Review Article

Rubella Virus and Birth Defects: Molecular Insights into the Viral Teratogenesis at the Cellular Level

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Received 16 March 2004; Accepted 22 April 2004

BACKGROUND: In utero rubella virus (RV) infection of a fetus can result in birth defects that are often collectively referred to as congenital rubella syndrome (CRS). In extreme cases, fetal death can occur. In spite of the availability of a safe and effective vaccine against rubella, recent worldwide estimates are that more than 100,000 infants are born with CRS annually. **RECENT PROGRESS:** Recently, several significant findings in the field of cell biology, as well as in the RV replication and virus-cell interactions, have originated from the authors' laboratory, and other researchers have provided insights into RV teratogenesis. It has been shown that 1) an RV protein induces cell-cycle arrest by generating a subpopulation of tetraploid nuclei (i.e., 4N DNA) cells, perhaps representative of the tetraploid state following S phase in the cell cycle, due to its interaction with citron-K kinase (CK); 2) RV infection induces apoptosis in cell culture, and 3) CK functional perturbations lead to tetraploidy, followed by apoptosis, in specific cell types. **CONCLUSIONS:** Based on several similarities between known RV-associated fetal and cellular manifestations and CK deficiency–associated phenotypes, it is reasonable to postulate that P90-CK interaction in RV-infected cells interferes with CK function and induces cell-cycle arrest following S phase in a subpopulation, perhaps representative of tetraploid stage, which could lead to subsequent apoptosis in RV infection. Taking all these observations to the fetal organogenesis level, it is plausible that P90-CK interaction could perhaps be one of the initial steps in RV infection–induced apoptosis-associated fetal birth defects in utero. *Birth Defects Research (Part A) 70:431–437, 2004.*

INTRODUCTION

Rubella virus (RV) is a human teratogen (Gregg, 1941; Frey, 1994; Lee and Bowden, 2000; Chantler et al., 2001) that can cause a spectrum of birth defects in the developing fetus, if the viral infection is acquired in the early months (first trimester) of pregnancy. Some rubella-associated defects are associated with second trimester infection and the types and frequency of defects are associated with the time of maternal infection. Also, the rubella infection of the fetus persists for months and infection of the eye may persist for months after birth. Preventing rubella infections, therefore, is a significant aspect of public health policy. In utero infection of a fetus with RV, in extreme cases, causes death of the fetus. The spectrum of birth defects range from blindness, deafness, and congenital heart disease, to mental retardation and central nervous system (CNS) complications (Table 1), which are often collectively referred to as congenital rubella syndrome (CRS) (Lambert et al., 1965). Estimates are that 10–25% of nonimmunized women of childbearing age are susceptible to RV infection (Ray, 1987), and the incidence of birth defects in babies born to women infected with RV during the first trimester of gestation reaches 80–90% (Frey, 1994; Webster, 1998; Dwyer et al., 2001). On the other hand, rubella causes only a mild, self-limited illness with fever

Grant sponsor: Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (US FDA) (laboratory and research support to CDA).
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Published online 25 June 2004 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bdra.20045

Table 1
Congenital Rubella Clinical Manifestations

Eyes
Retinopathy
Cataracts
Microphthalmos
Ears
Uni- or bi-lateral deafness
Deafness-associated speech defects
Heart
Patent ductus arteriosus
Pulmonary arterial stenosis
Ventricular septal defects
Central nervous system
Mental retardation
Central auditory defects
Sensoneural defects
General
Low birth weight
Micrognathia
Fetal loss (rare)
Transient neonatal effects
Adenopathies
Bony radiolucencies
Hematospenomegaly
Meningoencephalitis
Thrombocytopenia with or, without purpura
Late-emerging/developmental
Chronic diarrhea
Late onset of intestinal pneumonitis (3–12 months age)
Insulin-dependent diabetes mellitus

Adapted from Dudgeon (1975); Cooper (1985); Cutts et al. (1997).

and rash in infected children and adults (Cutts et al., 1997; Chantler et al., 2001).

The worldwide rubella pandemic in 1962–1965 clearly demonstrated the public health importance of reducing CRS and paved the way for development of a rubella vaccine. This rubella pandemic caused 11,000 fetal deaths and 20,000 cases of CRS-affected infants in the United States alone (Orenstein et al., 1984). With the introduction of a safe and effective rubella vaccine into childhood immunization schedules, the incidence of CRS in the United States and other developed nations has gone down substantially in the postvaccine era. On the contrary, the incidence of CRS remains very high in developing countries. Worldwide, it is estimated that there are more than 100,000 infants born with CRS annually (Robertson et al., 2003). Many developing countries around the world still do not have a mandatory program to vaccinate women of childbearing age, or have initiated such programs only recently. A recent report suggests that rubella has disproportionately affected the non–U.S.-born Hispanic population in the United States due to poor immunization levels in the population. This is evident, as 23 (77%) of the 30 infants with CRS reported between 1997 and 2000 were born to non–U.S.-born Hispanic mothers (Centers for Disease Control and Prevention, 1998, 2000, 2001; Reef et al., 2000; Danovaro-Holliday et al., 2003). Moreover, it is estimated that foreign-born workers in certain U.S. industries appear to be at increased risk for rubella, which clearly suggests that overall, rubella is still a public health concern in the United States as well. Although the consequences of RV infections in the first trimester have been well documented, the specific virus-induced effects have remained undefined and the explicit pathway leading to teratogenicity remains unclear (Lee and Bowden, 2000).

Clinical Features and Pathogenesis of Rubella Virus

Humans are the only known natural hosts for RV, and primary infection from person-to-person occurs by respiratory transmission. The virus replicates primarily in the nasopharynx and viremia begins five to seven days postinfection. The placenta and fetus can be infected during viremia. Rubella infection persists for 12–23 days (average, 14 days) with a prodrome of low-grade fever and lymphadenopathy in the second week, followed by macropapular rash 14–17 days after exposure. Complications associated with rubella infection are uncommon but tend to occur more frequently in adults than in children, and range from arthritis and arthralgia to encephalitis and hemorrhagic manifestations. There are several excellent reviews and book chapters in textbooks that provide detailed information on the clinical manifestations of CRS (Hanshaw et al., 1985; South and Sever, 1985; Gilbert, 1991; Best and Banatvala, 1995).

Genetic Organization of Rubella Virus

The identification, classification, and characterization of the RV genome in a number of laboratories enhanced our understanding of the basic functions of RV structural and nonstructural proteins (Frey, 1994). The virus belongs to the *Rubivirus* genus of the *Togaviridae* family of viruses (Chantler et al., 2001). The virion has a ~10-kb long single-stranded positive sense polyadenylated RNA genome, which also serves as an mRNA following a series of events that lead to the uncoating of the viral particle upon entry into cells (Frey, 1994; Atreya et al., 1996). This genomic mRNA (Fig. 1) is translated to provide two nonstructural proteins (P150 and P90), which upon viral infection, allow the synthesis of a subgenomic mRNA required for the synthesis of the viral capsid and two surface glycoproteins, E1 and E2 (Forng and Frey, 1995; Chantler et al., 2001). In the infected cell, new virus particles are assembled following encapsidation of the ribonucleocapsid (viral RNA-capsid protein complex) within a lipid bilayer envelope containing the two viral structural surface glycoproteins (E1 and E2) (Frey, 1994; Chantler et al., 2001).

Animal Models

Attempts to develop an animal model of CRS have not contributed much to the understanding of the underlying mechanisms of CRS and its pathogenesis (Parkman et al., 1965; Sever et al., 1966; Delahunt and Rieser, 1967; Cotlier et al., 1968; Rorke et al., 1968; Amstey, 1969; Kono et al., 1969; Avila et al., 1972; Patterson et al., 1973; Machado et al., 1976; Menser et al., 1978; Rayfield et al., 1986; Atkins et al., 1994; Webster, 1998). There are no reliable animal model systems for the study of clinically symptomatic RV infection (Domegan and Atkins, 2002). In contrast, recent studies focusing on RV gene/protein interactions with the host have provided some initial insight into how a "simple" RNA virus could cause devastating birth defects in the infected fetus during early development.

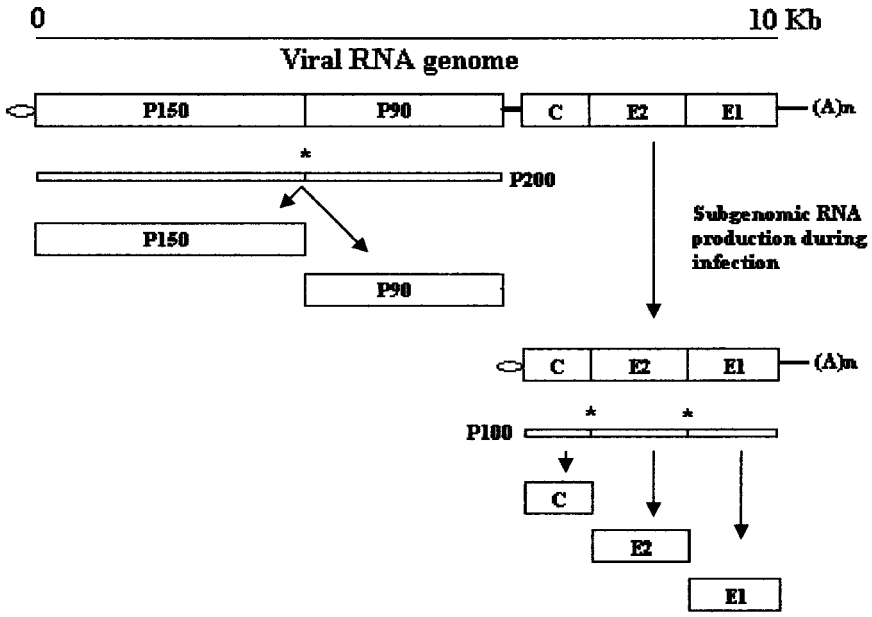

Figure 1. Schematic representation of rubella virus (RV) genome organization. A polyprotein, P200, is encoded by the 5′ nonstructural open reading frame of the RV genomic RNA. Upon translation of this 5′ portion of the RNA in the cell, P200 is produced, which undergoes *cis* cleavage due to the protease activity associated with P150 C-terminal region. This process produces two nonstructural proteins, P150 and P90. The P150 contains the amino acid motifs for methyltransferase, papain-like cysteine protease, and helicase. The P90 is the viral replicase protein replicase. The RV structural proteins are synthesized from a 24-S subgenomic RNA in the infected cell. The subgenomic RNA is translated in to a polyprotein, P100. The P100 undergoes distinct posttranslational modifications and is processed in the cell to produce functional capsid (C), E2, and E1 structural proteins. The protruding structure depicted on the left in genomic and subgenomic RNA is the cap structure.

Recent Advances in Rubella Virus Molecular and Cellular Biology Relevant to Teratogenesis

Viruses are intracellular pathogens whose survival in nature is dependent upon their successful intricate relationships with their cognate hosts. Elucidating the specifics of these viral-host interactions at the molecular level is currently a topic of great interest. Research from the authors' laboratory and others have examined how RV interacts with the host cells (Atreya et al., 1996, 1998, 2004; Duncan et al., 1999, 2000; Forng and Atreya, 1999; Hofmann et al., 1999; Megyeri et al., 1999; Pugachev and Frey, 1998b; Beatch and Hobman, 2000; Matrinez and Zapata, 2002; Mohan et al., 2002; Domegan and Atkins, 2002). RV, which encodes a limited number of proteins (two nonstructural and three structural proteins), may provide a simple system with which to study how molecular interactions of individual viral genes with host cell components can lead to teratogenesis at the cellular level.

Current research is proceeding along two major complementary lines. The first line of research, originated from the authors' laboratory, has identified two key cellular proteins (which normally regulate host cell-cycle and cytokinesis) that interact with a RV nonstructural protein (P90) during infection. The second line of research, from other laboratories, is the identification of RV infection-associated programmed cell death, or apoptosis, in virus-infected cells in culture. Since a role for programmed cell death in CRS has been proposed (Pugachev and Frey, 1998a), the above two lines of research suggest a possible overall mechanism whereby perturbation of cellular events and loss of cells could form the basis for teratogenesis. These two lines of research and their potential implications in CRS are described herein.

Rubella Virus–Host Cell Interactions

Several studies have suggested that RV-infected cells grow and divide slowly compared to uninfected cells. RV-infected diploid cell lines derived from human lung, kidney, or pituitary cease to grow within a few passages, which has been attributed to mitotic inhibition either due to an unknown protein present in the culture supernatants of the infected cells (Frey, 1994; Plotkin et al., 1965; Hoskins and Plotkin, 1967; Plotkin and Vaheri, 1967; Boue and Boue, 1969) or perhaps due to rubella-induced chromosomal breaks (Chang et al., 1966). Subsequently, it has been reported that RV-infected cells in culture exhibit disruption of actin filaments, which could explain mitotic inhibition associated with RV infection (Bowden et al., 1987). Relevant to these observations, we recently identified two cellular proteins that interact with the nonstructural RV protein, P90 (Atreya et al., 1996, 1998, 2004; Forng and Atreya, 1999). One is a cell-cycle regulatory protein, the retinoblastoma protein (RB), and the other is a cytokinesis regulatory protein, the citron-K kinase (CK). Details of these viral-cellular protein interactions and possible relevance to teratogenesis are discussed below.

Rubella Virus P90 Interacts with the Cellular Retinoblastoma Tumor Suppressor Protein (RB) during the Viral Infection

Histopathological analysis of the tissues from different organs of fetuses with CRS revealed teratogenic effects, such as reduced cell size and number compared to controls, which was suggestive of mitotic inhibition (Frey, 1994; Lee and Bowden, 2000). Malformation and pathological changes similar to those observed in human cases were also found in experimentally infected animals such as rabbits, hamsters, rats, and ferrets (Cotlier et al., 1968: Kono et al., 1969; London et al., 1970). A number of studies have shown that cells infected with RV grow more slowly than uninfected cells, perhaps due to inhibition of mitosis and exhibit chromosomal breakage (Plotkin and Vaheri, 1967). However, in spite of the accumulated evidence that RV can affect growth of cultured cells as well as the fetus, the mode of action and the actual molecular mechanism by which the virus causes cell growth retardation still remains

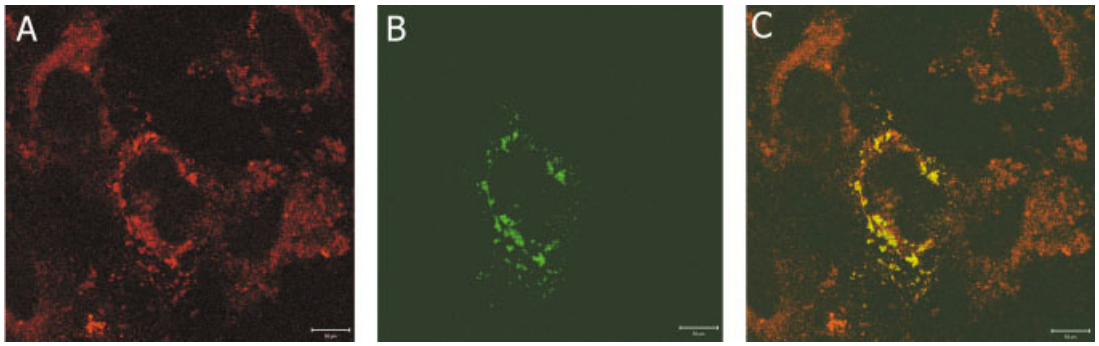

Figure 2. Demonstration of association of rubella virus (RV) P90 with GFP-tagged CK in RV-infected cells. Vero 76 cells were infected with RV at a multiplicity of infection (MOI) of one plaque-forming unit (pfu) per cell in T75 culture flasks for four to five days, and then trypsinized and transferred to chamber slides for transfection with GFP-CK plasmid and subsequent immunostaining and confocal image analysis as described (Mohan et al., 2002; Atreya et al., 2004). The P90 demonstrated a cytoplasmic particulate distribution (**A**; red). The transfected GFP-CK expression pattern was also cytoplasmic and particulate in nature (**B**; green). Both proteins demonstrated colocalization as shown (**C**; yellow), indicating that P90 expressed during RV infection clearly associates with CK under physiological conditions in the cells. Details are as described in Atreya et al. (2004). Scale bar, 50 μm; GFP, green fluorescent protein.

unknown. Birth defects are also common when human cytomegalovirus (HCMV) infects fetuses (Pass, 2001). One of the HCMV proteins (IE2 86) interacts with RB (Fortunato et al., 1997). HCMV infection in cell culture stimulates premature cellular DNA synthesis, leading to chromosomal damage and mitotic arrest of the cell, possibly in G_2/M (Kamiya et al., 1986; Jault et al., 1995). These effects have been implicated in HCMV-induced teratogenesis. Because RV and HCMV both cause teratogenesis, we postulated that a RV-encoded protein might also interact with RB in the cytoplasm and disrupt fetal growth. In a comparative sequence analysis of RV nonstructural proteins, we identified a functional RB pocket-binding motif (LxCxE) on rubella viral protein, P90 (the viral replicase) and demonstrated that P90 binds RB both in vitro and in vivo. The LxCxE motif in P90 is required for efficient viral replication and deletion of this motif from the rubella genome was found to be lethal (Atreya et al., 1998; Forng and Atreya, 1999). In a RB null (−/−) mouse fibroblast cell line, virus production was lower than in a RB wild-type (+/+) mouse cell line (Atreya et al., 1998), suggesting that P90 can interact with other members of the RB family of proteins when RB was not expressed in a cell (Atreya et al., 1998). These results suggest that RB, or one of its family members, serves as an obligatory host factor in RV replication; as a consequence of this obligate nature of the interaction, when the viral P90 binds to RB, it perturbs normal cell cycle regulatory functions of RB, resulting in cell growth aberrations. One caveat is that RV is a cytoplasmic virus with no nuclear phase and the RB protein is mostly confined to the nucleus, although a diffused cytoplasmic location of RB has also been reported in mitotic cells at a frequency of less than 3% (Mittnacht and Weinberg, 1991). Therefore, it remains to be experimentally proven that either RV P90 undergoes a short, transient nuclear phase, or the reportedly small quantity of cytoplasmic RB and/or its family members is in fact sufficient to bring about the perturbations described above. If this is resolved, the specific P90-RB interaction could explain, in molecular terms, the previous observations by several groups that RV infected cells cease to grow after few passages (Plotkin et al., 1965; Hoskins and Plotkin, 1967; Plotkin and Vaheri, 1967; Boue and Boue, 1969).

P90 Associates with the Cellular Cytokinesis Regulator, Citron-K Kinase during RV Infection and Induces Cell Cycle Aberrations

We recently identified a novel interaction between RV replicase P90 protein and a cytokinesis-regulatory protein, CK, which is cytoplasmic in location (Atreya et al., 2004). Our analysis found that full-length P90 binds CK and in RV-infected cells, P90 colocalizes with CK in the cytoplasm (Fig. 2). Furthermore, during RV infection as well as cellular expression of P90 alone, a discrete subpopulation of cells containing tetraploid nuclei (i.e., 4N DNA) content were identified, representative of tetraploid status, indicating that these cells are arrested in the cell cycle following S phase, suggesting that cellular expression of viral P90 during RV infection perturbs cytokinesis. Aberrations in cytokinesis and subsequent apoptosis do occur in specific cell types when the CK gene is knocked out, or its regulatory function is perturbed. CK is the only known downstream target of cellular Rho, and the small GTPases of the Rho family are crucial regulators of cytoplasmic formation of actin structures during cytokinesis (Di Cunto et al., 1998, 2000, 2002; Madaule et al., 1998; Fujisawa et al., 1998; Eda et al., 2001; Glotzer, 2001; Liu et al., 2003). CK-knockout mice exhibit programmed cell death of neuroblasts and a subset of malformative syndromes of the CNS, possibly as a direct consequence of the cell division–associated abnormalities due to lack of CK expression (Di Cunto et al., 2000, 2002). Loss of CK function either due to the gene deletion as in one-week-old *flathead* mutant rat, or in CK-knockout mice, causes significant increases of G2 tetraploid nuclei (i.e., 4N DNA) in their hepatocytes, prior to apoptosis (Liu et al., 2003). Because RV P90 binds CK and also induces a 4N state representative of tetraploidy, it is conceivable that downstream events could culminate in apoptosis of the virus-infected cells, as described below.

Rubella Virus Infection Induces Apoptosis in Cell Culture

A consistent hallmark of CRS is fetal growth retardation or underdevelopment (Frey, 1994; Lee and Bowden, 2000). In many primary and continuous cell lines, RV grows slowly to low titers with little or no sign of cytopathic

Table 2
Similarities in Cellular Manifestation of Citron-K Deficiency and Rubella Virus Infection

Citron-K deficiency	Reference	Rubella virus infection	Reference
Apoptosis	DiCunto et al. (2000, 2002)	Apoptosis	Pugachev and Frey (1998b); Duncan et al. (1999, 2000); Hofmann et al. (1999); Megyeri et al. (1999); Domegan and Atkins (2002)
Cytokinesis failure	DiCunto et al. (2000, 2002)	Cell growth inhibition	Chang et al. (1966); Hoskin and Plotkin (1967); Plotkin and Vaheri (1967); Bowden et al. (1987); Pugachev and Frey (1998a)
Reduction in central nervous system growth	DiCunto et al. (2000, 2002)	Central nervous system complications	Dudgeon (1975); Cooper (1985); Atkins et al. (1994); Lee and Bowden (2000)
Induces tetraploidy	DiCunto et al. (2000); Eda et al. (2001)	Induces tetraploidy	This study

effects (CPE), whereas it reaches high titers and causes CPE in Vero cells (African Green monkey kidney cells) (Frey, 1994). The virus-induced CPE and subsequent cell death were attributed to caspase-3–dependent programmed cell death (Pugachev and Frey, 1998a). The viral genetic determinants associated with cell death were mapped to the nonstructural proteins (NSP) and a speculation was made that the inducer of apoptosis may be one of the NSPs, or some virus function associated with the NSPs (Pugachev et al., 1997; Pugachev and Frey, 1998b). Following this first report of RV-induced apoptosis, several laboratories also reported caspase-3–dependent induction of apoptosis using several cell lines, including a human lung carcinoma cell line, and different laboratory strains of RV (Hoffmann et al., 1999; Megyeri et al., 1999; Duncan et al., 1999, 2000; Domegan and Atkins, 2002). In two of these reports, rubella-induced apoptosis has been suggested to be p53-independent (Hofmann et al., 1999; Domegan and Atkins, 2002). It has been proposed that during congenital rubella infection it is possible that RV destroys precursor cells by induction of apoptosis (Pugachev and Frey, 1998b). Thus, demonstrating RV-associated apoptosis at the cell culture level provides a link between the documented CRS pathology of fetuses exposed to rubella infection during the first trimester (Tondury and Smith, 1966) and cellular interactions between viral and cellular proteins.

Now, we know that 1) RV P90 protein induces cell-cycle arrest by generating a subpopulation of 4N cells, perhaps representative of the tetraploid state following S phase in the cell cycle, due to its interaction with CK; 2) RV infection induces apoptosis in cell culture; and 3) CK deficiencies by way of gene knockout or mutations in the gene leads to tetraploidy, followed by apoptosis in specific cell types. The similarities between known RV-associated fetal and cellular manifestations and CK deficiency–associated phenotype (Table 2) suggest that P90-CK interaction in the infected cells disrupts the normal function of CK and induces cell-cycle arrest following S phase in a subpopulation, perhaps representative of tetraploid stage, which could lead to subsequent apoptosis in RV infection. Data shown here, together with other studies on CK (Di Cunto et al., 2000, 2002; Liu et al., 2003), support the working hypothesis that the P90-CK interaction could be one of the initial steps in RV infection-induced apoptosis-associated fetal birth defects in utero. Apoptosis by RV requires high concentrations of RV nonstructural proteins (Pugachev and Frey, 1998a). One of these nonstructural proteins, P90, is capable of binding to cellular protein CK. This could induce cell-cycle arrest, followed by accumulation of cells with 4N DNA (suggestive of tetraploidy), which could trigger apoptosis. Drawing an analogy from the consequences of perturbation of the CK function, it is conceivable that accumulation of cells with 4N DNA, due to P90-CK interaction, is a "CK functional perturbation" event prior to their culmination in apoptosis. With this knowledge, one could now interpret the previous report of the correlation of RV replication on focal clones of cells during critical stages in the ontogeny of specific fetal organs and associated wide range of abnormalities as in fact being due to the loss of such focal clones as a result of apoptosis following cytokinesis aberrations.

As described above, there is considerable optimism that it may be possible to explain the teratogenic effects of a simple RNA virus in terms of molecular interactions between viral and cellular proteins. Progress in this area is attributable to the convergence of several independent lines of research. Knowing the kind of devastating lifelong effects that rubella can inflict on a developing fetus, it is exciting to know that some of the key cellular proteins that regulate cell growth and cell division are identified as the targets of the RV P90 protein. This opens a path for identifying drugs that could interdict the P90 cellular protein interactions in vivo, in appropriate cases, as a part of rubella-associated CRS intervention. The lack of an appropriate animal model makes it difficult to correlate CRS with some of the molecular surrogates. Given that rubella P90 interacts with cellular RB, it is reasonable to rationalize that this interaction perturbs cell growth by interfering with the interactions in which RB could have participated, if it were not associated with P90. A similar rationale is applicable to the fact that the P90 also interacts with the cytokinesis regulator, CK. It is possible that there may be more than one pathway by which RV affects cell growth. So far, there are no reports of physiological and functional relationships between cellular RB and CK, although it is quite plausible. These recent studies on RV may lead us a step closer toward understanding molecular mechanisms

underlying RV-associated teratogenesis at the cellular level.

ACKNOWLEDGMENTS

We thank Drs. R. Lundquist and P.L. Atreya for critical review of the manuscript.

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