ELUCIDATION OF ANTIGENIC EPITOPEs ON THE RUBELLA VIRUS E1 GLYCOPROTEIN

by

Helena Hojung Chaye

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We accept this thesis as conforming to the required standard.

The University of British Columbia 1993

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Department of PATHOLOGY (Ph.D. in GENETICS)

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Rubella virus (RV), a positive-stranded RNA virus, is the only member of the genus Rubivirus within the Togaviridae family. The virus consists of a host derived lipid bilayer membrane, membrane glycoprotein spikes, and the C protein which together with viral RNA forms the icosahedral nucleocapsid. Although clinical rubella is a relatively mild disease, RV remains an important human pathogen because of its teratogenic effects in utero which can result in new born infants with congenital rubella syndrome (CRS). Complications such as polyarticular arthralgia and arthritis following vaccination or infection are common and rare cases of progressive panencephalitis have been reported.

To obtain a better understanding of the immunopathology of RV infection, the degree of antigenicity of the structural proteins in humans were examined for both cellular and humoral immune responses. It was found that of the structural proteins, E1 glycoprotein was the dominant antigen recognized by the study population sampled from normal individuals with no history of rubella associated conditions. It was also found that the CRS patients had immune responses distinct from that of the normal population. In CRS patients E2 was the dominant antigen to which both cellular and humoral immune responses were elicited. The implications of these observation with respect to proposed mechanisms of persistent infection are discussed.

Twenty three synthetic peptides spanning the entire E1 sequence were screened with human peripheral blood lymphocytes and the corresponding sera to examine the distribution of antigenic domains. E1 glycoprotein was also the target of viral neutralizing (VN) and hemagglutinin (HA) epitope mapping studies. Deletion mutants of E1 were constructed from E1 cDNAs and expressed in vitro, in COS cells and in E.coli. The mutants were screened with monoclonal antibodies with VN and HA inhibiting activities. The mutants containing the functional epitopes were further studied by using synthetic peptides. HA epitope was mapped to amino acid residues E1\textsubscript{214} to E1\textsubscript{240} while two VN epitopes mapped to amino acid residues E1\textsubscript{214} to E1\textsubscript{233} and E1\textsubscript{219} to E1\textsubscript{233}. The potential use of the defined epitopes in the development of subunit vaccine is discussed.
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<th>Full Form</th>
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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary Units</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-Bromo-4-Chloro-3-indol Phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CB</td>
<td>conjugate buffer</td>
</tr>
<tr>
<td>CIC</td>
<td>circulating immune complex</td>
</tr>
<tr>
<td>CRS</td>
<td>Congenital Rubella Syndrome</td>
</tr>
<tr>
<td>CTP</td>
<td>cytidine triphosphate</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>ddNTPs</td>
<td>dideoxynucleoside triphosphates</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleoside triphosphates</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>endo H</td>
<td>endo-β-N-acetylglicosaminidase H</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FMDV</td>
<td>Foot and Mouth Disease Virus</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HAI</td>
<td>hemagglutination inhibiting</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis Virus</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility complex</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
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<td>millimolar</td>
</tr>
<tr>
<td>moi</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MMR</td>
<td>mumps, measles, rubella vaccine</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro-Blue Tetrazolium</td>
</tr>
<tr>
<td>ns</td>
<td>non-structural</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>RAA</td>
<td>Rubella Associated Arthritis</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory Syncytial Virus</td>
</tr>
<tr>
<td>RV</td>
<td>Rubella Virus</td>
</tr>
<tr>
<td>S</td>
<td>Svedberg Unit</td>
</tr>
<tr>
<td>SB</td>
<td>sample buffer</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest Virus</td>
</tr>
<tr>
<td>SI</td>
<td>stimulation index</td>
</tr>
<tr>
<td>SV</td>
<td>Sindbis Virus</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TTP</td>
<td>thymidine triphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
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This thesis is dedicated to my family ... Mom, Dad, John and Mikey.
1. INTRODUCTION

Rubella virus (RV) causes a relatively mild German measles disease most common in children. When contracted in utero, RV can cause a wide variety of birth defects, collectively described as congenital rubella syndrome (CRS) (Oxford and Obery, 1985). Moreover, there is an increasing evidence that RV is a significant human pathogen involved in panencephalitis (Townsend et al., 1975) and polyarticular arthritis (Chantler et al., 1981; 1982; 1985).

Presently available rubella vaccines were developed with limited information regarding the genetics of the virus and the molecular basis for its virulence. Complications such as polyarticular arthralgia and arthritis following vaccination are common (Chantler et al., 1982). Rare cases of progressive panencephalitis have also been attributed to adverse complications resulting from vaccination (Marvin, 1975; Townsend et al., 1975). In addition to these problems, RV grows to relatively low titre and its structural proteins are difficult to purify. Thus, in light of these problems, the need for a new approach to designing a safer and effective rubella vaccine is evident. This thesis will discuss the methodology with which immunologically functional epitopes were elucidated and the potential role of these epitopes in the RV vaccine development.

1.1 RUBELLA VIRUS

1.1.1. Classification

RV is the only member of the genus Rubivirus in the Togaviridae family (Porterfield et al., 1978) which also include Alphavirus, Pestivirus and Arterivirus. Togaviruses are
enveloped RNA viruses whose genome consists of a single molecule of single-stranded positive polarity RNA complexed with a single species protein to form an icosahedral nucleocapsid. Surrounding the nucleocapsid is a host cell-derived lipid bilayer in which glycoprotein spike complexes are embedded.

1.1.2. Morphology

Electron microscopy studies of RV have shown that the virus is spherical and approximately 60-70 nm in diameter with a 30 nm dense core enveloped by a lipid bilayer (Murphy et al., 1968; Von Bonsdorff and Vaheri, 1969). Spikes of 5-8 nm in length on the surface of the virion are associated with hemagglutinin activity (Holmes et al., 1969).

The site of budding seems to vary with the host cell type. Maturation in BHK-21 cells occur primarily in cytoplasmic vesicles, Golgi and vacuoles, and to a lesser extent at the plasma membrane. In Vero cells, however, virions bud exclusively from the plasma membrane (Bardeletti et al., 1979).

1.1.3. Nucleic Acid and Genome Organization and Replication

The RV genome is an infectious single-stranded RNA molecule with a sedimentation rate of 40S (Vaheri and Hovi, 1972). Infected cells contain a 40S genomic and a 24S subgenomic mRNA both of which are capped and polyadenylated (Oker-Blom et al., 1984; Kalkinnen et al., 1984). The subgenomic 24S mRNA corresponds to the 3' one-third of the 40S RNA and encodes the structural proteins E1, E2 and C (Oker-Blom et al., 1984). The sequence for the 24S subgenomic mRNA is known for wild type M33
(Clarke et al., 1987, 1988) and Therein strain (Frey et al., 1986; Frey and Marr, 1988) as well as for vaccine strains RA27/3 and HPV77 (Nakhasi et al., 1989a; Zheng et al., 1989). In comparison to M33 strain, 31 amino acid changes in RA27/3 and 5 amino acid changes in HPV77 were found. Whether these changes are important for attenuation is yet to be determined.

The RV genome is 9757 nucleotides in length and has a G/C content of 69.5% (Dominguez et al., 1990). It contains two long open reading frames (ORF’s): a 5’ proximal ORF of 6656 nucleotides and a 3’ proximal ORF of 3189 nucleotides. The genome organization is similar to that of alphaviruses. Sequences homologous to three highly conserved regions among alphaviruses were found on the RV genomic RNA: a stem-loop structure at the 5’ end of the genome, a 51 nucleotide sequence near the 5’ end of the genome and a 20 nucleotide sequence at the subgenomic RNA start site (Dominguez et al., 1990).

The 24S subgenomic mRNA is 3346 nucleotides in length and contains a 3189 nucleotide of open reading frame encoding NH$_2$-C- E2-E1-COOH (Frey and Marr, 1988). The nucleotide sequences of 24S mRNA from various RV strains indicate 95% homology, although little homology was found with the alphavirus subgenomic mRNA (Frey and Marr, 1988).

The first event leading to the expression of the structural proteins is the synthesis of 24S subgenomic mRNA from the negative sense template intermediate (Oker-Blom, 1984). The 24S mRNA encodes a 110,000 dalton polypeptide (p110) that is proteolytically processed to yield three structural proteins C, E2, and E1 (Oker-Blom et
al., 1983) (Fig. 2). The capsid protein C, a non-glycosylated protein of 33 kD, is rich in basic amino acids and proline (Clarke et al., 1987). E1 (58 kD) and E2 (42-47 kD) are both type I membrane glycoproteins comprise the viral spikes located on the virion surface (Oker-Blom et al., 1983). C protein remains in the cytoplasm whereas the E2 and E1 proteins are co-translationally translocated into the endoplasmic lumen.

![Genomic RNA Diagram](image)

**Fig. 1.** Schematic representation of the synthesis and processing of the structural proteins of RV. ■ = non-glycosylated E1 and E2; = glycosylated E2; = glycosylated E1 (from Wolinsky, 1990).

1.1.4. Non-structural Proteins

The non-structural proteins are encoded by the 5' two-thirds of the 40S genome (Dominguez et al., 1990). Amino acid comparisons between the non-structural proteins of RV and alphaviruses revealed only one short (122 amino acid) region of significant homology indicating that these viruses are distantly related. This region of homology is located at the N-terminus of nsP3 in the alphavirus genome (Fig. 1). RV non-structural
protein ORF contains two global amino acid motifs (helicase and replicase motifs) which are conserved in a large number of positive polarity RNA viruses (Dominguez et al., 1990). The order of the helicase motif and the nsP3 homology region in the RV and alphaviruses is reversed with respect to each other indicating a genetic rearrangement during the evolution of the viruses. To date there is no data pertaining to the RV non-structural proteins beyond the genetic organization. The RV non-structural proteins are yet to be defined and analyzed but are thought to be similar to those of alphaviruses.

Fig. 2. Comparative diagram of the genomes of RV (RUB) and Sindbis virus (SIN). The location within the non-structural protein ORF of regions of nucleotide homology (51 nucleotide conserved region and SG (structural genes) RNA start site conserved regions encoding homologous amino acid sequence (helicase motif, SIN and RUB homology, and replicase motif) are shown. ORFs are denoted by boxes and untranslated regions by lines (from Dominguez et al., 1990).
Nucleotide sequence analysis of Semliki Forest Virus (SFV) and Sindbis Virus (SV) genomic RNAs revealed that four genes encode the non-structural proteins (Strauss et al., 1984; Takkinen, 1986). Translation begins at an AUG codon, 60-80 nucleotides 5' to the cap site (Ou et al., 1983). The non-structural proteins are synthesized as two polyprotein precursors which are then cleaved to produce the four non-structural proteins (Schlessinger and Schlessinger, 1990). One or more of these non-structural proteins must possess an RNA dependent RNA polymerase activity whose functions include initiation and elongation of the full length positive and negative sense RNA and positive sense 26S subgenomic RNA (Lemm and Rice, 1993), as well as capping and methylation (Schlessinger and Schlessinger 1990).

Bowden and Westaway (1985) found other polypeptides of 150,000 and 87,000 daltons in RV infected cells. They also identified several other minor RV specific proteins with sizes ranging from 17,000 to 111,000 daltons. Much work is required to elucidate the identity and functions of these proteins before the molecular mechanism for replication can be defined.

1.1.5. Structural Proteins

1.1.5.1. Capsid Protein

The capsid (C) protein is associated with the 40S genomic RNA to form nucleocapsids (Vaheri and Hovi, 1972). Sequence analysis of RV cDNA indicate that C protein is rich in arginine and proline (Clarke et al., 1987). C is often detected as a doublet on SDS-PAGE under reducing conditions, differing in less than 1 kD. This
phenomenon may occur as a result of two closely spaced translation initiation sites
separated by 7 amino acid residues (Clarke et al., 1987). Under non-reducing conditions,
C runs in a dimeric form with an apparent molecular weight of 66 kD suggesting disulfide
bond linkages in native proteins (Mauracher et al., 1991).

Unlike alphaviruses, RV capsid has no inherent autoprotease activity (Clarke et al.,
1987; McDonald et al., 1991). Studies with monoclonal antibodies have mapped four non-
overlapping epitopes, the significance of which is not yet clear (Waxham and Wolinsky,
1985a, b).

1.1.5.2. E2 Glycoprotein

RV E2 glycoprotein forms the spike complex on the virion surface and migrates as
a diffuse band (42-47 kD) on a reducing SDS-PAGE. E2 synthesized in the presence of
tunicamycin has a molecular weight of 30 kD indicating that it is heavily glycosylated
(Oker-Blom et al., 1983; Clarke et al., 1988). The number of potential N-linked
glycosylation sites vary in different strains of RV as determined by nucleic acid
sequences. There are three N-linked glycosylation sites in M33 and HPV77, whereas
both Therein and RA27/3 contain four glycosylation sites (Clarke et al., 1987; Vidgren et
al., 1987; Frey and Marr, 1988; Nakhasi et al., 1989). The carbohydrate moieties on RV
glycoproteins are of the complex endo-H resistant type (Oker-Blom et al., 1983), and
sialiated O-linked sugars are also present on E2 (Lundstrom et al., 1991).

Biological functions of E2 are not clearly defined. E2 is necessary for transport of
E1 to the Golgi and cell surface (Hobman et al., 1990). In addition, strain specific
epitopes and a neutralizing epitope have been found on E2 (Dorsett et al., 1985; Green and Dorsett, 1986).

Due to the poor immunogenicity of E2, few monoclonal antibodies have been isolated (Green and Dorsett, 1986; Waxham and Wolinsky, 1985a, b). Digestion of intact virions with mixed glycosidases indicates that the carbohydrate moieties on E2 are less accessible than those on E1 (Ho-Terry and Cohen, 1984), suggesting that E2 may be buried under E1 in the spike complex.

1.1.5.3. E1 Glycoprotein

E1 (58 kD) is the most well studied RV structural protein. Post translational modifications of E1 include fatty acid acylation in the C-terminal region (Waxham and Wolinsky, 1985a; Hobman et al., 1990) as well as the N-linked glycans (Frey et al., 1986; Clarke et al., 1987; Terry et al., 1988; Hobman et al., 1991). The glycosylation of E1 is thought to stabilize the conformation of E1 in its biologically and immunologically functional state (Ho-Terry et al., 1984).

E1 contains the hemagglutinin activity as well as a number of neutralization domains (Waxham and Wolinsky, 1983, 1985a; Green and Dorsett, 1986). Using trypsin and staphylococcus V8 protease digestion, three non-overlapping hemagglutinin domains were mapped between residues 245-285 of E1 (Ho-Terry and Cohen, 1985; Terry et al., 1988). Competitive inhibition assays with a panel of monoclonal antibodies defined six non-overlapping epitopes on E1 with viral neutralizing and/or hemagglutinin activities (Waxham and Wolinsky, 1985a). There are three N-linked glycosylation sites on E1.
(Clarke et al., 1987). The role of carbohydrate in the presentation of antigenic and immunogenic epitopes on E1 were examined by Qiu et al. (1992). They have constructed a panel of vaccinia recombinants expressing glycosylation mutants of E1, and have shown that the single glycosylation mutants (G1, G2 and G3) but not the double mutants (G23) or the triple mutant (G123) were capable of inducing antibodies with viral neutralizing activities. Among the single glycosylation mutants, only G2 and G3 were active in producing antibodies with hemagglutination inhibiting activities. They also observed that all the E1 monoclonal antibodies to E1 used in the study recognized all the glycosylation mutants. This study indicates that although carbohydrate on E1 may not be directly involved in the antigenic structures of E1, it is important in maintaining a stable conformation for expression of immunogenic epitopes.

From the predicted topological structure of the glycoprotein spike complex (Fig. 3), and the localization of hemagglutination and viral neutralization activities on E1, it is thought that E1 mediates binding to the viral receptors on the host cell surface. In addition, immunological studies suggest that antibodies to E1 play a major role in protective immunity to RV (Katow and Suguira, 1985).
Fig 3. Model of the E1/E2 glycoprotein spike of RV. N-linked sugars are indicated by (○) located on both E1 and E2 whereas O-linked sugars (■) are located on E2 only (from Ph.D thesis of C. Mauracher, 1992).
1.1.6. RV Entry

Entry of togaviruses into the host cells is thought to occur via receptor mediated endocytosis (Helenius et. al.,1982). Bound virions accumulate in coated pits which are then endocytosed to form coated vesicles. The vesicles fuse with endosomes resulting in acidification of the vesicles. This causes a conformational change in the viral glycoprotein complex resulting in fusion of the viral envelope with the endosomal membrane (Tycko and Maxfield, 1982).

Although the entry and uncoating of RV have not been studied as extensively as alphaviruses, sufficient evidence suggest that RV also uses the endocytic pathway for cell entry (Vaanananen and Kaariainen, 1980). pH of <5.0 causes structural changes in RV E1 allowing RV membrane to fuse with endosomes (Katow and Suguirra, 1988). The acidic environment of the endosomes is also thought to cause the RV C protein to become hydrophobic resulting in nucleocapsid uncoating in the endosome and releasing the viral RNA into the cytoplasm for replication initiation (Mauracher et al., 1991). To date a specific membrane receptor has not been identified for RV. Regardless of the multiplicity of infection, it has not been possible to infect 100% of a cultured cell monolayer with RV and has led to the speculation that RV receptor is expressed in a cell cycle manner (Hemphill et al.,1988).

1.2. Rubella Pathogenesis and Pathology

1.2.1. Clinical Features

The clinical symptoms of RV infection can range from subclinical to the
characteristic features of adenopathy, general malaise, low grade fever and exanthem (Cooper and Buimovici-Klein, 1985). In more severe cases, the RV infection associated with progressive panencephalitis which manifests 10 to 20 years after the infection (Townsend et al., 1985). The general representation of the RV pathogenesis is shown in Fig. 4. The clinical infection spans only a short portion of the time scale, lasting from shortly before the onset of the rash to shortly after the recession of the characteristic rash. Furthermore, many cases of RV infection pass without clinical symptoms including a discernible rash (Wolinsky, 1990).

Fig. 4. Representation of the pathogenesis of RV infection from the time of infection. HAI=hemagglutinin inhibiting IgG; CF=complex fixing anti-RV IgG (from Cooper and Buimovici-Klein, 1985).
RV infection becomes a more serious medical concern when contracted during the first trimester of pregnancy leading to fetal infection. The virus is highly teratogenic and \textit{in utero} infection often results in a variety of birth defects collectively called congenital rubella syndrome (CRS) (Fig. 5). Cataracts, mental retardation, deafness, congenital heart disease are some of the more common defects (Table I) (Cooper and Krugman, 1969).

![Bar graph showing the relationship between the apparent time of maternal rubella infection and the consequence of the infection for fetal development. The data are based on 422 infants registered in the National Congenital Rubella (from Wolinsky, 1990).](image)

Fig. 5. The relationship between the apparent time of maternal rubella infection and the consequence of the infection for fetal development. The data are based on 422 infants registered in the National Congenital Rubella (from Wolinsky, 1990).
Table I. Congenital feature of Rubella (from Oxford and Obery, 1985).

<table>
<thead>
<tr>
<th>Feature</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrauterine growth retardation (low birth weight)</td>
<td>TC</td>
</tr>
<tr>
<td>Thrombocytopenic purpura</td>
<td>TC</td>
</tr>
<tr>
<td>Hepatosplenomegaly</td>
<td>TC</td>
</tr>
<tr>
<td>Meningoencephalitis</td>
<td>TC</td>
</tr>
<tr>
<td>Bone lesions (radiographic)</td>
<td>TC</td>
</tr>
<tr>
<td>Large anterior fontanelle</td>
<td>TC</td>
</tr>
<tr>
<td>Adenopathy, generalized</td>
<td>TU</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>TU</td>
</tr>
<tr>
<td>Cloudy cornea</td>
<td>TU</td>
</tr>
<tr>
<td>Hemolytic anemia</td>
<td>TU</td>
</tr>
<tr>
<td>Pneumonia due to rubella</td>
<td>TU</td>
</tr>
<tr>
<td>Myocarditis due to rubella</td>
<td>TU</td>
</tr>
<tr>
<td>Deafness, sensorineural</td>
<td>PC (DU)</td>
</tr>
<tr>
<td>Central language disorders</td>
<td>PDC</td>
</tr>
<tr>
<td>Mental retardation</td>
<td>PDC</td>
</tr>
<tr>
<td>Behavioral disorders</td>
<td>PDC</td>
</tr>
<tr>
<td>Spastic diplegia</td>
<td>PC</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>PC</td>
</tr>
<tr>
<td>Pulmonic stenosis</td>
<td>PC (DU)</td>
</tr>
<tr>
<td>Cataract (and microphthalmia)</td>
<td>PC</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>PC</td>
</tr>
<tr>
<td>Glaucoma</td>
<td>PU (DU)</td>
</tr>
<tr>
<td>Severe myopia</td>
<td>PU</td>
</tr>
<tr>
<td>Inguinal hernia</td>
<td>PU</td>
</tr>
<tr>
<td>Cryptorchidism</td>
<td>PU</td>
</tr>
</tbody>
</table>

* Transient (T); permanent (P); developmental (D); common (C); or uncommon (U).

1.2.2. Congenital Rubella Syndrome

Rubella was first described in 1814, but did not attract serious attention until 1942 when Dr. Norman Gregg recognized the teratogenicity of the virus (reviewed in Cooper and Buimovici-Klein, 1985). Following Gregg's initial observation, world wide studies established that a syndrome of defects resulted from intra-uterine infection. Congenital rubella, due to the multitude of symptoms, is clinically complex (Cooper and Buimovici-Klein, 1985).
Prospective studies show that infection during the first four weeks of pregnancy results in congenital defects up to 90% of the infants (Fig. 5). The incidence of congenital defects resulting from intrauterine infection in the first four months of pregnancy has been estimated at between 15% to 50%. Also, intrauterine infections during the first 8 weeks may result in spontaneous abortions at a rate of 20% (Oxford and Obery, 1985). The rate of fetal infection diminishes to an estimated 25-30% following the first trimester (Miller et al., 1982). The severity of birth defects and late onset sequelae correlate strongly with the gestational age of the fetus at infection. The rate of infection and the severity of the defects are independent of the severity of the rubella in the adult (Oxford and Obery, 1985).

The mechanism by which the virus interferes with fetal development is not clear. It has been suggested that RV infection interferes with regular mitotic division and that infected cells secrete factors that inhibit mitosis of cell in culture (Plotkin and Vaheri, 1967). Increased numbers of chromosomal breaks have also been observed in RV infected cells, but it is not clear whether this is a direct consequence of abnormal mitosis. Yoneda et al (1986) observed that cells persistently infected with RV responded poorly to growth factors and produced little collagen in comparison to uninfected cells. This finding is consistent with general observation of organ dysfunction and retarded growth in congenital rubella syndrome (CRS) patients.

The mechanisms proposed are predicated on the hypothesis that RV has established a persistent infection in CRS patients. Virus can be isolated from most organs at birth and is actively secreted in urine, stool and nasopharyngeal secretions in
more than 80% of CRS patients in the first month of life (Cooper and Krugman, 1967). The rate of viral excretion decreases as patients get older, however, viruses have been isolated from CRS patients 20 years and older (Menser et al., 1967; Weil et al., 1975).

Proposed mechanisms for viral persistence are as varied as those for the teratogenicity of the virus. First, Abernathy et al (1990) maintained a RV persistent Vero cell line in the presence of anti-RV antibodies for 45 weeks. The investigators suggest that since RV buds both at the cytoplasmic membrane and into the intracellular vacuoles, the passage of virus to daughter cells occurs during cell division. In this proposed model, the virus avoids exposure to antibodies and is thereby protected from antibody mediated clearance. The same study reported that the persistent virus population treated with antibody was less cytopathic. Accordingly, they proposed that in the presence of anti-serum, the only repository for virus was the infected cells. Those infected with cytopathic virus were killed, resulting in survival of less cytopathic virus population. These results may explain persistent infection of RV in some individuals regardless of the presence of vigorous humoral response.

Contrary to the above, second plausible mechanism for viral persistence arises from the observations of reduced immunity. This reduced immunity coined as immunological tolerance has also been observed in other viral infections. In utero infections leading to viral persistence have been documented with Lymphocytic Choriomeningitis Virus (LCMV) in mice (Traub, 1983), Borna agent in rats (Hirano et al., 1983) and Border Disease virus in sheep (Barlow, 1983). In case of LCMV or Borna agent, viral specific cytotoxic T cell response is absent. RV specific T cell response
studies have shown reduced levels in CRS patients (Buimovici-Klein and Cooper, 1985). The degree of impairment was greater in children infected during the first trimester of gestation. It is possible that the lack of a cytotoxic T cell response allows the virus to persist in vivo. Another study reported that CRS patients have reduced ratios of CD4+/CD8+ in addition to overall reduced levels of RV specific activated T-helper cells (Rabinow et al., 1986). Antigen specific proliferation assay revealed that CRS patients exhibited higher lymphocytic response to E2 than the normal population (Chaye et al., 1992b). The poor immunogenicity of E2 in the normal population may have evolved as a protective mechanism against the observed adverse effects arising from E2 immunity in the CRS patients. This is further supported by the E2 induced autoimmune lymphocytic hypophysitis-like syndrome in hamsters that could be prevented by neonatal thymectomy (Yoon et al., 1991). The inability of CRS patients to produce high affinity anti-rubella IgG (Fitzgerald et al., 1988), the reduced levels of E1 reactive IgG (Katow and Suguiru, 1985) and deletion on HAI IgG (Cooper et al., 1971) have all been proposed as possible mechanisms of decreased antibody mediated viral clearance of RV resulting in viral persistence.

1.2.3. Rubella Associated Arthritis

The pathogenesis of rubella associated arthritis (RAA) either from wild type infection or from vaccination remains a matter of speculation (Ford et al., 1986). RV has been isolated from synovial fluid and in peripheral lymphocytes in individuals with RAA years after infection or immunization (Ogra et al., 1975; Chantler et al., 1985). Such findings have led to the proposal that RV can persist in the cells of synovial fluid or the
synovial membrane. RAA is most commonly associated with peripheral joints (Sauter and Utsinger, 1978). The lower temperature of the peripheral joints may be critical with the establishment of persistent infection since it has been reported that RV readily establishes persistent infections of synovial cell cultures at 32°C but not at 36°C (Cunningham and Fraser, 1985). More recently, Miki and Chantler (1992) have shown that wild type and vaccine strains of RV replicate at different rates in human synovial cell lines and in human synovial membrane organ cultures. They also reported that strains which have been reported to have a higher incidence of RAA exhibited a high degree of synoviotropism. Although persistence of RV seems to be associated with RAA, the pathology remains unclear. RAA may either be a direct consequence of non-lytic strain of RV or an indirect consequence of persistent infection triggered immunopathogenesis.

Circulating immune complexes (CIC) and their deposition in the synovial space is one hypothesis that is favoured in describing the pathogenesis of RAA (Inman et al., 1987). CIC are involved in inflammatory responses of CRS patients (Tardieu et al., 1980) and are most likely responsible for the vessel damage which presents as a rubelliform rash in acute Rubella. Information regarding CIC involvement in RAA is incomplete. No significant increase of CIC was observed in RAA patients compared to healthy rubella vaccinees (Singh et al., 1986). Conversely, others have shown increase of CIC containing anti-RV IgG following RV vaccination (Coyle et al., 1982). However, since no comparative study was done between the healthy and RAA patients, this study is not conclusive.

Reactivity of the virus-specific immunity with self-antigen has been proposed
(personal communication with Dr. J.K. Chantler) describing cross-reactivity of rubella specific epitopes with an unidentified protein derived from synovial epithelium. Another study reported cross-reactivity between pituitary cell proteins and RV glycoproteins E1 and E2 (Yoon et al., 1991). They found that hamsters injected with either E1 or E2 vaccinia recombinants developed autoimmune lymphocytic hypophysitis evidenced by the induction of autoantibodies against pituitary cells and by lymphocytic infiltration of the pituitary. These studies suggest that molecular mimicry may be significant in the mechanism of RAA pathogenesis.

A recent model for the pathogenesis of RAA has involved the role of undefined infectious agents as superantigens (Paliard et al., 1991), and the role of RV in this context is being currently investigated. RV capsid molecule shares structural similarities with the S. aureus enterotoxin superantigen defined by Marrack and Kappler (1990), in that both contain a 8 membered β-pleated barrel as their core structure.

1.2.4. Rubella Vaccine

Between 1965 and 1967, several live attenuated RV strains were developed and tested as vaccines. Of these, HPV77/DE5 and RA27/3 have been licensed in Europe and North America. Currently RA27/3 strain is commonly used as vaccine in Canada and the USA. The mechanism of RV attenuation remains unclear. Sequences of the structural proteins of both the wild type and the vaccine strains revealed only limited differences (Nakhasi et al., 1989a, 1989b). The low virulence and slow replication rates of vaccine strains are thought to be due to a deficit in cell attachment and entry (Nakhasi et al.,
RA27/3 has served well in immunization of children since its licensure. However, it has been associated with significant adverse effects in a significant proportion of adolescent and adult female vaccinees (Polk et al., 1982). Acute arthritis is estimated to occur in 13-15% of adult RA27/3 vaccine recipients (Howson and Fineberg, 1992), and cases of severe, chronic arthritis have been reported following the administration of RA27/3 in adult females (Tingle et al., 1986).

1.2.5. Host Response to Viral Infections

Host response to viral infection occurs in two phases (for review see Stites et al., 1984). Initially the response is largely non-antigen specific and involves stimulating the production of interferons and the activation of natural killer (NK) cells. Virus-infected cells are more susceptible than normal cells to NK-lysis. Once virus starts replicating, antigen specific immune responses are triggered. This involves T-cells and B-cells, both of which are derived from bone marrow stem cells. In this section, only the antigen specific immune response to virus infection will be discussed.

B-cells undergo differentiation to plasma cells which secrete the various classes of immunoglobulins. This usually occurs under the influence of activated T-cells. T-cells undergo thymic differentiation and education, during which they mature into different subclasses identifiable by cell surface antigens and by function. The two major T-cell functional subclasses are cytotoxic T lymphocytes (CTLs) and helper T lymphocyte (T_h Cs). CTLs are usually CD8+ and lyse virus infected cells. T_h Cs, which are usually
CD4+, are divided further into two subsets T\(_H\)1 and T\(_H\)2. T\(_H\)1 cells produce gamma-interferon and IL-2 and promote cell-mediated effector responses; whereas T\(_H\)2 cells produce IL-4, IL-5, IL-6 and IL-10, cytokines which produce B-cell development and can augment humoral responses (Scott, 1993).

The immune response to virus infection is generally characterized by the induction of a response from both CTLs and T\(_h\)Cs (Whitton and Oldstone, 1990). Classically described antiviral CTLs are restricted by class I major histocompatibility (MHC) antigens in their recognition of viral antigens, while T\(_h\)Cs are restricted by class II MHC antigens. Expression of MHC class II molecules is restricted to specialized antigen-presenting cells (macrophages, dendritic cells, B-cells), whereas MHC class I molecules are expressed on most cells.

It is widely accepted that T-cell receptors recognize degraded form of viral antigens bound to class I or class II MHC molecules (Williams and Smith, 1990; Teyton et al., 1990; Parham 1990). The processing pathways utilized by endogenously derived and exogenously derived antigens are distinct (Germain, 1988). Briefly, exogenous protein antigens and polypeptides present in a virus (if entry is by endocytosis), are degraded in an acidic environment of the endosome and the resulting processed fragments associate with class II molecules in the trans-Golgi or an endosomal compartment. Endogenously derived viral antigens are synthesized and degraded into fragments in the cytoplasm and subsequently transported into the endoplasmic reticulum for association with class I MHC molecules.

B-cell receptor for antigen is immunoglobulin (Ig), initially expressed as a
membrane protein. During their development, B-cells express IgM on their surface, followed by expression of different subclasses IgG, IgA, IgE upon activation. Once B-cell has switched its subclass from IgM, it becomes committed to secrete the switched subclass of Ig. When B-cell is activated by association with an antigen and by lymphokines secreted by T-cells, it differentiates into mature plasma cells which secrete one class of Igs. Memory cells are also generated.

Antibodies play an important role in the control of virus infection (Whitton and Oldstone, 1990). IgM and IgG can be effective in neutralizing viral infectivity. This may result from the antibody preventing virus attachment to specific cellular receptors by associating with viral antigens that recognize cell receptors. Complexing of viruses with IgG antibody will also facilitate their phagocytosis by macrophages via Fc receptors on these cells.

Both humoral and cellular immune responses function together to clear viruses once infected and to protect the host from future infection. However, it is thought that cytotoxic T-cells are essential in viral clearance. For example, in persistently infected mice by lymphocytic choriomeningitis virus, CTLs were not readily detectable (Jamieson and Ahmed, 1985). This persistence, however, was terminated by adoptive transfer of syngeneic virus-specific MHC-restricted CTLs.
1.2.6. Immune Response to RV

Contact with RV by infection or vaccination initially elicits an IgM response, mostly to E1 (Salonen et al., 1985; Zhang et al., 1991). The IgM response is transient usually lasting for approximately a month and is followed by production of other immunoglobulin classes - IgG, IgE and IgA (Salonen et al., 1985; Zhang et al., 1991). IgG production is the dominant serological response to all three structural proteins. Analysis by both immunoprecipitation and immunoblotting revealed that majority of RV IgG is directed to E1 with lower levels specific for E2 and C (Katow and Suguira, 1985; deMazancourt et al., 1986; Zhang et al., 1991).

IgGs to RV may have hemagglutination inhibiting and viral neutralizing properties (Green and Dorsett, 1986; Waxham and Wolinsky, 1985). The levels of IgG to RV correlate well with hemagglutination inhibiting titres and with neutralizing antibody titres (Stokes et al., 1969) and it is assumed that these responses play a positive role in viral clearance and protection (Waxham and Wolinsky, 1985a). Circulating immune complexes containing RV specific antibody and antigen are frequently found after infection (Ziola et al., 1983) but, in most cases, their presence has not been associated with any of the complications following RV infection or vaccination (Singh et al., 1986).

Much less is known about the importance of cellular responses to RV infection. RV-specific cellular responses have been demonstrated using lymphocyte proliferation assays and lymphocyte mediated cytotoxicity assays (Buimovici-Klein and Cooper, 1985; Vesikari and Buimovici-Klein, 1974; Ilonen and Salmi, 1986). Cell-mediated cytotoxicity has been implicated in pathogenicity of RV infection (Martin et al., 1989). Ilonen and
Salmi (1986) noted that the RV-specific cellular responses are MHC restricted. Similarly, Ou et al. (1992a,b,c) isolated T-cell clones against the E2 glycoprotein and the C protein from RV seropositive individuals and found that HLA restrictions are associated with HLA DR7 and HLA DR4 for E2 and C epitopes respectively.

Early cellular studies have been limited to the responses to whole RV in immune and susceptible individuals (Buimovici-Klein et al., 1979; Vesikari and Buimovici-Klein, 1974; Kauffman et al., 1974). The results of these studies demonstrated development of cellular immune response over time. Only recently RV protein specific lymphocyte proliferation assays have become possible. Ou et al (1992a,b) have mapped T-cell specific epitopes on E2 and C using synthetic peptides. In addition to E2 and C, McCarthy et al (1993) also identified T-cell epitopes on the structural protein E1.

1.3. Epitope Mapping

1.3.1. Epitope mapping using expressed proteins from *E. coli*

Antigenic and/or immunogenic epitopes have been localized by expressing parts of the viral proteins using prokaryotic expression systems and determining the antigenic reactivity of the expressed products. The inserted viral genes are expressed in *E. coli* either as fusion proteins or on their own. The products are usually analyzed either by western blot techniques and/or radio-immunoprecipitations (Rosenberg et al., 1987; Terry et al., 1989; Studier et al., 1990; Wolinsky et al., 1991). A number of functional continuous epitopes on viral proteins have been identified by means of prokaryotic expression vectors such as RV (Terry et al., 1989; Wolinsky et al., 1991; Chaye et al.,
1992a), feline leukemia virus (Nunberg et al., 1984), hepatitis B virus (Offensperger et al., 1985; Milich, 1988), infectious bronchitis virus (Lenstra et al., 1990), and human cytomegalovirus (Kniess et al., 1991).

Prokaryotic expression system may not be the appropriate choice for epitope analysis of all viral proteins. First, because expression levels depend on the growth of the host bacteria, any interference by the viral proteins may result in low levels of expression. For example, inhibition of \textit{E. coli} growth have been observed upon expression of the vesicular stomatitis virus G protein and hepatitis B virus S protein which result in low expression levels (Rose and Shafferman, 1981). Second, the expressed products may lose their biological activity after purification (Kleid et al., 1981). Purification of recombinant proteins require denaturation in a chaotropic agents followed by renaturation. Furthermore, viral proteins expressed in \textit{E. coli} may lack the appropriate post-translational modifications which may influence the tertiary structure of the protein. Thus the lack of these modifications may result in conformation that is no longer functional. The prokaryotic system is therefore most useful for antigens where activity does not depend on conformation or post-translational modifications such as glycosylation or formation of disulfide bonds. However, for antigens which are conformation independent, prokaryotic expression systems can be valuable tools for expression. For example, hepatitis B virus HBcAg produced in \textit{E. coli} is immunogenically active and confers protection against HBV challenge in immunized animals (Milich, 1988).
1.3.2. Epitope mapping using expressed eukaryotic systems

Expression of viral proteins in eukaryotic cell lines is a commonly used system for the analysis of the viral protein, post-translation modifications and the viral assembly (reviewed in Rutgers, 1990). It is also important in the analysis of viral epitopes which are conformation dependent and are influenced by the post-translational modifications. However, for the purposes of subunit vaccine design, expression in eukaryotic cells has had limited success. First, the viral protein domains containing the immunologically functional epitopes should be produced in large quantities. Second, they should be produced in systems that will permit relatively simple purification. Third, the epitopes should be conformation independent such that once purified from the rest of the proteins, they will retain their biological functions.

A number of viral polypeptides have been expressed by using recombinant DNA techniques in a variety of eukaryotic cell systems (Cane and Gould, 1988; Emini et al., 1988; Luckow and Summers, 1989; Putnak et al., 1988). Genetically engineered vaccinia viruses have been used to express epitopes of hepatitis B virus, herpes simplex virus and influenza hemagglutinin (reviewed in Mackett, 1990). Recombinant baculoviruses containing genes for animal virus proteins have also been shown to induce protective immunity in rats and rabbits (Luckow and Summers, 1988). Manipulations of S. cerevisiae genome resulted in expressions of the hepatitis B virus core protein and the HIV gag p55 (reviewed in Rutgers et al., 1990).

The expression efficacy for a particular viral protein appear to depend on the compatibility of the foreign protein to be expressed and the system chosen as well as the
purpose for which the expressed protein will be used. RV structural proteins have been
expressed both in the eukaryotic and in the prokaryotic expression systems. The proteins
expressed in the mammalian cells were used to study processing and transport of the
structural proteins (Clarke et al., 1988; Hobman et al., 1989, 1990). Vaccinia
recombinants were used to study the peripheral blood lymphocyte proliferative response
to the individual structural proteins (Chaye et al., 1992b). Prokaryotic systems were
chosen for the expression of antigens for epitope mapping (Terry et al., 1989; Wolinsky
et al., 1991; Chaye et al., 1992a). Each system chosen were particularly useful for the
purposes of that study.

1.3.3. Epitope mapping using synthetic peptides

Since the advent of automated systems for rapid synthesis of peptides with high
yields, a plethora of antigenic and immunogenic studies of viral proteins have been
reported using synthetic peptides as antigens and as immunogens (Heber-Katz and
Dietzschold, 1986; Milich, 1988; Roehrig et al., 1989). Common methods used to localize
epitopes in proteins have included measuring the cross-reactivity of antibodies raised
against intact protein to synthetic peptides and measuring the cross-reactivity of
monoclonal antibodies to the peptides. These experiments are usually carried out in
enzyme linked immunoassay (ELISA) where peptides are either directly bound to the
plate or are used to competitively inhibit binding of antibodies to proteins already bound
to the plates.

Various approaches in the use of peptides for mapping antigenic sites are used.
One of the methods incorporate results of predictive algorithms that provide amino acid
sequences that will likely form secondary structures such as α-helices or β-turns (Jameson and Wolf, 1988). The most commonly used algorithm is the hydrophilicity plot (Hopp and Woods, 1982). While many of the known antigenic sites have structures that these algorithms predict, not all sites with these secondary structures are antigenic.

Another approach is the measurement of the affinity of antibodies elicited to the native antigen for synthetic peptides (Steward and Howard, 1987). The higher the affinity, the closer the resemblance of the peptide to the determinant expressed on the native protein. It is thought that the degree of affinity reflect the complementarity of the antibody to the peptide. A series of overlapping peptides spanning a region of interest have also been used to map antigenic epitopes. It was found that although B-cell epitopes only consist of 5-7 amino acids, longer peptides provided better binding capacity. Therefore, this approach allows for fine mapping without compromising the binding capacity of the peptides to the antibodies.

Synthetic peptides have also proven to be instrumental in the T-cell epitope mapping (Brett et al., 1991; Fayolle et al., 1991; Ou et al., 1992; Wallace et al., 1991). Generally, a series of overlapping peptides are screened with T-cell clones, T-cell lines or with peripheral blood lymphocytes, in the latter to determine immunodominant epitopes. As with the studies using antibodies, algorithms for T-cell epitopes have been developed (Margalit et al., 1987; Rothbard and Taylor, 1988). Again as with B-cell epitopes, these algorithms are predictive tools and thus the predicted epitopes are not necessarily antigenic, however, they can provide a measure of confirmation once epitopes have been localized.
1.3.4. Functional epitopes of Rubella virus structural proteins

In 1985, Waxham and Wolinsky (1985) mapped hemagglutination and viral neutralization activities to the E1 glycoprotein. Since this study, much effort has been focused on delineating functional epitopes on E1 (Terry et al., 1988, 1989; Lozzi et al., 1990; Wolinsky et al., 1991). There are two prevailing reasons for these efforts. First, at present, serological techniques with whole RV as a target antigen for detection of antibodies to RV are most commonly used for laboratory diagnosis of acute and congenital rubella infections and for determination of rubella immunity. These serological techniques lack defined specificity against antigenic determinants such as hemagglutinin and virus neutralizing epitopes of RV. For example, women, seronegative as measured by HI assay (<1:8), were shown to have moderate levels of RV specific antibodies, measurable by ELISAs using whole RV (Tingle et al., 1983). Sera from CRS patients have higher levels of antibodies directed against E2 but with low or no reactivity to E1 (Chaye et al., 1992b). Since ELISAs employing whole RV fails to distinguish between the various antibody specificities, it is necessary to define the functional epitopes of RV structural proteins for diagnostic assays to assess the immunity against RV.

Second, functional epitopes are essential in the development of a non-infectious rubella vaccine. Subunit vaccines containing only those epitopes which will elicit protective immunity without the adverse side effects can only be achieved by accurately defining important epitopes. Immunosuppressive epitopes and/or autoreactive epitopes can be deleted in the construction of vaccines to limit the potential adverse effects of vaccination.

Numerous methods of viral epitope localization have been developed (Table II)
Epitope analyses of RV E1 have utilized recombinant DNA technology (Terry et al., 1989; deMazancourt and Perricaudet, 1989; Wolinsky et al., 1991), peptide analyses (Lozzie et al., 1990; Terry et al., 1988; Mitchell et al., 1992) and competitive binding assays with monoclonal antibodies (Waxham and Wolinsky, 1985a). Six independent epitopes have been identified which are thought to be important for viral infectivity and hemagglutination (Green and Dorsett, 1986; Waxham and Wolinsky, 1985a). Epitopes that react with monoclonal antibodies that have hemagglutination and viral neutralization activities have been localized to E1 residues E1_{245} to E1_{285} (Terry et al., 1988) and residues E1_{202} to E1_{283} (Wolinsky et al., 1991). The latter group has subsequently narrowed the epitope to E1_{213} to E1_{239} using a set of nested synthetic peptides (Wolinsky et al., 1993).

Protective immunity to viral infection requires activation of helper T cells specific for viral antigens. A T-helper function is required for the production of neutralizing antibodies and the activation of cytotoxic precursors into cytotoxic effector cells (reviewed in Milich, 1989). Ou et al (1992a,b,c, 1993) have identified T-cell epitopes by screening overlapping synthetic peptides with peripheral blood lymphocytes (PBL) from immune donors and subsequently with T-cell lines/clones derived from the PBL of immune donors. They have identified E1_{356}-E1_{377}, E2_{54}-E2_{74} and C_{255}-C_{280} as the immunodominant T-cell epitopes. Furthermore, McCarthy et al (1993) applying essentially the same methodology have identified T-cell epitopes located on all of the structural proteins. List of all T-cell epitopes defined in these studies are summarized in Table III. These studies will provide the basis for future construction of an effective subunit vaccine for RV.
Table II. Methods used to localize epitopes in virus (from Van Regenmortel, 1990)

<table>
<thead>
<tr>
<th>Method</th>
<th>Type of epitope recognized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 X-ray crystallography of antigen-Fab complexes</td>
<td>Discontinuous epitope reacting with homologous antibody</td>
</tr>
<tr>
<td>2 Study of cross-reactive binding of natural or synthetic peptide</td>
<td>Continuous epitope cross-reacting with heterologous antibody</td>
</tr>
<tr>
<td>fragments with viral antibodies</td>
<td></td>
</tr>
<tr>
<td>3 Study of cross-reactive binding of fusion proteins with viral</td>
<td>Cross-reactive continuous epitope</td>
</tr>
<tr>
<td>antibodies</td>
<td></td>
</tr>
<tr>
<td>4 Study of cross-reactive binding of virus with anti-peptide antibodies</td>
<td>Cross-reactive continuous epitope</td>
</tr>
<tr>
<td>5 Analysis of viral mutants with monoclonal antibodies</td>
<td>Neutralization epitopes and discontinuous epitopes</td>
</tr>
<tr>
<td>6 Competitive binding assays with pairs of monoclonal antibodies</td>
<td>Only relative position of epitopes is defined</td>
</tr>
</tbody>
</table>

Table III. Summary of T-cell epitopes of the RV structural proteins.

| E1  | 202-207 | DLVEYYI |
| E1  | 226-239 | HGPDWASPVCQ |
| E1  | 240-247 | PDCSRLVG |
| E1  | 248-261 | ATPERPRLRLVDAD |
| E1  | 272-291 | GEVVWTPVIGSQARKCGLHI |
| E1  | 307-326 | IHAHTTSDPWHPGPGLGLKF |
| E1  | 358-377 | VEGLAPGGGNCHLTNVGEDV |
| E2  | 31-55   | QLPFLGHGDHHGGTLRVCQHYRNAS |
| E2  | 56-75   | DVKOGHWKQGGGWGCYNLS DW |
| E2  | 81-105  | VCHTKHMDFWCVEHDRPPPATPTPL |
| E2  | 54-74   | |
| C   | 9-18    | MEDLQKALEA |
| C   | 64-97   | GNRGRGQRDDWSRAPPPPEERQETRSQTPAPKPS |
| C   | 119-152 | PELGPPTNPQFAAVARGLRPLHDPDEAPTEAC |
| C   | 205-233 | VRAYNQPGACVVRGWGKGERTYAEQDFRV |
| C   | 255-280 | PLPPHTTERIETRSARHPWRIRFGAP |
1.4. Project Rationale and Thesis Objectives

Although the history of Rubella dates back to 1941, little is known about the humoral and cellular immune responses to RV. Due to lack of this information together with the limited understanding of the immunopathology of RV infection, the development of an effective vaccine has been difficult.

The objective of this thesis is to define functional epitopes on the E1 glycoprotein which may be used in the construction of subunit vaccines. Site specific deletion mutants of the E1 glycoprotein expressed both in the eukaryotic and the prokaryotic expression systems were screened with monoclonal antibodies with hemagglutination inhibiting and viral neutralizing activities. The deletion mutants containing HA and VN domains were further divided into synthetic peptides and screened on ELISAs. The human B- and T-cell epitopes on E1 glycoprotein were analyzed and the results were discussed in relation to the HA and the VN epitopes defined with the monoclonal antibodies. The potential use of these epitopes in the construction of subunit vaccines will be discussed.
2. Materials and Methods

2.1. Materials

DNA modifying enzymes and restriction endonucleases were purchased from Bethesda Research Laboratories, Promega Biotec, New England Biolabs, Boehringer Mannheim, Pharmacia and United States Biochemical Corporation. All enzymes were used as specified by the manufacturer unless indicated otherwise. The oligonucleotides HC-1, HC-2, HC-3, HC-4 and HC-5 were synthesized on an Applied Biosystems oligonucleotide synthesizer by T. Atkinson (Biotechnology Laboratory, UBC) (Table IV). Human anti-rubella serum was a gift from Dr. Aubrey Tingle (Department of Pediatrics, University of British Columbia). Mouse monoclonal antibodies to E1 were produced previously in this lab. Tissue culture agents were from Gibco and Sigma. Synthetic E1 peptides were provided by Dr. P. Chong at Connaught Laboratories (Table V). Human peripheral blood lymphocytes representative of asymptomatic immune study group were generously donated by volunteers and the patients samples were provided by Dr. Aubrey Tingle. COS cells were obtained from Dr. David Russell (Department of Molecular Genetics, University of Texas, Dallas). \textit{E.coli} strains DH5α and DH5α F' cells were purchased from BRL and BL21(DE3)/pLysS was given by Dr. William F. Studier (Biology Department, Brookhaven National Laboratory, Upton, New York).

2.2. Methods

2.2.1. Propagation of bacterial strains

\textit{E.coli} strains DH5α and DH5α F' from Bethesda Research Laboratories were used
for the propagation of recombinant clones. DH5α cells containing recombinant plasmids were grown in LB medium (1% tryptone; 0.5% yeast extract; 1% NaCl) containing 100 ug/ml ampicillin (AP) for selection of antibiotic resistance. DH5αF' cells were propagated in 2xYT (1.6% Tryptone, 1% Yeast Extract, 0.5% NaCl). *E.coli* strain BL21(DE3)/pLysS cells were grown in medium with 100 µg/ml ampicillin plus 25 µg/ml chloramphenicol to maintain the plasmid pLysS. For long term storage the bacterial strains were stored in 15% glycerol at -70°C.

### 2.2.2. Preparation of competent cells and transformation

Competent cells were prepared using a method described in Promega Biotec technical bulletin 018. Briefly, *E.coli* cells were grown in 20 ml of LB broth until the absorbance at 600 nm reached 0.15-0.3. Cells were centrifuged at 5000 rpm in a Sorvall SS34 rotor at 4°C for five minutes, and the supernatant was discarded. The bacterial pellet was resuspended in 10 ml of cold solution A (10 mM 3-[N-morpholino] propanesulfonic acid (MOPS) (pH 7.0); 10 mM RbCl), and centrifuged as above. Cells were then resuspended in 10 ml of cold solution B (10 mM MOPS (pH 6.5); 10 mM RbCl; 50 mM CaCl₂) and incubated in ice for 30 minutes. After pelleting the cells as above, cell pellets were resuspended in 1 ml of solution B plus 15% glycerol, and quick frozen in 0.2 ml aliquots in dry ice-ethanol and stored at -70°C.

For plasmid transformation, 0.2 ml of competent cells were incubated on ice with 10-50 ng of plasmid DNA for 30 minutes. After a two minute heat shock at 37°C, 1 ml of LB broth was added to the transformation mixture and the cells were allowed to recover at 37°C for one hour before plating onto selective media. For M13
**Table IV.** Oligonucleotides

<table>
<thead>
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<tbody>
<tr>
<td>HC-1</td>
<td>CCATGGGGGCGATGGCCGATTGGGGC</td>
</tr>
<tr>
<td>HC-2</td>
<td>CCATGGGGAACCAACAGTCCCCGTT</td>
</tr>
<tr>
<td>HC-3</td>
<td>CCATGGGGGCGATGGCCGATTGGCC</td>
</tr>
<tr>
<td>HC-4</td>
<td>CCATGGATGACAATTCGGGCTCC</td>
</tr>
<tr>
<td>HC-5</td>
<td>CCATGGGGGACGCTCTGCGGT</td>
</tr>
</tbody>
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**Table V.** Synthetic peptides screened with MAbs

<table>
<thead>
<tr>
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<th>Sequence</th>
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<tbody>
<tr>
<td>EP11</td>
<td>GQLEVQVPPDPGLVEYIMN</td>
</tr>
<tr>
<td>EP12</td>
<td>IMNYTGNQQRWGLGSPNCH</td>
</tr>
<tr>
<td>EP13</td>
<td>NCHGPDWASPVCQRHSPDCS</td>
</tr>
<tr>
<td>EP14</td>
<td>PDCSRLVATPERPRLRLVDD</td>
</tr>
<tr>
<td>EP15</td>
<td>RLVADDPLLRTAPGPGEVW</td>
</tr>
<tr>
<td>EP24</td>
<td>VPPDPGLVEYIMNYTGN</td>
</tr>
<tr>
<td>EP25</td>
<td>DPGDLVEYIMNYTGNQSRWGLGSPNCHGPDWASP</td>
</tr>
<tr>
<td>EP26</td>
<td>GLGSPNCHGPDWASP</td>
</tr>
</tbody>
</table>
transformations, the cells were plated with 50 µl of 5-bromo-4-chloro-3-indoyl-β-D-galactoside (Xgal) (2%), 10 µl isopropylthio-β-D-galactoside (IPTG) (100 mM), 50 µl fresh exponential DH5αF', and 3 ml soft agarose (0.7% in 2YT at 55°C) on YT without antibiotic.

2.2.3. Growth of transformants and preparation of plasmid DNA

2.2.3.1. Small scale plasmid preparation

Colonies containing plasmids were picked into 2 ml of 100 µg LB-ampicillin (AP) per ml or LB with 100 µg/ml AP and 25 µg/ml chloramphenicol (Cam) and the bacteria were grown to saturation. M13 transformants were grown in 5 ml of YT containing 50 µl of an overnight culture of DH5αF' for 6 hours at 37°C. Plasmid and M13 RF DNA were isolated by the alkaline lysis method. Briefly, bacterial cells from 1.5 ml culture were pelleted for one minute in a microfuge. The pellet was resuspended in 100 µl of 50 mM glucose; 10 mM EDTA; 25 mM Tris-HCl (pH 8.0) and lysed by the addition of 200 µl of 0.2 N NaOH/1% SDS for five minutes at 0°C. Chromosomal DNA and proteins were precipitated by incubating the lysis mixture with 150 µl of cold potassium acetate (3MK⁺; 5M CH₃COO⁻ (pH 4.8)) at 0°C for five minutes, followed by centrifuging in a microfuge for five minutes at 4°C. The supernatant was extracted with an equal volume of phenol:chloroform (1:1), and the DNA was precipitated with two volumes of ethanol at room temperature for five minutes. Plasmid DNA was recovered by centrifugation in a microfuge for five minutes at room temperature, washed in 70% ethanol, dried in a Speed Vac Concentrator, and resuspended in 50 µl of TE containing 20 µg/ml RNase A.
Aliquots were used for restriction analysis or subcloning.

2.2.3.2. Large scale plasmid DNA preparations

The protocol is a procedure obtained from Promega Biotec technical bulletin 009 (developed by Dr. P. Krieg and Dr. D. Melton of Harvard University) with modifications. Cells grown in selective media overnight in 250 ml cultures were pelleted by centrifugation at 5000 rpm in a Sorvall GSA rotor at 4°C for five minutes. The supernatant was discarded and each pellet was resuspended in five ml of 50 mM Glucose; 10 mM EDTA; 25 mM Tris-HCl (pH 8.0) containing 20 mg lysozyme followed by 20 minute incubation on ice. Cells were lysed by addition of 12 ml of 0.2 N NaOH; 1% SDS and incubation on ice for 10 minutes. Chromosomal DNA and proteins were precipitated with eight ml of cold potassium acetate solution (see mini-prep procedure) on ice for 20 minutes, followed by centrifugation at 10,000 rpm in a SS34 rotor at 4°C for 15 minutes. RNase A (100 ug) was added to the cleared lysate followed by incubation at 37°C for 30-45 minutes. The lysate was extracted twice with equal volumes of phenol:chloroform, and the nucleic acids were recovered in siliconized Corex tubes by centrifuging at 10,000 rpm for 10 minutes at RT in a SS34 rotor. The pellet was dried, and dissolved in 1.60 ml of sterile water. DNA was selectively precipitated by addition of 0.4 ml of 4 M NaCl and 2.0 ml 13% polyethylene glycol (PEG mw 8,000), mixing and incubation on ice for 60 minutes. The plasmid DNA was pelleted in siliconized Corex tubes at 10,000 rpm for 15 minutes at 4°C in a SS34 rotor, washed with 70% ethanol, dried and dissolved in TE.
2.2.3.3. Isolation of single-stranded DNA

Five ml of YT media containing 50 ul of overnight culture of DH5αF' cells was inoculated with cored plaques of recombinant M13 phage and was grown for 6 hours at 37°C. 1.5 ml cultures of DH5αF' containing M13mp18 or M13mp19 recombinants were pelleted by centrifugation in a microfuge for 5 minutes at room temperature. The pellet was subjected to the plasmid mini-prep procedure for isolation of RF DNA. Phage were precipitated from the supernatant by addition of 300 µl of 20% PEG6000/2.5 M NaCl, vortexing, and incubating at room temperature for 15 minutes. Phage were pelleted by centrifugation for 15 minutes in the microfuge. The supernatant was discarded and the pellet was resuspended in 0.1 ml TE and extracted once with 50 µl of phenol, once with 50 µl phenol:chloroform (1:1) and finally once with 100 µl of chloroform. The aqueous layer was ethanol precipitated with one tenth volume of 3 M sodium acetate (pH 5.5). The DNA was spun down in a microfuge, washed with 70% ethanol, dried, and resuspended in 30 µl TE.

2.2.4. Expression vectors

The multiple cloning sites of pSPT18 and pSPT19 (Pharmacia) are flanked by oppositely oriented T7 and SP6 RNA polymerase promoters which allow transcription of either strand of inserted DNA (Fig. 6). Synthetic mRNAs from cDNAs cloned into these vectors were used to direct translation in a rabbit reticulocyte lysate system.

For transient expression of RV cDNA in COS cells, pCMV5 (D. Russell, Texas) was used (Fig. 6). This vector directs transcription by the human cytomegalovirus major
immediate early gene promoter. pCMV5 contains the SV40 origin of replication allowing replication in COS cells as well as a prokaryotic origin of replication and ampicillin resistance gene for growth and selection in \textit{E.coli}.

\textit{E.coli} expression vectors pET8c, pET3a,b,c and pET3xa,xb,xc were supplied by Dr. F.W. Studier (New York) (Fig. 7). All pET translation vectors place the cloned cDNA under the control of T7 promoter and an efficient translation initiation signal for the gene 10 protein of T7 phage. The letters ‘a’, ‘b’ and ‘c’ with the pET3 and pET3x vector denote the three reading frames relative to the gene 10 initiation codon. Vectors pET3a,b,c carry a fragment that codes for the first 11 amino acids of the gene 10 resulting in a fusion protein. Translation products from vectors pET3xa, xb, xc are hybrid protein with 261 amino acid fusion domain from the amino-terminus of the gene 10. Vector pET8c allows direct joining of the coding sequence to the gene 10 initiation codon at the Ncol site.

\textit{Vaccinia} recombinants used in the T-cell proliferative studies have been constructed in the laboratory.
Fig. 6. Physical map of the expression vectors pSPt18/19 (Pharmacia) and pCMV5 (Andersson et al., 1989).
Fig. 7. Physical map of the expression vector pET and its derivative (Studier et al., 1990). Vectors ET3a,b,c carry fragment that codes for the first amino acids of the gene 10 and vectors pETxa, xb, xc (not shown in the figure) carry a fragment that codes for the first 261 amino acids of the gene 10.
2.2.5. Construction of deletion mutants

A series of in-frame deletions and truncations were generated by restriction endonuclease subfragment excisions using the natural sites available within the E1 coding sequence in p3’E2/E1 plasmid (Hobman et al., 1988). For expression in COS cells and in E.coli, the appropriate fragments were excised from p3’E2/E1 and subcloned into vectors pCMV5 and pET, respectively. Small fragments of E1 were amplified by polymerase chain reactions prior to subcloning. The constructions of mutant forms of E1 are as follows (Fig. 8):

1. m1: the XhoI fragment (450 nt) was excised from plasmid p3’E2/E1 and product religated.
2. m2: the fragment (560 nt) from BamHI to HindIII sites was removed from p3’E2/E1 and the product religated.
3. m3: the fragment (670 nt) from Smal to HindIII was removed, the ends filled by repair and religated.
4. m4: the fragment (1057 nt) from Ncol to Smal deletion, the ends filled by repair, and religated.
5. m5: the fragment (1147 nt) from Ncol site to BamHI was removed, ends filled by repair, and religated in the presence of BamHI linker (pGGGATCC) to introduce the correct reading frame.
6. m6: the fragment (670 nt) from Smal-HindIII sites was excised from m1, the ends filled by repair, and religated.
7. m7: 787 nucleotide NcoI-XhoI fragment removed from m6.

8. FP1-FP5: cDNA fragments were amplified by the polymerase chain reaction (Erlich, 1989) using synthetic oligonucleotides (Table IV) as shown below, subcloned into pET3xb vector (Fig 7), and sequenced to check for mutations which may have accumulated during the amplification. The synthetic oligonucleotides used in the amplifications were:

FP 1 (CCATGGGGGACACAGTCCCGGCTCTCTGGCGT),
FP 2 (CCATGGGGGAGGCTCTGCGT),
FP 3 (CCATGATGACACATCTGGCGT),
FP 4 (CCATGATGACACATCTGGCGT),
FP 5 (CCATGATGACACATCTGGCGT).

2.2.6. Polymerase chain reaction (PCR):

E1 sequences of FP1 to FP5 (Fig. 8) were amplified by PCR with the DNA thermal cycler (Perkin-Elmer Cetus) (Erlich, 1989). PCR mixtures contained 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 15 mM MgCl₂, 200 μM each of the four dNTP’s; 0.01% gelatin; 0.1% Triton-X-100; 2 units of Taq-Pol; and 1 μl of p3'E2/E1 plasmid; 1 μl of 10 mM primers per
50 µl reaction mix. Thermal cycle parameters were 95°C for two minutes, 60°C for 30 seconds, 72°C for one minute and total of 30 cycles. PCR amplified products were gel purified, T7 DNA polymerase treated and ligated to pET3xb vector restricted with BamHI and blunt ends filled by repaired.

2.2.7. Restriction endonuclease digestions and DNA modifications:

All restriction digestion reactions were performed according to assay conditions specified by the suppliers.

DNA fragments were ligated using T4 DNA ligase in 50 mM Tris-HCl (pH 7.6); 10 mM MgCl₂; 1 mM ATP; 1 mM DTT; 5% (w/v) polyethylene glycol for 2 hours at room temperature. Reactions were diluted five-fold with TE prior to transformation.

DNA fragments with 5' overhangs were blunt ended with E.coli DNA polymerase I Klenow enzyme in 50 mM Tris-HCl (pH 7.2); 10 mM MgSO₄; 10 mM DTT; 50 mM BSA; 80 μM dNTP's for 30 minutes at room temperature. The enzyme was inactivated by heating at 70°C for 5 minutes.

Removal of terminal 5' phosphates from DNA fragments with 5' overhangs was done using calf intestinal alkaline phosphatase (CIP) in 50 mM Tris-HCl (pH 9.0); 1 mM MgCl₂; 0.1 mM ZnCl₂; 1 mM spermidine for two successive 30 minute incubation periods of 15 minute at 37°C and 15 minutes at 56°C. CIP reactions were terminated by addition of 0.3% SDS and phenol:chloroform extraction followed by ethanol precipitation.

Purification of DNA fragments from agarose gels or enzyme reaction mixtures was routinely done using GENECLEAN™. Desired fragments were excised from ethidium
bromide stained TAE agarose gels and the gel matrix was solubilized in 2-3 volumes of saturated sodium iodide at 55°C. DNA was removed from the agarose solutions by vortexing the mixture with a suspension of glassmilk\textsuperscript{TM}, and a brief spin in a microfuge. Contaminants were washed away from the glass bound DNA by three successive washes with cold NaCl/ethanol/water (NEW) solution. The DNA was eluted from the glass beads with TE or water by incubating at 55°C for 3 minutes.

2.2.7. Purification of oligonucleotides:

Deoxyribo-oligonucleotides were synthesized by T. Atkinson (UBC) (Table III). The crude oligonucleotides were purified by electrophoresis through a 20\% acrylamide gel containing 7M urea and 50 mM TBE buffer. The gel slice containing the oligonucleotide was incubated overnight at 37°C in 0.5 M ammonium acetate; 10 mM Mg(OAc)\textsubscript{2}. The mixture was centrifuged to remove the gel slices and the supernatant was concentrated to 2 M ammonium acetate followed by ethanol precipitation. Oligonucleotides were resuspended in TE to use in polymerase chain reactions and as primers in the sequencing reactions. Some of the oligonucleotides were kinased with T4 polynucleotide kinase in 50 mM Tris-HCl (pH 7.5); 10 mM DTT; 10 mM MgCl\textsubscript{2} containing wither 30 μCi [\textsuperscript{32}P]-ATP for screening colonies for recombinants.

2.2.8. Identification of E1 recombinants

2.2.8.1. Colony hybridization

Five recombinants FP1 to FP5 were screened using the colony hybridization protocol described in Maniatis (1982). Using sterile toothpick, colonies were transferred
onto agar plate with Hybond-N™ (Amersham) filter and onto master plate. The plates were inverted and incubated overnight at 37°C. The bacteria colonies were lysed by treating the filter with 10% SDS for 3 minutes. The filter paper was then transferred to Whatman 3MM saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes followed by 5 minute incubation with the neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0)). The filter was air dried colony side up on a sheet of 3 MM paper. The dried filter was placed onto UV transilluminator for 3 minutes to chelate the DNA to the filter paper.

The filters were soaked in 6xSSC for 5 minutes and then transferred to prewashing solution (50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, 10% SDS) for 1 hour at 42°C. Following 1 hour prehybridization at 60°C, filters were hybridized with $^{32}$P-labelled oligonucleotide probes overnight in hybridization solution at 60°C. After three 10 minute washes in 6xSSC at 65°C, the filters were wrapped in saran wrap and autoradiographed. SSC: 0.15M NaCl, 0.015 M NaCitrate (pH 7.0); 10xDenhardt's solution: 0.1% each of ficoll, polyvinylpyrolidone, BSA; Prehybridization solution: 50% formamide; 6xSSC; 5x Denhardt's solution; 0.1% SDS; 100μg/ml denatured, salmon sperm DNA; Hybridization solution: prehybridization solution plus $^{32}$P-labelled probe.

2.2.8.2. Dideoxy sequencing of DNA

Sequenase™ (modified T7 DNA polymerase) was used for sequence determination of the cDNA constructs. The DNA template was annealed to 1 ul of primer in 2 ul of 5X sequencing buffer (5 X buffer=200 mM Tris-HCl (pH 7.5); 50 mM MgCl$_2$; 250 mM
NaCl). The tube was warmed to 65°C for 2 minutes and allowed to cool to room temperature slowly. Two μl of labelling mix (1.5 μM dGTP, 1.5 μM dCTP, 1.5 μM dTTP), 1 μl 0.1 M DTT, 0.5 μl [α-35S]dATP or [α-32P]dATP (10 μCi/μl), and 2 μl Sequenase™ (diluted 1:8 in TE) was added to the template/primer mixture and incubated for 5 minutes at room temperature. The template/primer mixture was distributed (3.5 μl to each tube) to 4 pre-warmed tubes containing 2.5 μl of each of the following:

G: 80 μM of each dATP, dTTP, dCTP, dGTP, 8 μM ddGTP
A: 80 μM of each dATP, dTTP, dCTP, dGTP, 8 μM ddATP
T: 80 μM of each dATP, dTTP, dCTP, dGTP, 8 μM ddTTP
C: 80 μM of each dATP, dTTP, dCTP, dGTP, 8 μM ddCTP

The tubes were incubated for 5 minutes at 37°C and the reactions were terminated by the addition of 4 μl of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The samples were heated to 72°C for 2 minutes immediately prior to loading onto a gel.

2.2.9. Separation of nucleotides (DNA sequencing gel)

Three ul of DNA sequencing reactions were loaded onto 6% and 8% polyacrylamide gels (19:1 acrylamide:bis-methylacrylamide, 8 M urea, 0.06% ammonium persulfate, 20 μl TEMED, 50 mM TBE). Electrophoresis was performed at 1600V, 37 watts. The gels were dried onto Whatman 3 MM paper using vacuum gel drier at 80°C and exposed to X-ray film at room temperature overnight for [α-35S] and for [α-32P], exposure was done at -70°C.
2.2.10. Expression of E1 recombinants

2.2.10.1. In vitro transcription

Plasmid constructs p3'E2/E1, m4, m5 were linearized with HindIII, m2 and m3 were linearized with BamHI and Smal, respectively. Linearized plasmid DNAs were purified using GENECLEAN and templates were added to 50 µl of transcription reaction mixture containing 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 500 U of RNasin (Promega Biotec) per ml, 100 µg of nuclease-free bovine serum albumin per ml, 0.5 mM each ATP, CTP, UTP and 0.05 mM GTP. After 60 minute incubation at 40°C, DNA template was digested with 15 units of DNase I at 37°C for 15 minutes. The newly transcribed RNA templates were extracted with phenol:chloroform, precipitated with ethanol, and stored in diethyl pyrocarbonate (DEPC)-treated water at -70°C.

2.2.10.2. In vitro translation

SP6-derived transcripts were translated in a nuclease-treated rabbit reticulocyte lysate system (Promega) containing 0.02 mM amino acid mixture minus methionine or minus cysteine; [³⁵S]-methionine or [³⁵S]-cysteine at 1200 µCi/ml; RNAsin at 1600 units/ml; and RNA at 40 µg/ml in a 25 µl volume. After incubation at 30°C for 1 hour, the translation products were immunoprecipitated with anti-RV human sera or monoclonal antibodies to E1.
2.2.10.3. Transfection of COS cells

COS cells were transfected with plasmid DNA containing deletion mutants of El (m1, m2, and m3) using method described by Adam and Rose (1985). Subconfluent monolayer of cell grown in Dulbecco modified Eagle medium (DMEM) plus 5% fetal calf serum were washed twice with Tris-saline (25 mM Tris-HCl (pH 7.4), 140 mM NaCl, 3 mM KCl, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$, 0.9 mM Na$_2$HPO$_4$). Cells were incubated with DEAE-dextran (Mr=$5 \times 10^5$; 500 µg/ml) and plasmid DNA (4 µg/ml) in Tris-saline at 37°C for 30 minutes. The DNA solution was then removed and replaced with DMEM plus 40 µM chloroquin for 3 hours at 37°C. After removal of chloroquin solution, the cells were shocked with 10% dimethylsulfoxide/DMEM for 3 minutes at room temperature. Finally, the monolayer was washed three times with Tris-saline and incubated at 37°C for 40 hours in DMEM plus 5% calf serum. The monolayer was scraped and lysed in lysate buffer (25 mM Tris-HCl (pH 7.4); 100 mM NaCl; 1 mM EDTA; 1% Nonidet P-40) containing 1 mM PMSF prior to immunoblotting.

2.2.10.4. Transformation of *E.coli* strain BL21(DE3)/pLysS

Expression of the truncated and deleted El constructs was directed by inducible T7 RNA polymerase engineered in the *E.coli* strain BL21(DE3)/pLysS. This strain contains a copy of T7 RNA polymerase gene located in the chromosome under the control of the inducible lacUV5 promoter, and a plasmid which encodes for constitutive expression of lysozyme and abrogates the need for sonication. Cultures were grown at 37°C in L-broth containing ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml) for selection of plasmid pLysS. T7 RNA polymerase was induced by addition of
isopropylthiogalactoside (IPTG) (0.04 mM) when the culture reached optical density of 0.8-0.99 at 600 nm. Induced cultures were allowed to grow for additional 2 hours at 37°C and were subsequently harvested by centrifugation. The pellets were resuspended in 1/50 volume of DNase I buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM MgSO₄), freeze/thawed twice to lyse the cells and then treated with DNaseI (1mg/ml) for 15 minutes at room temperature. 5-10 µl samples were analyzed by electrophoresis on 12% SDS-PAGE. Expressed proteins were detected by immunoblotting. Expression of recombinants from pET3x vectors were sufficiently high and can be visualized by coomasie brilliant blue staining. Bands corresponding to recombinant proteins were cut out and electroeluted for 3 hours at 10mA using Bio-Rad electroelution apparatus (Electroeluter 422, Bio-Rad, Richmond). The eluates were lyophilized and solubilized in 8M urea (50 mM Tris-HCl (pH 7.5), 5 mM EDTA) by incubating with rocking motion for 1 hour at room temperature. Supernatant was collected following 30 minute centrifugation and analyzed on immunoblots.

2.2.11. Detection of E1 recombinants

2.2.11.1. Monoclonal antibodies

E1 monoclonal antibodies were generated and characterized previously in this laboratory. Properties of the monoclonal antibodies used are summarized in Table VI. 3D9F, 3D9D, and 12B2D were characterized to have hemagglutinin inhibiting (HI) activity 1:16384, 1:8192, and 1:4096, respectively. 21B9H, 12B2D, and 16A10E were found to have viral neutralizing (VN) activity. 21B9H neutralizes both M33 and RA27/3 strains with the addition of complement whereas, 16A10E neutralizes M33 only.
2.2.11.2. Immunoprecipitation

Human polyclonal anti-rubella serum was preincubated with Protein A-Sepharose (Pharmacia) for at least four hours at 4°C in binding buffer (100 mM Tris-HCl (pH 7.4); 400 mM NaCl) with constant mixing. The serum-coated beads were washed twice with binding buffer, and once in lysate buffer (25 mM Tris-HCl (pH 7.4); 100 mM NaCl; 1 mM EDTA; 1% Nonidet P-40). [35S]-labelled antigen from in vitro translation was mixed overnight at 4°C with the coated beads in lysate buffer. Beads were washed once with lysate buffer, twice with wash buffer (25mM triethanolamine; 172 mM NaCl; 1% SDS; 1 mM EDTA), three times with 10 mM Tris-HCl (pH 7.4), and once with water. Antigen-antibody complexes were dissociated from the Protein A-Sepharose by boiling in 1 X SDS dissociation buffer for 5 minutes, vortexing and pelleting the beads by centrifugation. Supernatants were collected and separated on SDS-PAGE, and then fluorographed.
2.2.11.3. Immunoblotting/dot blotting

COS cell lysates and \textit{E.coli} bacterial lysates were separated by SDS-PAGE and transferred to nitrocellulose filters using a Bio-Rad Trans-Blot apparatus for 60 minutes at 65 volts in 25 mM Tris-HCl; 192 mM Glycine (pH 8.3); 20% methanol. The filters were blocked for 60 minutes to overnight in TBS containing 4% powdered skimmed milk. Membranes were then incubated with human anti-RV serum or monoclonal antibodies for 2 hours, washed with TBS/0.3% Tween-20 and treated with goat anti-human or goat anti-mouse IgG conjugated to alkaline phosphatase (BRL) for two hours. Blots were washed as above and developed with NBT (nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indoyl phosphate). All incubations were done at room temperature. For dot blot analysis one µl of purified recombinants expressed in \textit{E.coli} (m7, FP1-FP5) were dotted onto nitrocellulose filters presoaked in TBS. Following overnight air dry, the filter was handled as an immunoblot as described above.

2.2.12. Electrophoresis

2.2.12.1. Separation of DNA fragment

The buffers used in agarose gel electrophoresis were 1XTAE (40 mM Tris-acetate; 1 mM EDTA (pH 8.0) and 1XTBE (89 mM Tris-borate (pH 8.0); 89 mM boric acid; 2 mM EDTA) for separation of small fragments. The gel concentration varied from 1% to 2% agarose with 1 µg/ml ethidium bromide for visualization. DNA samples were diluted to 8% sucrose; 20 mM EDTA (pH 8.0); 0.05% bromophenol blue; 0.05% xylene cyanol and separated by electrophoresis on 10 cm submarine horizontal agarose gels.
2.2.12.2. Separation of protein

Proteins were separated using a discontinuous gel system described by Laemmli (1970). Samples were adjusted to 62.5 mM Tris-HCl (pH 6.8); 10% glycerol; 2% SDS; 2% 2-mercaptoethanol and denatured at 95°C for 3 minutes. Stacking gels consisted of 4% polyacrylamide, and separating gels contained either 10% or 12% polyacrylamide. Gels were run at constant voltage of 100 volts until the markers have run to the desired position. The stacking gel was trimmed away, and the proteins were either fixed in 10% acetic acid for 15 minutes for fluorograph or transferred to nitrocellulose membrane for immunoblot analysis. Fixed gels were immersed in the fluorographic agent Amplify (Amersham) for 15 minutes, dried under vacuum and exposed to X-ray film at -70°C.

Solutions used for electrophoresis:

5X Stacking gel buffer: 0.625 M Tris-HCl (pH 6.8), 0.5% SDS
5X Separating gel buffer: 1.875 M Tris-HCl (pH 8.8), 0.5% SDS
5X Gel running buffer: 0.125 M Tris-HCl; 0.96M glycine, 0.5% SDS (pH 8.3)
Polyacrylamide Stock: 30% acrylamide, 0.8% N’N’-bis methylene acrylamide

Gels were polymerized by adding ammonium persulfate to 0.05% and TEMED (N’N’N’N’-Tetramethylenediamine) to 0.1%.

2.2.12.3. Coomassie blue staining

E1 recombinants expressed in E.coli were separated by SDS-polyacrylamide gels and simultaneously fixed with methanol:glacial acetic acid and stained with Coomassie Brilliant Blue R250 (0.25 g of Coomassie Brilliant Blue R250 is dissolved in 90 ml of
methanol:H$_2$O (1:1 v/v) and 10 ml of glacial acetic acid). Gel was immersed in staining solution and placed on a shaker for 30 minutes. Gel was then destained (destaining solution: 90 ml of methanol:H$_2$O and 10 ml of glacial acetic acid) by changing the destaining solution 3 to 4 times.

2.2.12.4. Electroelution

Polyacrylamide strips of recombinants expressed in *E. coli* were cut into 1 cm sections and electroeluted using a model 422 electroelution attachment of the BioRad Protean II system. Elution buffer (25 mM Tris base, 192 mM Glycine, 0.1% SDS) was filtered through a 0.22 µm filter. Electroeluates were lyophilized and resuspended in 1x sample buffer. Each preparation was assayed by immunoblot for antigenicity.

2.2.12.5. Enzyme linked immunoadsorbant assay (ELISA)

E1 peptides were coated onto Immunon-2 plates (Dynatech, Chantilly VA, USA) in carbonate buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$ (pH 9.5)). Following one hour blocking in 0.5% milk-PBS, the plates were incubated with monoclonal antibodies diluted in 0.5% skim milk-PBS. The two hour incubation was followed by the addition of alkaline phosphatase-conjugated goat anti-mouse IgG antibody (BRL) diluted 1:3000. The plates were developed in substrate buffer (1M Diethanolamine, 5 mM MgCl, 2 mg/ml p-nitrophenylphosphate (pH 9.6)) and read at 405 nm on a Bio-Rad microplate reader (Bio-Rad, Richmond CA, USA).
2.2.13. T-cell proliferation assay

Peripheral blood lymphocytes (PBL) were isolated on Ficoll-Hypaque density gradient (Boyum, 1968) to give $5 \times 10^5$ cells/ml in RPMI 1640 supplemented with 10% autologous plasma. For antigen-specific response, $1 \times 10^5$ cells per well were incubated in 96-well flat bottom plates with varying concentrations of expressed antigens in triplicate. Following 7 day incubation at 37°C with antigen, the cells were pulse labelled with $[^3H]$-thymidine (1µCi/well) for 6 hours, harvested and washed onto glass-fibre filters with distilled water. After the filters were air dried overnight, 3 ml of ACS II (Amersham) scintillation fluid was added to determine the incorporation of $[^3H]$-thymidine.

2.2.14. Antigen preparations for T-cell proliferation assays

2.2.14.1. Vaccinia recombinants

CV-1 cells were infected with vaccinia virus recombinants (E1, E2 and C, prepared in this laboratory) at 5 PFU/cell (Chaye et al., 1992b). At 40 hours post infection, the monolayers were washed with phosphate-buffered saline (PBS), scraped and suspended in MEM (modified Eagle’s medium) media. The isolated cellular extracts ($1 \times 10^8$ PFU/ml) were irradiated with a germicidal lamp at a distance of 3 cm for 5 minutes to inactivate the virus. Inactivated wild-type vaccinia virus harvested in the same manner as the recombinants were used to monitor the proliferative responses to the vaccinia virus. Media alone was used as the control to monitor the spontaneous proliferations. No residual vaccinia virus infectivity was observed in the inactivated cellular extracts by plaque assay.
2.2.14.2. E1 peptides

E1 peptides EP1 to EP23 (Table VII) were synthesized and provided by Dr. P. Chong at Connaught, Canada. Each peptide was prepared by dissolving in ethanol and air drying in sterile tissue culture hood overnight and resuspended in sterile PBS (2 mg/ml). Peptides were diluted in RPMI 1640 before adding to the proliferation assays.

2.2.15. Statistical methods

The T-cell proliferation data were analyzed using a mixed effects analysis of variance model. Proliferative responses to each of the structural proteins E1, E2, and C, were compared within each group and in between the two groups.

2.2.16 Study group

a) T-cell epitope mapping:

Eleven (5 females; 7 males) healthy individuals with no known conditions associated with rubella virus infection.

b) Antigen specific lymphocyte proliferation study:

Group A: fourteen adults from the hospital staff (seven males; seven females) who exhibited no rubella associated symptoms. Their ages ranged from 24 to 48 years, with three individuals having previously received rubella vaccines. All donors had documented histories of having received smallpox vaccinations.

Group B: four CRS patients, with ages of 2, 5, 24, and 25 years. All had a CRS diagnosis confirmed by clinical and serological criteria, and for each, RV exposure in
uterine had occurred prior to 16 weeks of gestation. One patient had a late onset sequelae, presenting with progressive senso-neural deafness at age 16. None of the patients had received smallpox vaccinations; the oldest of the two individuals had received measles-rubella vaccines.
Table VII. E1 synthetic peptides.

<table>
<thead>
<tr>
<th>EP</th>
<th>Synthetical Peptide</th>
</tr>
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<tbody>
<tr>
<td>EP1</td>
<td>EEAFTYLCTAPGCATQTPVPVR</td>
</tr>
<tr>
<td>EP2</td>
<td>VPVRLAGVGFESKIVDGGCF</td>
</tr>
<tr>
<td>EP3</td>
<td>FAPWDLETGACICEIPTDV</td>
</tr>
<tr>
<td>EP4</td>
<td>PTDVSCEGLGAWVTAPCARI</td>
</tr>
<tr>
<td>EP5</td>
<td>CARIWNGTQRACTFWAVNAYS</td>
</tr>
<tr>
<td>EP6</td>
<td>GSYYKQHYPTACEVEPAFGH</td>
</tr>
<tr>
<td>EP7</td>
<td>AFGHSDAACWGFPTDTVMSV</td>
</tr>
<tr>
<td>EP8</td>
<td>SVFALASYVQHPHTKRVRK</td>
</tr>
<tr>
<td>EP9</td>
<td>VKFHTETRTVWQLSVAGVSC</td>
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<td>EP10</td>
<td>VSCNVTTEHPFCNTPHGQLE</td>
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<td>EP11</td>
<td>GQUEVQVPPDPGDVLEYIMN</td>
</tr>
<tr>
<td>EP12</td>
<td>IMNYTGNQOQSWGLGSPNCH</td>
</tr>
<tr>
<td>EP13</td>
<td>NCHGPDWASPVCQRHSPDCS</td>
</tr>
<tr>
<td>EP14</td>
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<tr>
<td>EP15</td>
<td>RLVDADDPLRRTAPPGPEVW</td>
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<td>EP16</td>
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<td>EP18</td>
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<td>EP19</td>
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<td>EP21</td>
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</tr>
<tr>
<td>EP22</td>
<td>GEDVGAFFPGKFTAAAL</td>
</tr>
<tr>
<td>EP23</td>
<td>LNTPPPYQVSGGSESDRASAGH</td>
</tr>
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</table>
3. RESULTS and DISCUSSION

3.1. Section I: Mapping the hemagglutinin and viral neutralizing epitopes of the RV E1 glycoprotein

Epitopes important for viral infectivity and hemagglutination have been mapped to the E1 glycoprotein (Green and Dorsett, 1986; Ho-Terry et al., 1985; Waxham and Wolinsky, 1985a). In this study, a panel of E1 deletion mutants and subset of E1-specific monoclonal antibodies were used for analysis of hemagglutination and viral neutralization epitopes of the E1 protein. The deletion mutants were expressed in the cell-free rabbit reticulocyte system, in COS cells and in E.coli and were subsequently screened with the monoclonal antibodies. Finally, synthetic peptides derived from the predicted E1 amino acid sequence were also screened.

3.1.1. In vitro transcription and translation of the E1 deletion mutants

All RV cDNA constructs for in vitro expression were derived from p3'E2/E1 described in Hobman et al (1988). In addition to containing the entire E1 gene, the wild-type E1 construct p3'E2/E1 also contains the capsid protein translation start site as well as nucleotides specifying the first eight amino acids of C and 69 carboxyl-terminal residues of E2, including the putative E1 signal sequence (Hobman et al., 1988). The in-frame deletions and truncations were generated by using the available restriction sites within the E1 coding sequence (Fig. 8). The mutants m2, m3, m4 and m5 were constructed as described in the Material and Methods (page 42). The constructs were linearized with HindIII for wild-type, E1, m4, m5, with Smal for m2 and BamHI for m3 (Fig. 8). The linearized constructs were transcribed with SP6 RNA polymerase and
subsequently translated in a rabbit reticulocyte lysate system without microsomes as described in Hobman et al. (1988). Fluorographs from translation products immunoprecipitated with monoclonal antibodies 21B9H and 3D9F and human anti-RV serum are shown in Fig. 9. Mutants m2 and m3 were immunoprecipitated by both hemagglutination inhibiting (3D9F) and viral neutralizing (21B9H) monoclonal antibodies (Fig. 9(i)). These results suggest that the binding capacity of both MAbs is independent of the presence of carbohydrate moieties on E1 fragments. Cell-free translation of RNAs from p3’E2/E1, m2, and m3 produced proteins with apparent molecular mass of 61, 40, and 35 kDa, respectively. The higher molecular weights observed in the translation products than predicted from amino acid sequences for m2 (33 kDa) and m3 (28 kDa) are due to the presence of eight amino acids of C protein and 69 carboxy-terminal residues of E2 in m2 and m3 which were not cleaved in the in vitro translation system. In contrast to m2 and m3, N-terminal deletion mutants m4 and m5 were not precipitated by either of the two monoclonal antibodies but were precipitated by the human serum (Fig. 9(ii)). These results suggest that the epitopes recognized by hemagglutination inhibiting and viral neutralizing monoclonal antibodies are present within the N-terminal E1 peptide domain upstream from the Smal site at position. The results from these experiments indicate that the epitopes for 21B9H and 3D9F are contained within the N-terminal half of E1, m3 (Fig. 8), from amino acid residues 1 to 253.
Fig. 8. Schematic representation of the cDNA fragments used for construction of E1 mutants. The deletions are denoted in terms of translated amino acid residues for mutants m1 to m7. The sizes of PCR products (FP1 to FP5) are also given in amino acid residues. E=EcoRl; X=Xhol; S=Smal; B=BamHI; H=HindIll; N=Ncol. N-linked glycosylations are indicated by Y. The top line shows the cDNA fragment encoding RV E1.
Figure 9. Translation of SP6-derived E1 mRNA and deletion mutant mRNAs in rabbit reticulocyte lysates. mRNAs were translated in presence of $^{35}$S-methionine, and the translated products were separated on 12% Laemmli gels and fluographed. (i) Immunoprecipitation of E1 and the mutants m2, m3 with MAbs 21B9H (A) or 3D9F (B). Neg denotes transcription/translation of a construct with no RV E1 sequence. (ii) Immunoprecipitation of mutants m4 and m5 with MAb 21B9H (A), 3D9F (B) or human anti-RV serum (C). Protein molecular weight standards are indicated (kDa).
3.1.2. Expression in COS cells

The cDNA inserts from p3'E2/E1, m1, m2, and m3 were subcloned into the eukaryotic expression vector pCMV5, downstream from the human cytomegalovirus immediate early gene promoter (Andersson et al., 1989). COS cells were transfected with recombinant plasmids and cell lysates were isolated at 48 hours post-transfection for immunoblot analysis. Wild-type E1 and mutants (m1, m2, and m3) all reacted with monoclonal antibodies that exhibit viral neutralizing (Fig. 10A) and hemagglutination inhibiting activities (Fig. 10B). Mutant 1 lacks the 450 amino acid Xhol fragment (E145 to E1481) but was still recognized by both monoclonal antibodies suggesting that the respective epitopes are not contained within this fragment. Similarly, both viral neutralizing and hemagglutination inhibiting monoclonal antibodies recognized the mutant m2 with the 560 amino acid deletion at the carboxy-terminus and the mutant m3 with 670 amino acid deletion also at the carboxy-terminus. Thus, the recognition of mutants m1, m2 and m3 by both monoclonal antibodies suggests that the corresponding epitopes are not contained within the Xhol fragment (E145 to E1481) or the fragment between Smal and HindIII (E1270 to E1481) (Fig. 8).

E1 protein contains three functional N-linked glycosylation sites (Hobman et al., 1991). In mutant m1, one glycosylation site is retained, while in mutants m2 and m3, all three glycosylation sites are retained. The observed apparent molecular weights of m1 (37 kDa), m2 (42 kDa), and m3 (38 kDa) suggest that they were translocated and glycosylated, as the estimated molecular weights based on the predicted amino acid sequence for m1, m2 and m3 are 36, 33 and 28 kDa, respectively. The presence of
oligosaccharides on the mutants did not appear to affect the recognition of epitopes by 21B9H and 3D9F suggesting that the binding capacity of both MAbs is independent of carbohydrate moieties on E1 fragments.
Fig. 10. Immunoblot analysis of E1 mutants expressed in COS cells. COS cells were transfected as described in Hobman et al., 1988. After 48 hour transfection, the transfected COS cells were scraped off the plates and analyzed by immunoblotting. E1 antigens were detected using MAb 21B9H (A) and 3D9F (B). The relative mobilities of protein standards (kDa) are indicated. VEC = COS cells transfected with pCMV5 vector.

3.1.3. Expression in *E.coli* strain BL21(DE3)/pLysS

Since the expression of the smaller mutants were not detected in transfected COS cells, *E. coli* pET vectors were used to express these mutants. Mutant cDNAs (m4 and m6) were inserted into the Ncol site of the pET8c vector (Rosenberg et al., 1987) and expressed in *E.coli* as non-fusion proteins. The cell lysates from induced *E.coli* cultures
were separated on a 0.1% SDS-12% PAGE and RV E1-specific polypeptides were detected by immunoblotting using monoclonal antibodies (Fig. 11). Lack of recognition of m4 but recognition of m6 (band at 20 KDa) by hemagglutination inhibiting and viral neutralizing monoclonal antibodies confirm that hemagglutination and viral neutralizing epitopes are located within these two regions (E1, to E1 \textsubscript{44} and E1 \textsubscript{193} to E1 \textsubscript{269}). To determine which contains these epitopes, mutant m7 was constructed (Fig. 8) and expressed in \textit{E.coli} as a fusion protein using vector pET3xa (Studier et al., 1990).

Expressed fusion protein from m7 was recognized by both hemagglutination inhibiting and viral neutralizing monoclonal antibodies (Fig. 11), suggesting that hemagglutinin and viral neutralizing epitopes are located within the region E1 \textsubscript{193} and E1 \textsubscript{269} and not in the region E1, to E1 \textsubscript{44}. The region E1 \textsubscript{193} to E1 \textsubscript{269} was further divided into two smaller fragments (FP-1 and FP-2) using the polymerase chain reactions with synthetic oligonucleotides (Table IV). Mutants FP-1 (E1 \textsubscript{214} to E1 \textsubscript{264}) (33.4 KDa) and FP-2 (E1 \textsubscript{193} to E1 \textsubscript{226}) (32.6 KDa) were expressed as fusion proteins in \textit{E.coli} using pET3xa vector (Studier et al., 1990) (Fig. 12). The combined apparent molecular mass of the fusion partner and the E1 fragments are 33.4 KDa for FP-1 and 32.6 KDa for FP-2. Both hemagglutination inhibiting and viral neutralizing monoclonal antibodies reacted with mutant FP-1 but not with mutant FP-2 (Fig. 11) suggesting that the corresponding epitopes are contained within the regions E1 \textsubscript{214} to E1 \textsubscript{254}. Mutant FP-1 was further subdivided into three small constructs: FP-3 (E1 \textsubscript{226} to E1 \textsubscript{254}) (31.7 KDa), FP-4 (E1 \textsubscript{214} to E1 \textsubscript{240}) (31.9 KDa) and FP-5 (E1 \textsubscript{214} to E1 \textsubscript{226}) (30.3 KDa). The denoted molecular masses are of the fusion partner and the E1 fragment. Binding of viral neutralizing and hemagglutination inhibiting monoclonal
antibodies to the expressed fusion proteins is shown in Fig. 11A and 11B, respectively. Both hemagglutination inhibiting and viral neutralizing monoclonal antibodies recognized mutant FP-4, but not mutants FP-3 and FP-5. The failure of FP-3 and FP-5 to react with the monoclonal antibodies is not due to the low levels of expression, as abundant expressed proteins were observed in SDS-PAGE stained with Coomassie brilliant blue (Fig. 12). It is possible that the hemagglutination and viral neutralizing epitopes may be interrupted by the break at the amino acids around residues E1226, or the epitopes on FP-5 and FP-3 may be buried under the large fusion partners and inaccessible to the MAbs. Thus it is concluded from these results that the epitopes defined by monoclonal antibodies 3D9F (HI) and 21B9H (VN) map to a domain of 27 amino acids (E1214 to E1240).
Fig. 11. Immunoblot analysis of E1 fusion proteins expressed in E.coli. Expression of E1 mutants was induced with the addition of isopropylthiogalactoside when the culture reached an optical density of 0.8-0.9 at 600 nm. Induced cultures were harvested 2-4 hours after induction. Expressed proteins were separated on 12% Laemmll gels and detected by immunoblotting. Blots were detected with MAbs 21B9H (A) and 3D9F (B). RV = RV antigen preparation; UNI = uninduced E.coli culture.
3.1.4. Synthetic peptide ELISA

To define the epitopes further, six overlapping synthetic peptides (EP11 to EP15 and EP25) spanning the m7 region (Fig. 13) were synthesized and coated onto ELISA plates and probed with MAbs. Rabbit anti-peptide sera were used as positive controls to ensure that the peptides were sufficiently bound to the plates. Peptide-specific ELISA results were observed only with the 35 amino acid peptide EP25 by VN MAb 21B9H (Fig. 14). EP25 was then divided into two smaller peptides, EP24 (17 aa) and EP26 (15 aa) (Fig. 13). Although, MAb 21B9H reacted strongly with EP25, it failed to recognize EP24 or EP26 in a peptide specific ELISA (Fig. 15). However, another viral neutralizing MAb (16A10E) recognized both EP25 and EP26 (Fig. 15), suggesting that there are two distinct viral neutralizing epitopes on E1. Three HI MAbs (3D9F, 3D5D and 12B2D) failed to recognize any of the synthetic peptides tested.

3.1.5. Summary of HI and VN epitope mapping

Combining the data obtained from the studies of the truncated forms of E1 and the peptide analysis, the results are summarized as follows (Fig. 16):

1) The viral neutralizing epitope defined by MAb 21B9H mapped to amino acid residues 214 to 233 (QQSRWGLGLSPNCHGPDWASP).
2) The viral neutralizing epitope defined by MAb 16A10E mapped to amino acid residues 219 to 233 (GLGSPNCHGPDWASP).
3) The hemagglutinin epitope defined by MAb 3D9F mapped to amino acid residues 214 to 240 (QQSRWGLGLSPNCHGPDWASPVQCQRHSP).
Fig. 12. Coomassie brilliant blue stained SDS-PAGE of E1 fusion proteins expressed in E.coli. 2-4 hours after induction, cultures were centrifuged and resuspended in 1/10 the volume of lysate buffer. 5μl of the concentrated cell lysate loaded per well. uni=uninduced; VEC=induced fusion partner without E1 sequences; M=molecular weight marker (kDa).

Fig. 13. Position of peptides relative to the mutant m7. The numbers indicate the positions of each peptide in E1 protein.
Fig. 14. Recognition of E1 peptides EP11 to EP15 and EP25 by viral neutralizing MAb 21B9H and hemagglutinin inhibiting MAb 3D9F. 100 ng/ul of synthetic peptides were bound to immulon-2 plates and probed with monoclonal antibodies at 1:2000 dilutions. The negative sera are normal Balb/C mouse sera not exposed to RV.

Fig. 15. Recognition of E1 peptides EP24, EP25 and EP26 by VN monoclonal antibodies 21B9H and 16A10E at 1:2000 dilutions. The negative sera are normal Balb/C mouse sera not exposed to RV.
Fig. 16. Summary of the results using peptides (EP11 to EP14, EP24 to EP26) and PCR products expressed as fusion proteins (FP-1 to FP-5). VN = viral neutralizing monoclonal antibody 21B9H; HAI = hemagglutinin inhibiting monoclonal antibody 3D9F.
3.1.6. Discussion of Section I

Using *in vitro* and *in vivo* expression systems, twelve E1 mutants were constructed and expressed in order to identify the location of epitopes recognized by E1-specific monoclonal antibodies. Due to the nature of the experiments used in this study, the epitopes that have been mapped are linear in structure and conformational-independent. Any epitopes that are dependent on native conformation may not have been located. There appears to be no general rule whether neutralizing epitopes are linear or conformational (Alexander and Elder, 1984; Long et al., 1986; Wright et al., 1989). For construction of synthetic peptide vaccines, it is necessary to define functional epitopes which can be mimicked by linear polypeptides fragments (Dietzschold et al., 1990).

In general, the oligosaccharide side chains of viral glycoproteins do not act as epitopes per se, but only modulate the expression of neighboring epitopes constituted by residues of the underlying polypeptide backbone. The presence of carbohydrates preserves the conformational integrity of some epitopes that lose antigenicity upon deglycosylation (van Regenmortel, 1990). In addition, attachment of additional oligosaccharide may prevent monoclonal antibody from binding to its underlying epitope. However, the majority of neutralizing antibodies are not dependent on the presence of carbohydrates. Deglycosylated virus adsorbs neutralizing antibody from sera as efficiently as glycosylated virus (van Regenmortel, 1990). The likelihood of epitopes to be either hidden under carbohydrate moiety or by the folding induced by carbohydrate moiety is less for those epitopes which are conformation independent. This characteristic is important in the design of subunit vaccines. The epitopes contained in the vaccines
should be accessible to circulating immunoglobulins to elicit protective immunoglobulin production. The monoclonal antibodies in this study recognize epitopes regardless of the presence or absence of carbohydrates on E1 and its mutants. This suggests that vaccines containing epitopes defined in this study will likely be accessible by the host’s immunoglobulins.

Fig. 16 summarizes all the data obtained in this section. Epitopes for viral neutralizing monoclonal antibodies 21B9H and 16A10E mapped to amino acid residues 214 to 233 and 219 to 233, respectively. The hemagglutination epitope defined by 3D9F mapped to amino acids 214 to 240. The inability of the monoclonal antibody 21B9H to recognize peptides EP12, EP13 and EP26 as well as the expressed mutant proteins FP2, FP3 and FP5 suggests that residues 214 to 219 and 226 to 233 are critical for antibody-peptide interaction. Alternatively, the epitope, upon binding to the ELISA plate, may have been altered such that the monoclonal antibody no longer recognized its epitope (Tang et al., 1988). The structural data suggest that epitopes on native proteins consist of 15-20 residues with a smaller subset of 5-6 of these residues contributing most of the binding energy (Laver et al., 1990). Since EP26 is only 15 amino acids in length, it is possible that the critical 5-6 amino acid residues are not available for binding to the solid support. EP25 (35-mer) is recognized by the monoclonal antibody 21B9H suggesting that the epitope on this larger peptide is in the appropriate form. The surrounding extra amino acid residues may be required for appropriate recognition of the epitope by monoclonal antibody 21B9H.

In contrast to monoclonal antibody 21B9H, EP26 reacted positively with
monoclonal antibody 16A10E. This result suggests that there are two distinct viral neutralizing epitopes close together or overlapping on a linear peptide. However, monoclonal antibody 16A10E failed to recognize peptides EP12 or EP13 and the mutant proteins FP2, FP3, and FP5. This result implies that the epitope for monoclonal antibody 16A10E overlaps the break regions of the above mentioned peptide (Fig. 13) and the deletion products and is, hence, mapped to residues E1_{219} to E1_{233}.

Using various fusion protein constructs the hemagglutination epitope, as defined by monoclonal antibody 3D9F, mapped to FP4 (E1_{214} to E1_{240}). Since monoclonal antibody 3D9F failed to recognize any synthetic peptides that included peptide EP25 (E1_{198} to E1_{233}), this implies that the epitope recognized by monoclonal antibody 3D9F requires additional residues at the C-terminus of peptide EP25. Positive recognition of FP1 (E1_{214} to E1_{254}) and FP4 (E1_{214} to E1_{240}) by monoclonal antibodies further supports this conclusion. However, the absence of positive identification of EP13 (E1_{224} to E1_{243}) and FP3 (E1_{226} to E1_{264}) by the monoclonal antibody makes conclusion difficult. As with the epitopes for the viral neutralizing monoclonal antibodies used in this study, the hemagglutination epitope may have been altered during the binding of the smaller peptides to the plates, resulting in negative data for EP13. On mutant FP3, the epitope may not have been retained due to the fusion partner. On the other hand, proper recognition of the monoclonal antibody 3D9F epitope may require additional residues at the N-terminus of EP13 and FP3. Though the antibody binding residues may only be within 5-6 amino acids of FP4, the surrounding residues may be required to maintain the stability of the antibody-antigen complex (Laver et al., 1990).
Terry et al. (1988) have identified three epitopes (EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3}) within the E1 region E\textsubscript{1245} to E\textsubscript{1285}. Monoclonal antibodies recognizing epitopes EP\textsubscript{1} and EP\textsubscript{2} show both hemagglutination inhibiting and viral neutralizing activity, while monoclonal antibody recognizing EP\textsubscript{3} epitope show only viral neutralizing activity. It is not unexpected to observe that the epitopes mapped in this study do not overlap the epitopes mapped by Terry et al. (1988), but are adjacent to the EP\textsubscript{2} epitope. It is possible that this study and the study of Terry et al. (1988) have independently mapped three distinct epitopes on E1. The mechanisms of viral neutralization by these monoclonal antibodies are not yet clear. Neutralization by monoclonal antibodies may prevent infection directly or indirectly by binding to the glycoprotein and preventing receptor recognition or by binding to a site in proximity to the receptor binding site, causing steric hinderance or a conformational change that the receptor-binding domain is masked or altered (Ioro, 1988).

Waxham and Wolinsky (1985a) have defined six non-overlapping antigenic epitopes on E1 using monoclonal antibodies in competitive inhibition studies. They further mapped the hemagglutination and viral neutralizing epitopes within the 82 amino acid domain of E1 (E\textsubscript{1202} to E\textsubscript{1283}) using viral neutralizing monoclonal antibodies E1-18 and E1-20 (Wolinsky et al., 1991). Subsequent to this study, the same group screened a series of five overlapping synthetic peptides from this region (Wolinsky et al., 1993). The epitope was mapped to E\textsubscript{1208} to E\textsubscript{1239} (peptide SP15) with the minimal amino-terminal requirements of E\textsubscript{1221} and E\textsubscript{1223} for monoclonal antibodies E1-18 and E1-20, respectively. The peptide SP15 induced viral neutralizing antibodies in mice and rabbits which recognized SP15, peptides overlapping SP15 and rubella virus. The findings in this study
are consistent with the epitopes defined by the monoclonal antibodies 21B9H and 16A10E, and further support the hypothesis that these epitopes may be critical for protective host humoral immune response.

The significance of the epitopes within the region $E_{1214}$ to $E_{1240}$ defined in this study is further noted by Mitchell et al. (1992). A synthetic peptide corresponding to residues 213 to 239 was used as a target antigen in ELISA to assess the antibody responses of patients during acute and convalescent phases of wild rubella infection. It was found that the E1 peptide-reactive antibodies closely paralleled the RV-specific antibodies measured by RV ELISA, hemagglutination inhibiting and viral neutralizing assays (Mitchell et al., 1992). This result suggests that the epitopes defined may be hemagglutination and viral neutralizing epitopes for human antibodies of RV E1 and may prove useful in determining effective RV immunity in diagnostic assay for rubella in addition to their usefulness in the construction of subunit vaccines.
3.2. Section II: Cellular response to RV structural proteins

3.2.1. Proliferative responses to RV structural proteins

This study was designed to identify an RV structural protein that may play a pivotal role in eliciting a T-cell immune response and to determine the difference, if any in the proliferative profile between the normal individuals with no rubella-associated symptoms and those suffering from CRS. Peripheral blood lymphocytes were stimulated in vitro with recombinant vaccinia virus expressing E1, E2 or C protein. Cellular extracts from wild-type vaccinia virus were used as the control to monitor the proliferative responses specific to the vaccinia virus. As shown in Fig. 17, depending on the background response to wild-type vaccinia virus, the optimal concentration of each antigen inducing the greatest response varied with each subject in the study. The counts per minute value used in the statistical analysis were those obtained at the optimal concentration of each antigen. The proliferative responses were expressed as stimulation index (SI). The results indicated that each individual in both study groups exhibited differential response to E1, E2 and C (Fig. 18). E1 was the predominant antigen to which a majority of the subjects elicited lymphocyte proliferative responses. Relatively large proportions of individuals had proliferative responses to C which is an internal protein. Proliferative responses to E2 was observed mostly for the individuals with CRS (Fig. 18).

The response levels to each structural protein in the two subject groups were compared (Fig. 19). Statistical analyses by using nonparametric statistics was undertaken to determine the significant levels. For group A (control), it was found that the lymphoproliferative response to E1 was dominant, followed by the response to C, with the
weakest response being directed to E2. The response to E1 was significantly higher than the response to E2 (p=0.002), whereas statistical differences between the E1 and C responses were weak (p=0.1). For group B (CRS), no significant differences were observed in the responses to the individual viral proteins; however, it was observed that the proliferative response to E2 was the dominant response of the three proteins in these individuals. For CRS patients, a decrease in the E1-specific response and an increase in the E2-specific response were observed contrary to the statistically significant different responses observed for E1 and E2 in the control population.

3.2.2. Antibody response to RV structural proteins

Separated RV structural proteins were used in an ELISA system to quantitate IgG responses directed to whole RV as well as to E1, E2 and C. The results of the antigenic specific ELISA (Table VIII) showed that circulating anti-RV IgG in the control population was predominantly directed to E1, at levels significantly higher than those of anti-E2 (p < 0.002) and anti-C IgG (p < 0.0001). CRS patients had significantly elevated levels of anti-E2 IgG (p < 0.05) in comparison to those of the control, whereas no significant difference was detected in their anti-E1 responses (p < 0.3). The increase in the E2/E1 ratio, which had been previously reported for CRS patients (Katow and Suguiru, 1985), seems to be due primarily to an increase in the serological response to E2 in this patient population.
Fig. 17. Proliferative response to recombinant vaccinia virus expressing RV antigens E1, E2 or C. Peripheral blood lymphocytes were stimulated with inactivated vaccinia recombinant expressing RV antigen. Stimulated lymphocytes were harvested at day 7 following a 6 hour pulse with $[^3]H$-thymidine. Representative proliferation response as shown by incorporation of [3H]-thymidine into DNA at different antigen dilutions when background response to wild-type vaccinia virus was either high (A) or low (B). □ = E1 recombinant; ■ = E2 recombinant; ○ = C recombinant; △ = wild type.
Preceding the calculation of stimulation index (SI), the raw values were corrected for the spontaneous background response, which ranged from 100 to 500 cpm. SI = cpm(vaccinia recombinant - spontaneous) - cpm(wild type - spontaneous), where spontaneous refers to spontaneous background response. High background response to wild type vaccinia virus was observed for six individuals (no. 3, 4, 5, 11, 12, and 13) in group A. The optimal concentration of antigen used in the statistical analysis was a $10^2$ dilution for high background response and a $10^1$ to $10^2$ dilution for low background response. Control = individuals without rubella associated symptoms; CRS = patients with congenital rubella syndrome.
Comparison of the B-cell and lymphoproliferative responses in the two patient populations showed similarities in their protein specificities. The control population showed the immunodominance of E1 in both the cellular and humoral responses over both E2 and C (Fig. 19, top panel). The increase of the serological response to E2 observed for the CRS population was also reflected by an increase in E2-specific proliferative responses (Fig. 19, lower panel). However, in the analysis of the lymphoproliferative and B-cell responses of individual CRS patients or control individuals, a poor correlation between circulating IgG levels and stimulation indices to the individual proteins was observed, as demonstrated by the results for patients 2 and 14 (Fig. 18; Table VIII).
Fig. 19. Comparison of proliferative and IgG responses to E1, E2 and C in the control and CRS study group. Values of SI and arbitrary unite per minute are based on a natural logarithm scale. AU = arbitrary units of IgG to each structural protein; • = means; error bars indicate the standard errors of the means. The dots indicate measuring point averages for each patient.
Table VIII. ELISA immunoreactivity of individual structural proteins and whole virus by using sera from subjects in group A (control) and group B (CRS patients) (from Chaye et al., 1992b).

<table>
<thead>
<tr>
<th>Group and subject</th>
<th>IgG response (IU/ml) to whole RV</th>
<th>IgG response (AU/ml) to:</th>
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<td></td>
<td></td>
<td>E1</td>
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</tr>
<tr>
<td>2</td>
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3.2.3. Discussion of Section II

Studies of immune response to RV infection in humans have indicated that of the structural proteins studied, envelope glycoproteins, in particular E1, are the primary targets for induction of humoral immune responses (deMazancourt et al., 1986; Katow and Sugiura, 1985; Zhang et al., 1991). Immune responses to non-structural proteins may be important in RV immunity; however, such proteins are not available for immunological studies. Thus, this study has been limited to the analyses of the structural proteins. The results of early studies with rubella subunit vaccine composed of only viral envelope proteins showed that the vaccine was capable of stimulating both humoral and cellular responses in rabbits (Cappel and DeCuyper, 1976). The present study showed that the E1 glycoprotein is the most antigenic of the three structural proteins in eliciting both cellular and humoral responses. This parallel pattern in the cellular and humoral responses is not unexpected. T-cell and B-cell cooperation for antibody production is not random in that the B-cell selects the T-cell with which to cooperate (Celada and Sercarz, 1988). This selection is postulated to take place at the level of antigen processing and presentation by the B-cells. The region of the antigen bound by the paratope of the immunoglobulin may be protected from enzymatic degradation during processing and thus will be presented intact. Therefore, B-cell epitope and T-cell epitopes may be found on the same antigen (French et al., 1989; Nicholas et al., 1988). This hypothesis is further supported by the pattern observed for the individuals with CRS, in whom both cellular and humoral responses to E2 were relatively elevated when compared to the responses of normal individuals. The relatively reduced levels of immune response, both humoral and
cellular, against E2 glycoprotein in the control individuals may be a result of antigenic competition between the glycoproteins E1 and E2. In the natural infection by influenza virus, suppression of the anti-neuraminidase response has been observed to be due to antigenic competition (Johansson et al., 1987). This competition is affected by the relative amounts of competing immunogens, resulting in an immune response that is predominantly directed against hemagglutinin. The low level of immune response to E2 in RV infection may be due to the inaccessibility of the polypeptide chain to the host’s immune system because of masking by glycosylation (Ho-Terry and Cohen, 1984). Katow and Sugiura proposed that the elevated level of immune response to E2 is a direct consequence of CRS (Katow and Sugiura, 1985). This is further supported by work reported by Williams et al. (1992). This group found that patients with the retinal degeneration of retinitis pigmentosa had elevated levels of E2 antibodies than the normal individuals.

Persistent RV infection have been reported by a number of investigators (Chantler et al., 1982; Cunningham and Fraser, 1985; Oxford and Potter, 1971), though the mechanism for establishing persistence is still not clear. Recently, Williams et al. (1993) observed defective phagocytosis by cultured human retinal pigment epithelial cells with persistent rubella virus infection, which may be a possible mechanism of establishing persistence. Extrathymic tolerance resulting in the absence of neutralizing antibodies to the virus may interfere with viral clearance and contribute to persistence (Nossal, 1991). Persistent infection may cause slow release of the viral antigens, leading to continual immunostimulation and increased immune response to antigens that are not seen by the
immune system for rapidly cleared viral infection (Oldstone, 1989). RV infections have been implicated in a number of disorders, such as arthritis, diabetes, and encephalitis (Ginsberg-Fellner et al., 1985; Martin et al., 1989; Marvin, 1975). Williams et al. (1993) Nath and Wolinsky (1990) found that irrespective of the antibody titre to whole RV, the relative proportion of the IgG response to E1 was diminished and that to E2 was elevated in MS (multiple sclerosis) patients when compared to the control population. No difference in C was observed. Perhaps in susceptible individuals, vigorous immune responses directed against E2 may result in autoimmunity as an indirect consequence of persistent infection and may result in autoimmune conditions.

In addition to the cellular responses, humoral responses were also studied. A lack of correlation between the proliferative responses to RV structural proteins and the presence of RV-specific IgG in sera was observed (Table VIII). This discrepancy in protein recognition between T- and B-cell repertoires has also been detected in bovine herpesvirus infection (Hutchings et al., 1990) and in RV infection (Williams et al., 1992). The presence of lymphocyte responsiveness in the absence of circulating antibody is of clinical importance because it is a direct evidence of previous exposure to antigen. The absence of detectable antibody titre does not always correlate well with susceptibility to clinical infection (Brody, 1966; Horstman et al., 1970). Consequently, it is possible to have no circulating antibody levels and be protected against clinical reinfection. Whether the individuals who showed conflicting T- and B-cell assay results are susceptible to clinical infection was not determined here in this study. But what this study does indicate is that serology studies alone are not accurate indicators of whether an individual is
susceptible or not. Lymphocyte proliferation assays, in addition to immunoblots and/or ELISAs may, provide more accurate information on whether an individual is immune than immunoblot analysis and/or ELISAs alone. At present, laboratory diagnosis for RV infection employs techniques such as virus neutralization assays, hemagglutinin assays and ELISAs which rely on the presence of anti-RV antibodies. The results of these assays are used to determine whether an individual should be immunized. These results are especially important to women planning to become pregnant since the risks of fetal damage associated with re-exposure to RV are not fully understood. It was also observed that immunization of those who are sero-negative but have been previously exposed to the RV may suffer symptoms similar to RAA (personal communication with Dr. A. Tingle). No experimentally substantiated explanations have been forwarded to explain these observations. Thus, serology studies alone may not be sufficient to determine whether an individual is protected from future infection. It would also be informative to investigate the changes with time in the T-cell proliferative profiles towards each of the structural proteins in parallel with humoral analysis. These studies would chart the development of immunity of RV infection or vaccination in greater detail and allow a better understanding of the clinical development of RV infection and immunity.
3.3. Section III: Human T- and B-cell epitopes of E1 glycoprotein

3.3.1. Lymphocyte proliferative response to E1 peptides

23 overlapping peptides (EP1 to EP23) covering approximately 90% of the E1 protein sequence have been synthesized and provided by Dr. P. Chong at Connaught Laboratories (Fig. 20, Table VII). The lengths of the peptides were chosen based on the high index of hydrophilic β-turns and α-helix as judged by secondary structure prediction analysis according to Hopp and Woods (1981). These regions are likely to be exposed and antigenic. Long peptides (17 to 22 residues) were synthesized to mimic epitopes on native E1 protein because they have more chance of having a conformation similar to that of the corresponding portions of the native protein than short peptides (Rothbard and Taylor, 1988).

Sera and peripheral blood lymphocytes were isolated from 10 seropositive healthy donors with no history of clinical rubella. Nine of the ten donors were found to react to RV structural proteins in immunoblot analysis using non-reducing conditions (Fig. 21). This immunoblot suggests that 9 of the 10 donors have RV-specific circulating antibodies. Lymphocyte proliferative responses of human PBL were determined in vitro by direct stimulation with RV or with E1 peptides. All individuals responded to RV, although the levels of response varied considerably between different donors (Table X). Each individual showed an unique response profile to the E1 peptides (Table X). Peptides EP12 (residues 207-226), EP17 (residues 289-308), EP19 (residues 324-343), and EP21 (residues 358-377) were recognized by six or more individuals, while EP8 (residues 140-159) and EP9 (residues 157-176) were not recognized by any of the 10 individuals in the
study group (Table X and Fig. 22). Since the regions of overlap between adjacent peptides are generally one to three amino acids, it is possible that epitopes presented in the overlap regions were not detected in this study.

T-cell antigenic sites are postulated to be amphipathic helices, with one face predominantly polar and the opposite face predominantly apolar (Margalit et al., 1987). Amphipathic scores of domains present in peptides EP8, EP9, EP12, EP17, and EP19 are listed in Table IX. Amphipathic score is a method of predicting T-cell epitope and a high amphipathic score does not necessarily indicate the presence of a T-cell epitope, as it is true in converse, where a low score does not exclude a particular domain as a T-cell epitope. The high amphipathic score and lack of response observed for peptides EP8 and EP9 are not unexpected. Since the algorithm used to ascertain the amphipathic score does not take into account of MHC restrictions (Margalit et al., 1987). MHC restriction is an important factor in T-cell epitope selection in vivo and thus this type of predictive methods has inherent limitations.
Fig. 20. Predicted structure of RV E1 protein by conventional structural analysis algorithms. (A) Secondary structure analysis of local average α-helix and β-turns potentials according to Chou and Fasman (1978); (B) Hydrophilicity plots predicted by the method of Hopp and Woods (1981). The values are derived from the average of heptapeptide windows and are plotted at the midpoint of each segment.

Fig. 21. Immunoblot analysis of participants' sera against M33 RV antigens under non-reducing conditions. Sera 1 to 10 were used to at a 1:80 dilution, M is the mixture of negative control serum form four individuals. E1, E2 and C refer to the structural proteins of RV. Relative mobilities of protein standards (kDa) are indicated at the left.
Table IX. Amphipathic scores of E1 peptides

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<tr>
<td>EP17 (289–308)</td>
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<tr>
<td>EP19 (324–343)</td>
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</tr>
<tr>
<td>EP21 (358–377)</td>
<td>VEGLAPGGGNCHLTVNGEDV</td>
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Fig. 22. Peptide stimulated T-cell proliferative responses. Each bar indicates the number of peripheral blood lymphocyte samples which had a stimulation index of greater than 2 for each peptide. Peptides are numbered 1 to 23 spanning the entire E1 sequence.
Table X. Lymphocyte proliferative responses to E1 synthetic peptides

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*Cell proliferation indices were calculated as described under Materials and Methods. Boldface numbers represent proliferative indices greater than 2.

Table XI. B-cell response to E1 peptides

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*Responses of sera from 10 individuals who displayed positive reactivity indicative of antibody binding to peptide are expressed in terms of relative reactivity as described under Materials and Methods. IgG antibody reactions were tested at a serum dilution of 1:64.
3.3.2. Antibody response to E1 peptides

B-cell epitopes may be linear as well as conformational dependent. Using synthetic peptides, only the linear B-cell epitopes are detected. The purpose of this part of the study was to ascertain a crude picture and to see whether a correlation exists between human B-cell epitopes and the epitopes mapped in the Part I of the study. The frequently recognized B-cell epitopes were EP2 (residues 19-38), EP4 (residues 54-74), EP9 (residues 157-176), EP20 (residues 341-360), and EP22 (residues 374-390) (Table XI). No reactivity was observed with peptides EP6, EP7, EP8, EP10, EP18, and EP21 (Table X). Since individual synthetic peptides are not covalently bound to the plates, the peptide ELISA may not work as well as for all peptides. To verify the assay, individual peptides were coated onto microtitre plates and probed with peptide-specific rabbit antisera raised against individual E1 peptides. All peptides were recognized by their respective antipeptide antisera at the reactivity titre >1/1600. These results establish that all peptides are adsorbed to the microtitre plates and their antigenic determinants are accessible to antibodies and synthetic peptides. To check the possibility of non-specific binding between synthetic peptides and antibodies, synthetic peptides were tested by using normal IgG’s from rabbits. No non-specific binding was found. Detection of RV-specific IgM antibodies is an important criterion for diagnosis of acute RV infection. All donors in this study are seropositive healthy adults, having no history of acute RV infection within the last 5 years. Since no RV-specific IgM response in the sera was detected in the immunoblot analysis, peptide-specific IgM responses were not determined.
3.3.3. Discussion of Section III

Ideal synthetic vaccines should have the following characteristics: (i) contain epitopes important for protective antibody production; (ii) contain T-cell recognition sites that can induce antibody production, cellular immunity, and prime memory T-cells to the pathogen; (iii) be silent for cross reactive domains to self antigen/and or for immunosuppressive domains; and (iv) provide long lasting immunity and not require frequent boosters. Synthetic peptides representing only B-cell epitopes are generally poor immunogens and need to be coupled to carrier proteins. However, such complexes are limited in application due to carrier-induced suppression and failure to prime T-cell memory response to the pathogen. Suitable carrier proteins for human use are not yet known. Studies of hepatitis B virus and foot and mouth disease virus showed that composite peptides containing both T- and B-cell sites yields a more efficient immunogen than B-cell epitopes alone (Milich, 1988; Milich et al., 1988).

At the time the synthetic peptides were being designed the minimum length of T-cell epitopes was thought to be 8-12 amino acids and 6-10 residues for a B-cell epitope (Dyson et al., 1988; Rothbard and Taylor, 1988). Accordingly, peptides of 17 or more amino acids were synthesized. Using 23 overlapping synthetic peptides, several immunodominant T- and B-cell epitopes of E1 were identified via in vitro proliferation assay and peptide specific ELISA. The frequently recognized T-cell and the common B-cell epitopes were EP2, EP4, EP9, EP21 and EP22 (Table XI). Comparing T- and B-cell responses to E1 peptides in each individual, a poor correlation between the reactivity of the antibody and the stimulation index of peptides was observed (Tables X and XI). This
is not surprising since overlapping T- and B-cell sites are generally unusual (Milich, 1988) though viral-specific peptides containing overlapping domains have been documented (Cohen et al., 1984; Milich, 1988). The identified viral neutralizing epitopes on E1 are located within the peptides EP12, EP13, EP14, EP15 and EP16 (Chaye et al., 1992; Wolinsky et al., 1992, 1993). However none of these epitopes was found to be a site frequently recognized by B cells. The studies of Rothbard and Taylor (1988), and more recent studies of Rammensee et al. (1993) and Germain (1993) found that peptide length for Class I antigen is 8 or 9 residues (reviewed in Rammensee et al., 1993) and for Class II antigen is 12 to 20 residues (reviewed in Germain, 1993). Since, the design of the assay preferentially selects for Class II restricted CD4+ (Ou et al., 1992a)there may have been epitopes that were missed by this panel of peptides.

A hierarchy of immune responsiveness to the individual peptides was expected in the population depending on MHC restriction (Milich, 1988b). This was observed in this study with the 23 overlapping peptides. Individuals 1, 4, 5, and 7 responded to 50% or more of the peptides. The order of response to peptides differed between individuals. Peptides EP12, EP17, EP19 and EP21 induced proliferation responses in the majority of the individuals in the study and indicated substantial T-cell stimulating activities of these peptides. Unfortunately the phenotypes of the donors' MHC antigens have not been determined. Whether the T-cell responses of these peptides are restricted by a common class II MHC antigen or whether they are compatible with more than one antigen type are not known. The role of MHC class I or class II restricted RV-specific T cells in RV infection and protection has not been reported. A low RV antigen-specific response is
shown to be associated with HLA-DW2 (Ilonen and Salmi, 1986). Recently, Ou et al. (1992b, 1992c) have isolated human CD4+ cytotoxic T-cell clones reactive to E2 and C proteins of RV. Recognition of capsid epitopes by T-cell clones is associated with HLA-DR4 or DRw9 (Ou et al., 1992c), whereas the E2 epitope is associated with HLA-DR7 (Ou et al., 1992b).

Defining immunodominant epitopes on an antigen is critical for determining domains to be included in subunit vaccines. Undesirable determinants such as those responsible for eliciting autoimmunity should be eliminated in constructing subunit vaccine. RV infection is known to result in complications of autoimmunity in nature. If determinants which induce responses which cross react with self-antigen in context with MHC can be eliminated and such complications would be limited.

Another advantage of elucidating T-helper sites is that they can be attached to antibody binding sites of other RV viral proteins, E2 and C. Viral neutralizing epitopes have been mapped to E2 as well as to E1 (Waxham and Wolinsky, 1985). In hepatitis B virus and influenza virus, cytotoxic T cell sites that are crucial for viral clearance have been mapped to non-envelope viral proteins. Recently identified immunodominant epitopes on C protein of RV can be incorporated in the peptide vaccine (Ou et al., 1992c). Composite vaccine containing all potential immunologically functional domains could provide protective immunity and aid in priming a subset of lymphocytes important for viral clearance. Subunit vaccine for hepatitis B virus containing intramolecular/interstructural T-cell epitopes and B-cell epitopes was found to be efficient in producing protective antibodies (Milich et al., 1988). By selecting immunodominant epitopes for both T and
B cells, potentially harmful or immunosuppressive domains can be eliminated. Rubella virus has been associated with autoimmune conditions (Chantler et al., 1982; Ginsberg-Fellner et al., 1985). If domains that induce such responses can be excluded, the dangers of vaccination can be reduced.
4. SUMMARY AND PERSPECTIVES

Peripheral blood lymphocytes from volunteers with no history of rubella associated conditions and patients with congenital rubella syndrome were studied for recognition of RV E1, E2 and C structural proteins. The corresponding sera were also tested using antigen specific ELISAs. Two interesting phenomena were observed from these studies. In normal individuals, of the RV structural proteins, E1 was the dominant antigen for both humoral and cellular immune responses while in CRS patients, immune responses to E2 were dominant. These studies confirmed the hypothesis that E1 is the dominant immunogen for protective immunity against RV infection.

The implication of these results with respect to differential immunity to E1 and E2 in CRS patients is less clear than the role of E1 immunity in the normal population. Studies in the past have shown reduced levels of cell mediated immune response in CRS patients than in the normal population (Buimovici-Klein and Cooper, 1985). Others have shown the reduced E1 specific IgG (Katow and Suguira, 1985), inability to produce high-affinity IgG (Fitzgerald et al., 1988) and lack of HAI IgG (Cooper et al., 1971) in CRS patients. The results of our study contribute to the overall understanding of the immune responses observed in CRS patients. Different profiles of immune responses observed in CRS patients than that of normal individuals may contribute to the manifestation of the symptoms suffered by CRS patients.

The remainder of the thesis focused on the E1 glycoprotein to define the immunogenically functional epitopes. First, E1 cDNAs with various deletions were expressed in vitro using cell-free rabbit reticulocyte expression system, in COS cells and
in *E. coli*. Appropriate vectors were manipulated to express the desired E1 cDNA sequences. The expressed proteins were screened with monoclonal antibodies with viral neutralizing and hemagglutination inhibiting activities. These studies showed that the epitopes recognized by these monoclonal antibodies were independent of carbohydrate moieties on the protein; ie, the epitopes were not affected by the changes in the conformation arising from glycosylation and/or no glycosylation. It was thus concluded that the epitopes were linear on the E1 glycoprotein. Synthetic peptides corresponding to the domain localized by using fusion proteins expressed in *E. coli* were synthesized and screened in ELISAs. The results defined the viral neutralizing epitopes to residues 214 to 233 and to residues 219 to 233. The epitope recognized by hemagglutination inhibiting monoclonal antibody was mapped to residues 214 to 240.

In addition to the epitopes defined by the monoclonal antibodies, the epitopes recognized by human T-cells and human antisera were also determined. Twenty-three overlapping synthetic peptides spanning the entire sequence of E1 were synthesized. These peptides were used as antigens in the lymphocyte proliferation assays using human peripheral lymphocytes. They were also used as antigens in antigen specific ELISA using the corresponding human antisera. Peptides EP12 (residues 207 to 226), EP17 (residues 289 to 308), EP19 (residues 324 to 343) and EP21 (residues 358 to 377) were recognized by greater than 50% of the individuals tested. The amphipathicity analysis showed that EP12, EP17, EP19 and EP21 have relatively high amphipathic scores (>15). ELISA experiments revealed that EP9 (residues 157 to 176), EP22 (374 to 390), EP2 (residues 19 to 38), EP4 (54 to 74) and EP20 (residues 341 to 360)
were frequently recognized by the sera tested. This study provides the basis for which a subunit vaccine may be designed. It has been shown that subunit vaccine comprising of both T and B cell epitopes is more effective in eliciting protective immunity. It also eliminates the need for carrier molecules, of which a suitable molecule has not been developed for humans.

In summary this project has confirmed the hypothesis that the E1 glycoprotein is the dominant antigen for which protective immunity is elicited. We have defined immunogenically functional epitopes using mouse monoclonal antibodies as well as the epitopes most frequently recognized by human T and B cells in the human population sample selected.

The results of this study have provided useful starting points for the future studies in the field of RV immunopathology. It is suggested by this study that distinct immunity profile exists with respect to the different structural proteins in the normal and CRS populations. Studies of larger samples of populations separately consisting of CRS, patients with rubella associated arthritis (RAA), and vaccine recipients would be pertinent to the greater understanding of the functions of each structural proteins in the immunopathology of rubella associated conditions. Furthermore, HLA studies of these various populations may provide information to link susceptibility of these rubella associated conditions to a MHC genotype. By comparative analysis, it is possible to define antigens which elicit the hostile immune responses in susceptible individuals. These epitopes then can be deleted from subunit vaccines. Both cellular and humoral immune responses should be studied.
In addition to defining immunosuppressive and autoreactive epitopes, it is also important to study the cellular mechanisms of these disorders; ie, whether the pathology of these disorders are driven by the cellular mechanism or humoral mechanism. Some autoimmune disorders are results of autoreactive immunoglobulins while others are due to autoreactive T-cells.

The epitopes defined using mouse monoclonal antibodies should be used to determine their immunogenicity. Antigenicity of a protein does not automatically translate into immunogenicity in vivo. It will be also important to the design of subunit vaccine to determine the optimum distance between the functional epitopes which will elicit the greatest protective immunity.

Although synthetic peptides are valuable in fine epitope mapping, their usefulness is inherently limited by the very same characteristic for which they are so valuable, their small size. Epitopes defined using synthetic peptides are linear and usually conformation independent. However, not all functional epitopes are linear and independent of the protein’s tertiary structure. We may have missed immunogenically significant epitopes which are conformation dependent. As it was suggested in the epitope mapping studies, the proper folding may be required for expression of epitopes important for hemagglutination. Moreover, glycosylation mutant studies (Qiu et al., 1992) showed that only single mutants induced viral neutralization antibodies. It is also possible that there may be epitopes consisting domains of both E1 and E2 glycoproteins which would be dependent on the correct quaternary structure of the glycoprotein spike. Epitopes such as these will not be detected using synthetic peptides alone. However, it is possible to
determine mimotopes on synthetic peptides. All these studies are required to determine
the correct combination of epitopes to be included in subunit vaccine for RV.
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