found that in BHK-C and BHK-E2E1 cells, no RV proteins were released into the medium (Fig. 2A). In BHK-24S cells, all three structural proteins were present in the medium as the result of subviral particle formation and egress (Fig. 2). These data strongly suggest that the interaction between glycoproteins and the nucleocapsid is the driving force for the assembly and release of the VLPs. This interaction has been defined to occur between the cytoplasmic tail of glycoprotein E1 and the capsid protein. Deletion of the cytoplasmic domain of RV E1 abolished the delivery of the VLPs into the medium (12A).

Besides being a useful tool to study RV assembly, BHK-24S cells in which VLPs are steadily assembled and released can be used as a potential source for mass production of rubella antigens at low cost under inducing conditions. BHK-24S cells continuously produce VLPs for up to 5 days without cell lysis when 30 μM ZnSO₄ is present in the medium and for up to 1 month in DME/F12 ( Gibco) medium. VLPs can be harvested daily from the medium, which is replaced with fresh medium after harvesting. Depending on the methods used to quantitate the yields of the VLPs and RV from culture medium, the yield of VLPs was found to be 2 times higher than that of RV in an HA assay, 5 times higher in ELISAs using human sera, and more than 10 times higher by protein quantitation using silver staining after gel electrophoresis (data not shown).

For immunogenicity studies, the VLPs were found to be significantly more active than the soluble E1 protein in inducing antibody responses in mice, especially for the production of VN and HA-inhibiting activity. The VLPs also evoked a cell-mediated immune response to RV and RV structural proteins. This is believed to be important in providing protective immunity against RV infection. Preliminary results have shown that CD4+ T cells may be the major effector in cell-mediated immune responses elicited by the VLPs in mice, although CD8+ T cells may be also involved (unpublished data). A study of the phenotype of the effector cells in proliferation assays is in progress. The VLPs are composed of all three structural proteins of RV, which makes them similar to RV regarding antigen presentation. Our studies suggest that the VLPs may serve as a candidate for safe vaccine development.

We are grateful to J. Wolinsky and J. Safford for providing anti-RV Mabs and to R. Palmeter for providing the pNUT vector. Special thanks to M. Weiss for technical assistance on electron microscopy and to C. Mauracher for performing solid-phase immunoassay using the VLPs.

This work was supported by a grant from the Medical Research Council of Canada (to S.G.). Z.Q. is the recipient of a British Columbia Children's Hospital Foundation studentship. T.C.H. is supported by a Medical Research Council of Canada postdoctoral fellowship. S.G. is an investigator of the British Columbia Children's Hospital Foundation.

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