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**COMPARATIVE ANALYSIS OF RUBELLA SPECIFIC ANTIBODY RESPONSES IN  
CONGENITALLY AND POSTNATALLY RUBELLA INFECTED HUMANS: A MODEL FOR  
SELECTIVE TOLERANCE**

by

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We accept this thesis as conforming  
to the required standard

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## ABSTRACT

Rubella virus (RV) is an enveloped, positive sense, single stranded RNA virus in the family *Togaviridae*. The virus consists of a host derived lipid bilayer membrane, two trans-membrane proteins E1 and E2, and the C protein which together with viral RNA forms the icosahedral nucleocapsid. RV causes natural infections in humans only, where it can lead to a variety of pathological conditions. The most severe outcome, Congenital Rubella Syndrome (CRS), occurs when the fetus is infected in the first trimester of gestation. RV can be isolated from CRS patients at birth and is believed to establish a persistent infection. Although congenital RV infection remains of clinical importance it also provides a rare opportunity for studying the effects of intrauterine viral infection on the human fetus. This thesis examines the hypothesis that intrauterine infection with RV can establish immunological tolerance which is reflected in the serological response to RV. The study on RV-specific tolerance may help in the understanding by which mechanism self-recognition is established in the human. The induction of immunological tolerance may also be an important mechanism in viral persistence and play a role in chronic inflammatory disorders such as Rubella Associated Arthritis (RAA).

A comprehensive study of the antibody mediated immunity of healthy individuals responding to postnatal infection with RV is compared to that of CRS patients. Assays were developed to measure antibody quantity, affinity and kinetics of the antibody response to whole virus as well as that to the individual RV structural proteins, E1, E2 and C. The viral proteins were purified from whole RV preparation by differential centrifugation followed by preparative SDS-PAGE under non-reducing conditions. Separated proteins were analyzed for their structural integrity by assaying their biological activity. RV protein-specific ELISAs were developed and used for antibody quantitation and in IgG affinity assays using the chaotropic elution technique. Biological activity of sera was assayed by HAI assay. The specificity of antibodies to linear and topographic epitopes was investigated by using reducing and non-reducing Western blotting. Observations made during the development of protein separation protocols have led to the description of an uncoating mechanism for RV. While establishing ELISA protocols, improved buffers were developed by including heat denatured blocking proteins and studies on a novel anti-RV IgG ELISA, which will give a more accurate assessment of protective immunity than ELISA technology used to date, were initiated.

Results indicate that CRS patients can produce similar amounts of IgG than control patients if measured by whole RV ELISA. Group differences were detected at the level of the protein specific responses. CRS patients exhibited significantly lower levels and had reduced affinities of E1-specific antibodies. The most consistent feature of this patient population was their inability to produce IgG to linear epitopes of the E1 protein. The potential role of the tolerization of anti-E1 Th cells due to the exposure of the immature fetal immune system to RV is discussed. A model is proposed which can accommodate the findings on serological and lymphoproliferative immune responses of CRS patients and which supports the hypothesis that congenital RV infection in the early gestational period leads to viral antigen tolerization. Tolerance to the immunodominant E1 protein may lead to a sufficient depression in responder T and B cells in order to allow chronic, low grade RV replication.

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## LIST OF ABBREVIATIONS

A <sub>405</sub>	Absorbance at 405 nm wavelength
aa	amino acid
AU	Arbitrary Units
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BDV	Border disease virus
Bis	N,N'-methylene-bis-acrylamide
bp	base pairs
BSA	Bovine serum albumin
#C	Degrees Celsius
cb	Conjugate Buffer
CIC	Circulating Immune Complex
CRBC	Chick Red Blood Cells
CRS	Congenital Rubella Syndrome
EIA	Enzyme linked immuno assay
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
et al.	et alii
HA	Hemagglutination
HAI	Hemagglutination Inhibition
Ig	Immunoglobulin
IU	International Units
kDa	kilodalton
LCMV	Lymphocytic Choriomeningitis Virus
M	molar
MBq	Megabecquerel
MEM	Minimum Essential Medium
ml	millilitre
l	microlitre
μm	micrometer
MMR	Mumps, Measles and Rubella vaccine
MW	Molecular Weight
n	sample number
NBT	Nitro Blue Tetrazolium
NGS	Normal goat serum
nm	nanometer
ns	non-structural
NT	Neutralization titre
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
pfu	Plaque forming units
pH	1/log [H <sup>+</sup> ]

pI	isoelectric point
rpm	Rotation per minute
RA	Rheumatoid Arthritis
RAA	Rubella Associated Arthritis
RT	Room temperature
RV	Rubella virus
S	Svedberg unit
sb	Sample Buffer
sb <sub>b</sub>	Sample Buffer (boiled)
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SFV	Semliki Forest virus
SI	Stimulation Index
subb	Substrate Buffer
SV	Sindbis virus
Tc	Cytotoxic T cell
Th	Helper T cell
TCS	Tissue culture supernatant
Tris	trishydroxymethylaminomethane
TX-100	Triton X-100
TX-114	Triton X-114
VSV	Vesicular Stomatitis virus
wb	Wash Buffer

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Foremost I would like to thank my family. To my parents who endured the tortuous path of digress and success and were there with both moral and financial support and to my grandmother, Berta Mauracher, who was behind me since I can remember - no matter what. To my wife, Aileen, who put every thing into perspective, failures, progress and life in general. To the life she gave me, my two beautiful daughters Andrea and Marita, I dedicate this thesis. By the time both of you chance to read these pages much time will have elapsed, and if I should have forgotten, remind me of what I promised at your births.

Much in research is doing the wrong thing at the right time, science begins when one finds the courage to admit it.

# 1. INTRODUCTION

Rubella virus (RV) causes a mild childhood illness but can cause severe birth defects if the fetus is infected in the first trimester of gestation. The teratogenic effect of RV can cause a wide range of birth defects and late onset sequelae collectively described as the Congenital Rubella Syndrome (CRS). RV persists *in utero* and can be isolated from virtually all CRS patients at birth and is believed to cause a low grade persistent infection in most patients over many years. The immunological dysfunction which allows the virus to persist and replicate in these individuals has not been defined. The investigations whose results are presented in this thesis examined the humoral responses of adults following the uncomplicated resolution of RV infection in comparison to the RV-specific humoral response of CRS patients to test the hypothesis that intrauterine exposure to a viral agent leads to virus specific immunological tolerance and that specific tolerance is reflected in the serological response to RV. Tolerance or immunological non-responsiveness leading to viral persistence is not only of interest to the pathology of CRS but may play an important role in the pathogenesis of other RV-associated syndromes such as Rubella Associated Arthritis or Chronic Progressive Rubella Panencephalitis, by the chronic low grade replication of virus in specific tissues such as the synovium or the brain.

This introduction contains three sections: a summary of rubella virus biology, rubella virus induced pathology and immunity, and a review of the use and development of diagnostic tools in the detection of RV and the RV-specific immune-responses. The rationale and objectives of this thesis will conclude the chapter.

## **1.1 Rubella Virus Biology**

### **1.1.1. Classification**

Rubella Virus (RV) is the only member of the genus Rubivirus in the family Togaviridae (Porterfield et al., 1978). The Togaviridae are defined as spherical, enveloped viruses of 60-70 nm diameter, with an icosahedral nucleocapsid. The genome is composed of a 40S (+)-sense-single stranded RNA molecule. Studies on the replication cycle of these viruses showed significant differences between flaviviruses and the other genera of the Togaviridae, resulting in the reclassification of the flaviviruses into their own family Flaviviridae in 1985

(Westaway et al., 1985). The Togaviridae are now composed of the rubivirus, alphavirus, pestivirus and arterivirus genera. The old classification of Group A and B Arboviruses is no longer officially recognized by the International Committee on Taxonomy and Nomenclature of Viruses.

### 1.1.2.RV Morphology and Physiochemical Properties

Early electron microscopic studies of RV have shown the virus to be spherical and of 60-70 nm in diameter (von Bonsdorff and Vaheri, 1969). An electron dense core of 30 nm diameter defines the nucleocapsid. An electron-lucent ring separating the capsid from the membrane is characteristic and allows this virus to be distinguished from other morphologically similar togaviruses (Murphy et al., 1968). Spikes of 5-8 nm length are observed on the virion surface which are associated with hemagglutination activity.

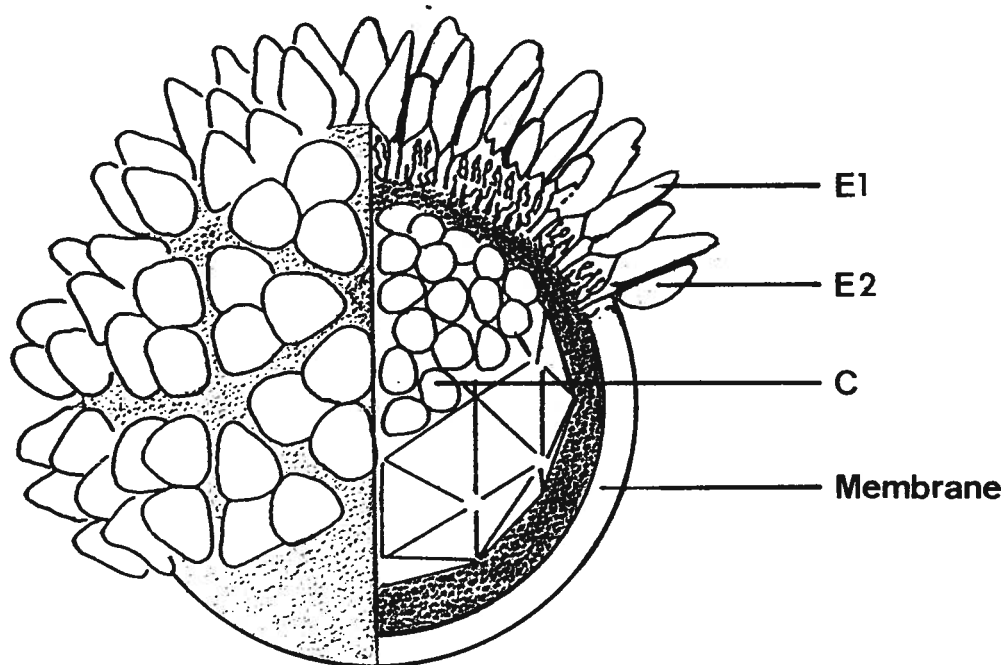


Figure 1. Model of Rubella Virus Structure. Capsid protein (33 kD) forms the icosahedral capsid surrounding the viral RNA (10 kb). The nucleocapsid is enveloped by a host derived lipid-bilayer membrane in which envelope proteins E1 (58 Kd) and E2 (42-47 kD) are embedded. The envelope proteins form E1-E1 homodimers and E2-E1 heterodimers to form surface spikes of 6-8 nm in length.

The virus is heat labile and can be rapidly inactivated by exposure to 56°C. The virus remains stable for several days at 4°C and can be stored for indefinite periods at -70°C. Agents which extract lipids, denature proteins or interfere with nucleic acids all lead to virus inactivation (Parkman et al., 1964).

RV is assembled from three structural proteins: E1, E2 and C (Vaheiri and Hovi, 1972; Payment et al., 1975). The envelope proteins E1 and E2 are both type 1 trans membrane proteins, acylated and highly glycosylated. In the intact virion these proteins form E1-E2 heterodimers and E1-E1 homodimers, visible by electron-microscopy. The capsid is composed of C protein and has an icosahedral symmetry of T=3. This icosahedron surrounds the positive sense, single strand RNA to form the nucleocapsid.

### 1.1.3. Structural Proteins

#### 1.1.3.1. E1 Glycoprotein

The E1 glycoprotein is the largest of three RV structural proteins with an apparent molecular mass of 58 kD on reducing SDS-PAGE and an acidic pI of 6.5 (Ho-Terry and Cohen, 1982). The protein is a type 1 transmembrane protein, containing a 14 residue cytoplasmic tail and a 27 residue trans membrane spanning region (Vidgren et al., 1987). The remaining 440 residues form the bulk of this protein's surface region. Post translational modifications include both fatty acid acylation in the C-terminal region (Waxham and Wolinsky, 1985a) as well as N-linked glycosylation at all three potential glycosylation sites, predicted by sequence analysis of all RV strains studied (Frey et al. 1986; Clarke et al., 1987; Terry et al., 1988). The glycosylation with these endo-H resistant glycans adds 5 kD to the molecular mass of this protein and although no direct biological function for this modification has been described, it is believed that it stabilizes E1 in its biologically functional and immunologically reactive conformation (Ho-Terry et al., 1984).

E1 contains the hemagglutinin activity as well as the majority of the defined viral neutralization domains. The hemagglutination of day-old chick erythrocytes and pigeon erythrocytes is a function of the E1 monomer (Ho-Terry and Cohen, 1985) but does not correlate completely with neutralization domains (Green and Dorsett, 1986). By using trypsin and *S.aureus* cleavage product analysis the HA activity was localized to a 13 kD fragment of E1. This region, containing amino acid residues 245-285, encodes three separate epitopes conferring both neutralizing and HAI antibody binding sites (Terry et al., 1988), showing that these two functions are



closely situated on the E1 protein. Using competitive inhibition assays with a panel of monoclonal antibodies it was further shown that at least six non-overlapping epitopes exist on this protein (See summary in table 1). Because of the concentration of 6 of the known 7 neutralizing epitopes and the receptor-like activity of HA, it is believed that E1 contains the as of yet undefined viral attachment site.

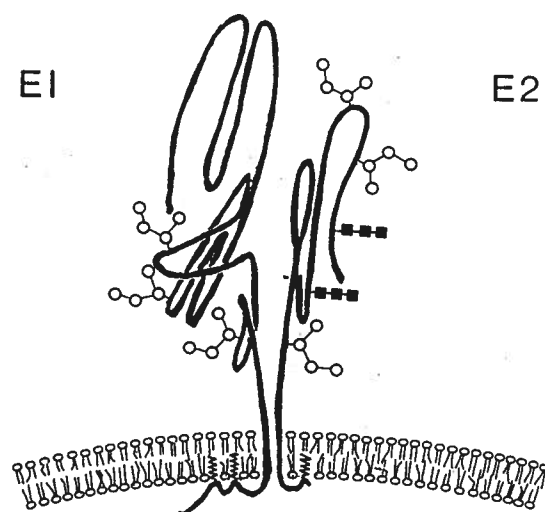


Figure 2. Model of the E1/E2 Glycoprotein Spike of RV. N-linked sugars are indicated by (MfM) located on both E1 and E2. O-linked sugars (■f■) are located on E2 only. Fatty acylation ( ) on cysteine residues are possible on both E1 and E2 cytoplasmic domains.

#### 1.1.3.2.E2 Glycoprotein

E2 is the smaller of the two envelope proteins and travels as a diffuse band in electrophoresis with an apparent molecular mass of 42-48 kD, under reducing conditions. In Therien strain and to a lesser extent in M-33 this diffuse band can be resolved into species termed E2a and E2b (Oker-Bloom et al., 1983). The protein has a wide range of isoelectric points ranging from pH 5.0 to 8.6 (Ho-Terry and Cohen, 1982) which is believed to be due to heterogeneous glycosylation with terminal sialic acids. The protein is highly glycosylated and if Vero

cells are infected with RV, in the presence of tunicamycin, a 30 kD species of E2 is isolated (Oker-Blom et al., 1983), indicating that more than one third of this protein's mass is derived from oligosaccharide chains. Depending on the RV strain, E2 contains 3 (M 33 and HPV 77) to 4 (Therien and RA 27/3) N-linked glycosylation sites (Clarke et al., 1987; Vidgren et al., 1987; Frey and Marr, 1988; Nakhasi et al., 1989) which are thought to be of the complex type, as judged by their resistance to endo-H glycosidase (Oker-Blom et al., 1983). Recently the presence of sialylated O-linked sugars on E2 has been described (Lundström et al., 1991), clarifying the existence of the 15 or more isoelectric variants of this protein.

No direct biological function has been assigned to this protein although the co-expression of E2 with E1 is necessary for the transport of E1 into the assembling virion (Hobman et al., 1990). Also one neutralizing domain has been described for this protein (Green and Dorsett, 1986). It has to be borne in mind that E2 occurs in a heterodimeric form with E1 and in that function might confer support or protection to the spike structure.

#### 1.1.3.3.C Protein

Capsid is a non-glycosylated protein of apparent molecular mass of 33'000 under reducing SDS-PAGE. On these gels, C is often observed to run as a doublet, differing in less than 1 kD in mass. This is thought to be due to the selective usage of two 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 mass of 66 kD (Mauracher et al., 1991a). C has two distinct pI forms at pH 8.8 and 9.5. This protein specifically interacts with the 40 S viral RNA (Weiss et al., 1989) and initiates the assembly of the nucleocapsid. The exact site and mechanism of nucleocapsid assembly has remained controversial, as distinct differences in RV are observed in comparison with the well studied alphavirus assembly pathway. Alphavirus capsid contains protease activity and cleaves itself, by an autolytic event, from the E1/E2/E3 polypeptide, followed by capsomere assembly in the cytoplasm (Malancon and Garoff, 1987). The capsid of RV has no inherent protease activity (Oker-Blom et al., 1984) and assembly of the capsomers has been observed to be dependent on membranes (Horzinek, 1981) suggesting that RV carries with it the signal peptide of the E2/E1 polypeptide (Suomalainen et al., 1990).

Four epitopes have been defined on this protein, none of which induce neutralizing antibodies (Waxham and Wolinsky., 1985a), although capsid has been implicated in the events of viral penetration (Mauracher et al.,

1991a).

Protein	MW	Glycosylation	Epitopes <sub>1</sub>	Neutralizing Domains <sub>2</sub>	HA Activity <sub>3</sub>	Capacity to Induce T Cells <sub>4</sub>
E1	58'000	3 N-linked	^ 6	strong	yes	strong
E2	42-48'000	3-4 N-linked/O-linked	^ 2	weak	no	weak
C	33'000	None	^ 4	no	no	medium

Table 1: Summary of Physical and Immunological Properties of RV Structural Proteins. <sub>1</sub> Epitopes are defined by competitive inhibition using monoclonal antibodies (Waxham and Wolinsky, 1985b). <sub>2</sub> Defined by monoclonal antibodies which can inhibit viral replication on Vero cells (Waxham and Wolinsky, 1985b; Green and Dorsett, 1986). <sub>3</sub> The ability of RV to agglutinate day old chick- or pigeon erythrocytes has been mapped to E1 by using purified protein (Ho-Terry and Cohen, 1980) or by monoclonal antibody (Green and Dorsett, 1986). <sub>4</sub> Defined by studying lymphoproliferative responses of healthy adults to RV proteins E1, E2 and C expressed individually in vaccinia virus (Chaye et al., 1992).

#### 1.1.4.Non-Structural Proteins

Little is known about the nature of RV non-structural (ns) proteins although their genes occupy two thirds of the RV genome. Most work on togavirus enzymes has been performed in SFV and SV and by analogy with these well studied viruses and findings of RV gene arrangement and sequence, it is believed that RV encodes 4 ns proteins (Schlesinger, 1987). One of these ns proteins is likely functioning as an RNA dependent RNA polymerase. The catalytic steps performed by this protein or protein complex include: initiation and elongation of full length (+) and (-) sense RNA, (+) sense sub-genomic RNA synthesis as well as capping and methylation (Schlesinger and Schlesinger, 1990). Furthermore it is believed that one ns protein acts as a virus specific protease involved in the cleavage of the viral polyprotein. Host cell proteins may also be involved in the enzymatic activities during viral replication as prolonged inhibition of host cell RNA synthesis prior to virus infection interferes with togavirus replication (Baric et al. 1983).

#### 1.1.5.Genome Organization

The RV genome exists as an infectious, single-stranded RNA molecule with a sedimentation rate of 40S (Hovi and Vaheri, 1970). In the infected cell, an additional 24S subgenomic RNA can be isolated, similarly poly-

adenylated and capped as the full-length genome and with an identical 3' sequence. This mRNA gives rise to a polyprotein from which the RV structural proteins are derived (Oker-Blom et al., 1984a).

The sequence for the 24S subgenomic RNA is known for wild type isolates M-33 and Therien strain (Clarke et al., 1987; Frey et al., 1986) as well as for vaccine strains RA 27/3 and HPV 77 (Nakhasi et al., 1989; Zheng et al., 1989). The aligned sequences of the 24S RNA reveal a 95% homology, whereas little homology exists between the genomes of RV strains and members of the alphavirus genus (Frey and Marr, 1988).

#### **1.1.6.RV Life Cycle**

The life cycle of RV is very similar to the replication strategy of the alphaviruses. In particular, the biology of SFV and SV have been studied in much detail and much of the knowledge obtained by the study of these two viruses has been shown to apply to RV as well, although differences in some of the details of morphology and replication exist.

##### **1.1.6.1.Entry and Uncoating**

Togaviruses are believed to enter the target cell by receptor mediated endocytosis, a step which can be selectively inhibited by lysosomotropic drugs (Helenius et al., 1984). The entry mechanism of the alphaviruses has been described in detail (Kielian and Helenius, 1986) and there is evidence that RV also uses the endocytic pathway for cell entry (Vaananen and Kaariainen, 1980). A specific cell surface receptor has not been defined for RV, but it has been speculated that membrane phospholipids might be involved in virus attachment (Mastromarino et al., 1990); while others interpret an infection frequency of less than 10% on a cultured cell monolayer as an indication that the RV receptor is a cell cycle dependent protein (Hemphill et al., 1988). Nevertheless, electron microscopic evidence shows RV particles being taken up by endocytosis and delivered to the endosome system (Dr.S.Katow, NIH, Japan, personal communication). Upon acidification of the endosome below pH 5.5 the RV E1 undergoes a structural change to become fusogenic (Katow and Sugiura, 1988), a step which allows the limiting membranes of the RV and endosome to fuse. The low pH environment of the endosome also causes a shift in RV C protein solubility, leading to nucleocapsid uncoating in the endosome (Mauracher et al., 1991). Upon membrane fusion the viral RNA penetrates into the cytoplasm and viral replication can be initiated.

### 1.1.6.2. Replication and Protein Expression

Naked RV RNA is infectious (Maes et al. 1966). Hence, the first event in replication is the translation of the non-structural proteins needed to produce full length 40S (+)-sense RNA and 24S (+)-sense subgenomic RNA, via a (-)-sense RNA intermediate (Oker-Blom, 1984b). The 24S subgenomic RNA is required as a template for translation of the structural proteins. This message is capped, polyadenylated and from the 5' end encodes for: C, E2 and E1 (Figure 3). The molecular mechanism which controls the production of 24S mRNA for structural protein translation, or full length 40S RNA for nucleocapsid packaging remains unclear.

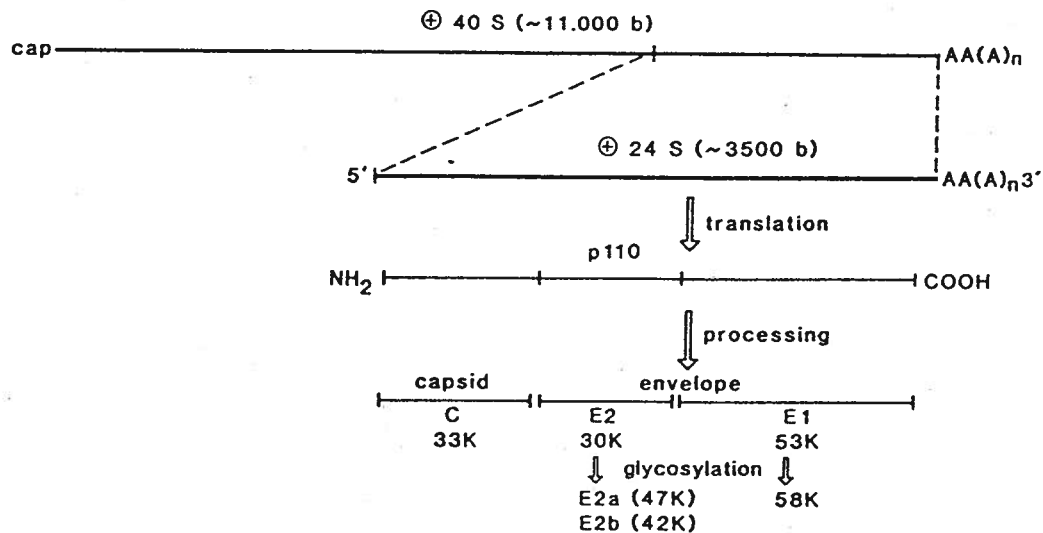


Figure 3. Strategy for the expression and processing of RV structural proteins from a 24S + sense RNA subgenomic fragment. (From: Oker-Blom, 1984b)

C protein remains in the cytoplasm whereas the E2 and E1 proteins are transported to the endoplasmic lumen and processed as transmembrane proteins. Therefore it is believed that translation, proteolytic cleavage and post-translational modifications occur simultaneously at the different sites of this polyprotein. A translocation signal sequence at the COO<sup>-</sup> terminal of the C protein allows the entry of the polyprotein into the lumen of the ER where a series of trans-membrane and signal sequences allow for the correct insertion of E1 and E2 (illustrated in detail in figure 4).

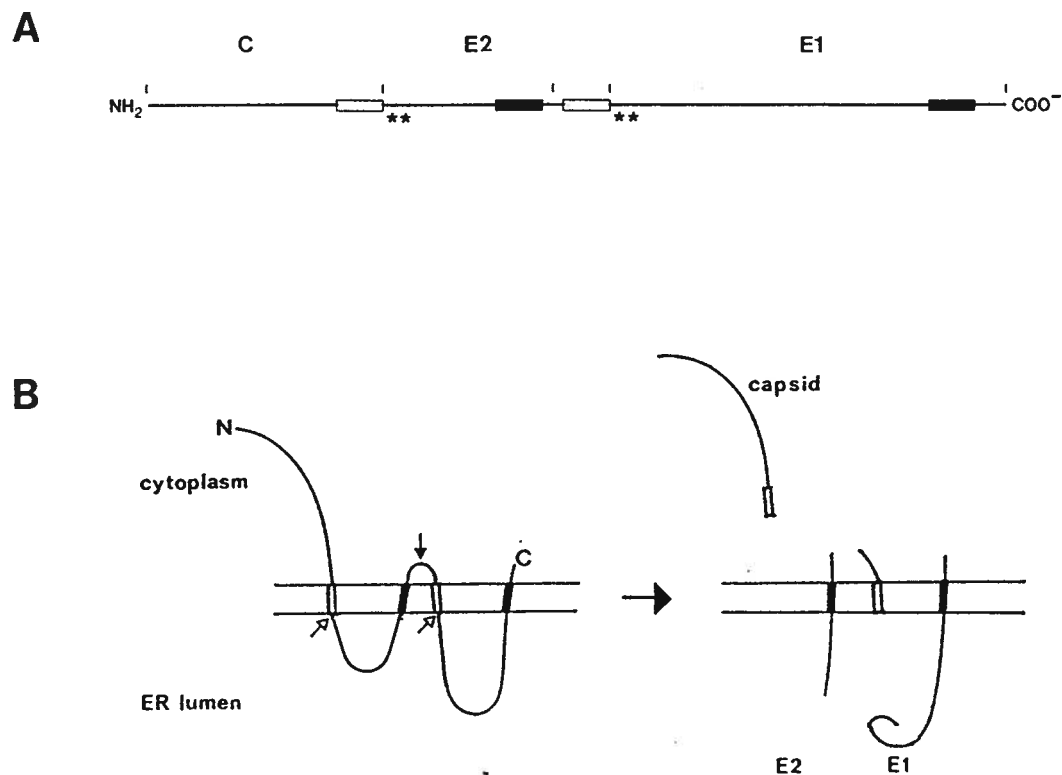


Figure 4. Model for the Topogenesis of the Rubella Virus Polyprotein. A) The 110 kD polyprotein is shown with the location of signal peptide sequences (white boxes) at the COOH termini of C and E2 and the transmembrane anchor sequences of E2 and E1 (black boxes). The asterisks (\*\*) indicate published N-terminus sequences of E1 and E2 (Kalkkinen et al., 1984). The polyprotein is not drawn to scale. B) Two cleavages by signal peptidase (white arrow head) release the capsid and separate the E2 and E1 proteins. A third cleavage site (black arrow head) has been proposed to occur in an arginine-rich region in the cytoplasmic tail of E2 (Vidgren et al., 1987). (Figure B adapted from: McDonald, 1991).

The membrane proteins are extensively post-translationally modified. Both E1 and E2 are acylated and N-glycosylated (Waxham and Wolinsky, 1985a; Hobman and Gillam, 1989). E2 is additionally glycosylated with O-linked saccharides (Lundström et al., 1991). Although E1 and E2 are co-translationally inserted into the membrane, their fates in intracellular transporting are quite different. E2 can be fully processed alone whereas E1 is dependent on E2 for complete processing and for transport to the cell surface (Hobman, 1989).

#### 1.1.6.3.Assembly

Depending on the host cell, the RV envelope proteins may either congregate in the plasma membrane or in the Golgi apparatus membrane to await assembled nucleocapsid for the budding process (Bardeletti et al., 1979, Hobman, 1989). Capsid protein of RV differs from the C of alphaviruses in that it does not contain auto-

proteolytic capacity and it is therefore believed that the E2 signal peptide remains with the RV C protein (Suomalainen et al., 1990; McDonald et al., 1991). Although this explains the microscopic observations that capsid and assembling capsomere units are found in association with cellular membrane systems (Horzinek, 1981), the C protein and the assembled nucleocapsid have complete hydrophobic properties under physiological pH conditions (Mauracher et al., 1991). In SV, the C protein specifically interacts with viral RNA sequences (Weiss et al., 1989) and the encapsidation process is thought to be catalyzed by such an RNA-protein binding event in RV also. The assembled nucleocapsids can be readily detected in the cytoplasm and seen to bud into either the Golgi or from the plasma membrane (Oshiro et al., 1969) thereby completing the virus life cycle. Capsid protein has recently been shown to also bind to ribosome subunits (Singh et al., 1991) and such binding activity could possibly be involved in the control of the transition between protein synthesis and virion assembly.

## **1.2. Rubella Pathogenesis and Pathology**

### **1.2.1. History**

The first description of a disease induced by RV infection was described as "Rötheln" by a German physician in the 18th century (in: Forbes, 1969), likely leading to the common name of "German Measles". In 1866 the English-speaking medical establishment (maybe because of the annoyance of having to pronounce an umlaut), finally followed the lead of the Scottish physician H. Veale in renaming this exanthematous rash and febrile disease to "Rubella" (Veale, 1866). To complicate matters, this disease obtained a third English identifier, following the attempt to classify acute exanthematous diseases into "First" through "Sixth" disease; Rubella, henceforth was known also as "Third disease" (Marcy and Kibrick, 1972). The viral etiology of rubella was confirmed in 1938 by human to human transmission studies (Hiro and Tasaka, 1938), thereby fulfilling Koch's postulates.

### **1.2.2. Clinical and Immunological Features**

#### **1.2.2.1 Rubella**

##### **a) Clinical Features**

The clinical features of this disease can range from a completely inapparent infection to the characteristic childhood presentation of low grade fever, adenopathy, malaise and exanthem. The exanthematous rash evolves

rapidly and in most cases spreads from the head to the trunk, where by the second day it may appear as a pinpoint or reticular pattern. The rash usually disappears by day four causing no scarring. Other epithelial surfaces such as the conjunctivae or the oro-pharyngeal region may also show signs of inflammation. In more severe cases, the infection results in encephalitis and in rare cases ( $< 0.05\%$ ) severe encephalitis can have a fatal outcome (Center for Disease Control, 1975). In cases of post-pubertal infection, the disease generally has a more severe outcome. Especially in young women the disease is associated with articular complications ranging from acute arthralgia to chronic arthritis (Smith et al., 1987). This outcome will be discussed in more detail in section 1.3.4.. The common symptoms of Rubella can be readily confused with similar illnesses presenting with a maculopapular rash, malaise and adenopathy. In one particular study of patients having a rubelliform rash, adenopathy, generalized malaise and joint pain only 36% were confirmed by laboratory tests to have had rubella. The remaining patients were infected with parvovirus (7%) or had an unknown etiology (57%) (Shirley et al., 1987). Definite diagnosis can therefore only be made following laboratory studies involving either virus isolation or serological analysis.

Rubella is highly communicable with spread, most probably, occurring by droplet infection or direct mucosal contact. Mucosal epithelial cells and submucosal lymphoid tissue serve as the initial infection site from where the virus spreads to regional lymph nodes. Rapid viral replication in affected lymph nodes leads to enlargement and tenderness which may start 5-10 days prior to the outbreak of a rash (Green et al., 1965). An incubation period of 7-9 days precedes the onset of viremia and shedding of the virus from nasopharyngeal secretions and stool (Heggie and Robbins, 1969). The exanthem commences at 16-21 days following initial exposure and coincides with the appearance of IgG in the blood stream. As the rash onset coincides with the generation of anti-RV IgG it has been hypothesised that the exanthem is caused by immune complex mediated vessel injury (Heggie and Robbins, 1969). The viremic phase quickly subsides with the mounting of the specific immune response and in general virus can not be isolated from biological fluids one week after initiation of rash (Davies et al., 1971). In cases of experimental RV infection or following vaccination these symptoms may occur up to a week earlier than natural infections (Anderson, 1949).

#### b) Immunological Features

The initial antibody response to RV infection or RV vaccination is the IgM response, which in most cases is



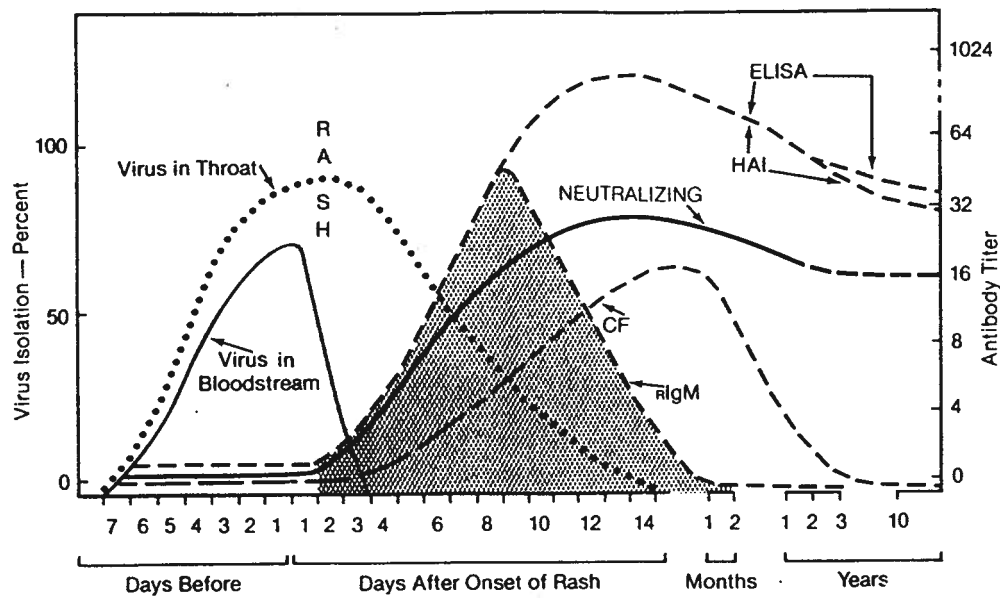


Figure 5. Clinical Features of Rubella. Patterns and kinetics of viral excretion, rash (shaded area) and antibody responses during primary Rubella. (HAI) Hemagglutinating antibody, (CF) Complement Fixing antibody, (rIgM) IgM treated with reducing agent. (From: Cooper and Buimovici-Klein, 1985)

specific to the E1 protein (Partanen et al., 1985). This response is transient, lasting on average for one month (see Fig. 5) but cases have been reported where IgM responses are detected for periods of months or years following initial Rubella infection (Al-Nakib et al., 1975). IgM persistence is not necessarily correlated with specific pathology but the occurrence of persisting IgM can make it difficult to confidently time initial RV infection by serological means. All remaining immunoglobulin classes are stimulated within weeks of infection including IgG, IgD and IgE (Salonen et al., 1985). The IgA response is felt to be transient, restricted to IgA1 and restricted to C protein (Partanen et al., 1985; Stokes et al., 1986). However more recent studies have shown that the serum IgA response may be long lasting and directed to all three proteins of RV (Chapter 3; Zhang et al., 1992). The IgG response is by far the dominant serological response and is directed to all three structural proteins of RV. When analyzed by immunoprecipitation techniques it is observed that the majority of RV-specific IgG is directed to E1 with significantly lower levels being directed to E2 and C (Katow and Sugiura, 1985; de Mazancourt et al., 1986). This holds true also if sera are analyzed by immunoblot or protein specific ELISA (Zhang et al., 1992; Chaye et al., 1992). The RV specific IgG response is predominantly made up by the IgG1 subclass with small amounts of IgG3 and IgG4 (Sarnesto et al., 1985).

The levels of IgG and its subclasses correlate well with HAI titres (Stokes et al., 1986) and with neutralizing

antibody titres (Stewart et al., 1967) and it has been assumed that these responses play a major role in viral clearance. The affinity of RV-specific IgG increases rapidly in the months following infection or vaccination (Hedman and Rousseau, 1989) and these biologically active antibodies are maintained indefinitely if infection occurs in childhood and contributes to the long term resistance to RV reinfection (Wolinsky, 1990). Circulating immune complexes (CIC) containing RV-specific antibodies and antigen are frequently detected following infection (Ziola et al., 1983).

The cellular response to RV is vigorous and in healthy responders is maintained at low levels over an indefinite period (Buimovici-Klein et al., 1977). RV-specific cellular responses are demonstrable by lymphoproliferative responses, lymphocyte-mediated cytotoxicity and are MHC restricted (Ilonen et al., 1986). Recent work has shown that E1 shows the highest reactivity in RV protein specific lymphoproliferative responses in adult humans, followed by C protein with weakest reactivities measured to E2 (Chaye et al., 1992).

The predominance of both anti-E1 B- and T-cell responses in RV specific immunity have led to the conclusion that immunity to E1 is central to protection from viral reinfection. This is further supported by the almost exclusive distribution of neutralizing domains on the E1 protein.

#### 1.2.2.2. Congenital Rubella Syndrome

##### a) Clinical Features

The characteristically uneventful outcome of childhood rubella stands in marked contrast to the devastating outcome of *in utero* RV infection. The connection between maternal rubella in the first trimester of pregnancy and the high incidence of congenital malformations, especially cataract formation, was first recognized in 1941 by an Australian ophthalmologist in the months following a Rubella epidemic (Gregg, 1941). Following this initial description, the teratogenic effects of this virus were quickly established and instigated the global effort for vaccine development and production.

Maternal RV viremia prior to conception is not associated with an infection risk to the fetus (Enders et al., 1988), however once gestation commences the risk of *in utero* infection becomes high, with few fetuses escaping infection if maternal Rubella occurs in the first month of gestation (South and Sever, 1988). 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 strongly correlate with the gestational age of the fetus at infection. In general it is believed that the closer infection occurs to conception the more severe the outcome will be. Congenital malformations are expected if infection occurs prior to 16 weeks. Birth defects are rarely described if infection occurs later than 17 weeks; late onset sequelae such as neurological deficits or endocrine disorders (hypothyroidism or diabetes mellitus) are however commonly described in congenitally infected patients and may be attributed to RV exposure (Munro et al. 1987).

RV readily infects placental tissue and virus can usually be isolated from this site (Alford et al., 1964). During early gestation, the placenta is observed to contain scattered foci of necrotic trophoblast tissue as well as vascular endothelium (Tondury and Smith, 1966). The invasion of the virus into the fetomaternal circulation allows for an embolic mode of transmission. This can explain the disseminated infection of the fetus affecting almost every organ system (Bellanti et al., 1965). The mechanism by which the virus interferes with organogenesis is not clear but several theories have been proposed to explain the observations of organ dysfunction and lowered cell numbers in organs which otherwise appear anatomically normal. It has been suggested that virus can directly damage cells or can persistently infect fetal cells and thereby interfere with the ontogeny of organs (Rawls and Melnick, 1966). A more indirect mechanism has been proposed to be mediated by soluble factors, secreted by RV infected cells, inhibiting regular mitotic division (Plotkin et al., 1967). More recently it has been described that cells, persistently infected with RV, do not react well to growth factors and produce little collagen in comparison to uninfected cells (Yoneda et al., 1986). The general observations of damaged larger vessels both in the placenta and the CRS fetus can explain lowered cranial size and birth weight as well as ischemic necrosis observed in neural tissues of some CRS patients. Immune complex mediated injury may also contribute much to the morbidity and mortality observed in perinatal and late onset sequelae. Inflammatory damage, ranging from acute neutrophil infiltration to chronic inflammatory lesions, is commonly described in lung or the brain at autopsy in CRS patients (Desmond et al., 1967). Increased loads of CIC, containing anti-RV IgG, have also been described in CRS patients with active clinical complications and have been interpreted as being indicative for the systemic, low grade replication of RV in these patients (Coyle et al., 1982). Table 2 lists some of the common clinical features of CRS.

Viral persistence is one of the hallmarks of the CRS. Virus may be isolated from most organs at birth and RV is actively secreted in urine, stool and naso-pharyngeal secretions in more than 80% of CRS patients in their

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

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

Table 2. Clinical Features of Congenital Rubella (From: Cooper and Buimovici-Klein, 1985).

first month of life (See Fig. 6). Thereafter, the rate of viral excretion diminishes and is only rarely detected once the patient has reached the age of 2 years. However it is felt that RV persists in organs of most CRS patients as virus has been isolated from patients 20 years and older (Menser et al., 1967; Weil et al., 1975). With the advent of PCR technology a more accurate estimate of the incidence and preferred organ distribution of persisting virus in this patient group may be forthcoming.

Persistence of RV in these infants has remained a paradox, as this virus readily resists clearance despite the presence of maternally acquired immunity as well as the autologous anti-RV IgM response. As CRS patients do not exhibit generalized immunosuppression after infancy and are capable of responding to the routine pediatric vaccines it has been proposed that a form of RV-specific tolerance exists. In several animal models it has been shown that *in utero* infection lead to immunological tolerance resulting in the persistence of the virus.

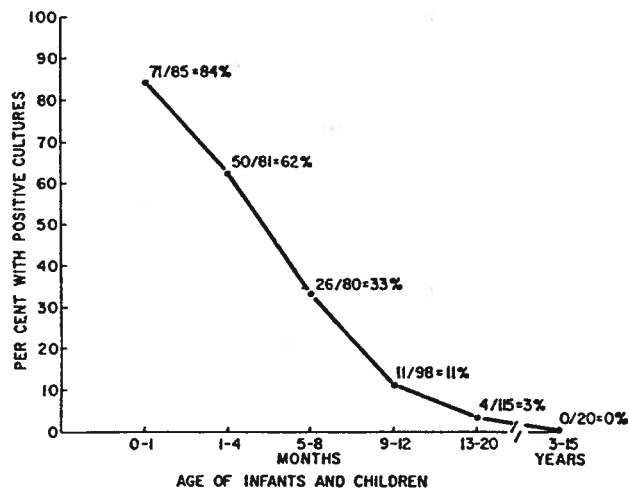


Figure 6: Incidence of virus shedding by age in CRS patients (Cooper and Krugman, 1967).

Examples such as LCMV in mice (Traub, 1938), Borna agent in rats (Hirano et al., 1983) and Border disease virus (BDV) in sheep (Barlow, 1983), have described that infection during the early ontogeny of the immune-system can lead to immunologic non-responsiveness of the fetus leading to a lifelong, low grade persistent infection of the individual. Immunological tolerance to these agents is rarely complete but rather a state of split tolerance is established. In the case of LCMV or the Borna agent, cytotoxic T cells (Tc) and delayed type hypersensitivity (DTH) reactions to viral antigens are not detectable. However helper T cell (Th) cell responses are believed to remain intact as IgG specific to the three LCMV structural proteins is produced; this hypothesis is supported by the observation that adult athymic nude mice, infected with LCMV, only respond with a transient IgM response (Buchmeier et al., 1980). However the IgG antibody in the tolerant animals is of low affinity and shows weak or no *in vitro* neutralization activity. The similarities between the immunity of LCMV tolerized mice and the CRS patients will be further discussed in section 1.2.2.2.c. Other explanations for viral persistence may include cryptic viral infections in immunologically preferred sites such as lens, salivary glands, cartilage or neural tissue. Virus might re-emerge from these sites following the waning of passively acquired IgG immunity. A more plausible explanation is that the presence of *in vitro* neutralizing antibody, transferred from mother to fetus, may be of no consequence for the replication of virus *in vivo*. This suggests that the cytotoxic responses

are most important in protective immunity and as the cellular response is not transferred from mother to fetus, the viral infection can not be cleared until an autologous cellular responses is mounted. Recent evidence has shown (Chaye et al., 1992) that viral-specific T cell responses to the E1 protein are significantly reduced in CRS patients and that this perturbation in the viral specific T cell repertoire may lead to deficiency in viral clearance.

#### b) Immunological Features

The congenitally infected fetus actively produces RV-specific IgM at 15 to 20 weeks of gestation (Daffos et al., 1984). A positive RV IgM titre in cord blood is therefore interpreted as serologic evidence for intrauterine infection with RV. However this does not correlate with the development of birth defects nor does the absence of virus-specific IgM preclude this syndrome (Enders and Jonatha, 1985). The newborn carries passively transmitted maternal IgG, which in the first year will be removed at an average half-life rate of 21 days (Hamilton, 1987). IgG of all subclasses can cross the placenta (McNabb et al., 1986) and CRS patients or their cord blood normally test positive for HAI and neutralizing IgG, reflecting the passively acquired maternal IgG (Sato et al., 1979). Nevertheless neither the maternal IgG nor colostrum/breast milk derived IgA is capable of eliminating viral replication. Over the first year, the infants mount their own RV-specific antibody response, switching from the endogenous IgM response to IgG and IgA. Because of the reported long term persistence of RV, it has long been speculated that a form of immunological tolerance must exist to allow viral persistence. This RV-specific tolerance can not be complete as CRS patients produce a RV-specific IgG to both envelope and capsid proteins and by inference must therefore also possess Th cell responses. Much controversy exists in the literature on the quantitative levels and protein-specific responses of both the humoral and cellular RV responses in the CRS patients. Initially it was observed that CRS patients quickly lost HAI antibodies in their first year of life, indicating a specific loss of anti-E1 antibodies (Cooper et al., 1971; Ueda et al., 1987). It was then proposed that tolerance in RV patients was indicated by their inability to produce IgG to RV E1, while maintaining reactivity to E2 and C. A 1985 study comparing the protein-specific antibody reactivity of CRS- and control patients using immunoprecipitation technique showed that CRS patients had significantly lower levels of E1- and elevated levels of E2-specific IgG (Katow and Sugiura, 1985), although these findings were not confirmed by others (de Mezancourt et al., 1986). The affinity of antibody directed to RV has been found to be low in CRS children, in comparison to age-matched RV sero-positive controls (Fitzgerald et al., 1988). Both

lowered affinity and non-responsiveness to key proteins or epitopes may explain viral persistence by deficient antibody mediated viral clearance mechanisms.

The study of RV-specific T-cell responses, measured by  $^3\text{H}$ -thymidine uptake or interferon release, has been described to be selectively reduced in CRS patients (Buimovici-Klein and Cooper, 1985). Moreover, these authors report that the degree of tolerization is dependent on the gestational age at infection, with infection in the first 16 weeks leading to the most pronounced reduction in T cell responses to whole RV. Others have reported that CRS patients have reduced ratios of CD4+/CD8+ cells, as well as an overall reduction of activated Th cells as indicated by significantly lower Ia positive cells than age-matched control patients (Rabinowe et al., 1986). Although suppression of RV-specific responses are suggested in CRS patients, a rudimentary Th cell response must exist to support anti-RV IgG production to the viral antigens. Recent evidence from studies using recombinant RV proteins or RV peptides in lymphoproliferative assays, suggests that only responses to the E1 protein are selectively suppressed, whereas responses to the E2 and C protein are normal or elevated in CRS patient groups (Chaye et al., 1992; Ou et al. 1992). This thesis will propose a model which explains the selective loss of anti-E1 T cells in the presence of IgG specific to all three RV proteins for the CRS patient group.

### **1.2.3.Tolerance following Intrauterine Virus Infection**

Both RNA and DNA viruses have developed a variety of means of producing persistent infections in their hosts (Table 3). Indeed it can be speculated that the earliest viral pathogens of the emerging human species had to be capable of inducing persistent infections as the human hosts were distributed in small pockets of population which rarely intermingled. This is supported by epidemiological studies on isolated populations in the Amazon, where it has been shown that the dominant viral infections are caused by persistently infecting viral species (Black et al., 1970). Only with the onset of organized agriculture did human populations reach basic community sizes which were large enough to support viruses which do not establish persistence in the host (ie: influenza, smallpox) (Fenner et al, 1974). Immunological tolerance to a virus (defined as the virus-specific refractoriness in the immune response following prior exposure to this virus) may be one mechanism which viruses have evolved to establish persistence in the vertebrate host. Animal models such as the neonatally LCMV infected

## Mechanisms of Viral Persistence

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### Avoiding Immunological Surveillance

- 1) Lowering or avoiding the presentation of antigen on MHC I and II molecules
  - a) Down-regulation by direct virus infection [Adenovirus (Tanaka et al., 1985)].
  - b) Infection of cells devoid of MHC-I, eg: neural cells [HSV I (Fitzpatrick et al., 1991)].
- 2) Infection of cellular constituents of the immune system [HIV (Hirsch and Curran, 1990)].
- 3) Generalized Immunosuppression [Measles virus (McChesney and Oldstone, 1987), HIV (Hirsch and Curran, 1990)].
- 4) Induction of Tolerance [Lymphocytic Choriomeningitis virus (LCMV) in mice (Buchmeier et al., 1980); Rubella virus (Coyle et al., 1982; Mauracher et al., 1992c)].
- 5) Infection of an immunologically privileged site [Papilloma viruses (Carson et al., 1986)].

### Alter Replication and Transcription of Virus

- 1) Production of Defective Interfering Particles [Adenovirus, Rhabdovirus (Huang, 1988)].
- 2) Generation of immunological variants [HIV (Ruscher et al., 1988)].
- 3) Loss of viral protein from the surface of the infected cell.
  - a) Capping and antigen modulation by anti-viral IgG [Measles (Fujinami and Oldstone, 1984)].
  - b) Budding of virion into Golgi or ER [HIV (Narayan et al., 1988); Rubella (Hobman, 1989)].
- 4) Insertion of genomic information, with transient expression (latency) [Herpes viridae (Fitzpatrick and Bielefeldt, 1991)].

### Other Mechanisms

- 1) Poor antigenicity [Scrapie and other agents causing spongiform encephalopathies (Chesebro, 1985)].
  - 2) Continuous infection of host or population by vector mediated infection from exogenous pool or zoonotic infections [Dengue virus (Burke et al., 1987)].
- 

Table 3. Mechanisms of Viral Persistence. For each mechanism, an example of a human or other vertebrate virus is given. Potential mechanisms for RV persistence are listed, however this virus may use other mechanisms in its persistence.

mouse, which leads to a life-long infection (Traub, 1938), have led to the concept of immunological tolerance (Burnet and Fenner, 1949) and the formulation of Burnet's clonal selection theory. These have also inspired extensive studies of tolerance induction by LCMV infection in the mouse model as well as in other virus/animal



models (Nash, 1985). The persistently LCMV-infected mouse was initially reported to be completely tolerant to the virus, but it was later reported that these animals were capable of producing antibody to all three LCMV proteins. These however were found as deposited IC in spleen and kidney (Oldstone and Dixon, 1969) where they could induce IC-mediated immunopathology. These virus-specific IgGs were found to be non-neutralizing and of low functional affinity (Buchmeier et al., 1980). The Tc and DTH responses are absent in these animals. Although studies on *in utero* infected animal models has clarified many concepts in immunological tolerance it was the introduction of the transgenic mouse model which allowed a more detailed interpretation of tolerance induction in both B and T cells. With the controlled expression of transgenic proteins in specific organs the role of both peripheral and central tolerance could be investigated, as well as the time point in lymphocyte ontogeny when tolerization occurred. Once it was recognized that the Th cell was an essential regulator of B cell progression, the issue was raised whether B cell tolerance was essential for self-education or if tolerance of the T cell responses was sufficient in the regulation of antibody responses. Mice which have been made double transgenic for hen egg lysozyme (HEL) and for high affinity anti-HEL antibody have provided evidence for B cell anergy (Goodnow et al., 1990), independent of T cell control. The model using MHC transgenic mice has shown that exposure of the pre-B cell to high levels of high density toleragen can lead to clonal B-cell deletion in the bone marrow (Nemazee and Bürki, 1989). On the other hand studies using a peripherally expressed, soluble transgene product (human insulin) have shown that B cell anergy does not occur but that antibody non-responsiveness is solely due to peripheral T cell tolerance (Whiteley and Kapp, 1989). Recent work using a transgenic G membrane protein of Vesicular Stomatitis virus (VSV) has also provided supportive evidence that B cell tolerance to a monovalent, peripherally expressed protein is regulated by Th cells (Zinkernagel et al., 1990). In these transgenic mice B cell tolerance remained complete when animals were challenged with recombinant G protein but tolerance was broken if whole VSV was used as an immunogen. Presumably the anergic anti-G protein B cells could be stimulated into IgG production by T cells specific to other viral proteins. Toleragen concentration, tissue distribution and multivalency clearly play a key role in B cell tolerance, with exposure of the immature B cell to multivalent antigens (ie: MHC) leading to clonal deletion. In encountering soluble monomeric proteins or membrane proteins of low copy number clonal anergy may occur but it now seems evident that the fate of the B cell is controlled by the underlying Th cells repertoire (Nossal, 1991). Suppressor

T cells in B cell tolerance (Theopold and Köhler, 1990) and the anti-idiotypic network theory (Jerne, 1974) may also play a role in the regulation of self-recognition but are beyond the scope of this introduction.

Many similarities exist between the tolerant state of mice following neonatal or intrauterine LCMV exposure and humans infected with RV *in utero* during the first trimester of gestation. In both cases there is an IgG response which is of low affinity and which lack *in vitro* neutralizing activity. IC formation and deposition followed by IC-mediated inflammation has been described in both models. Whether CRS patients show a complete lack of virus-specific Tc and DTH reactivity, as is the case in the murine model, remains speculative. Recent data suggests that lymphoproliferative responses to the E2 and C protein of RV remain intact in CRS patients and it is likely that Tc responses to these minor T cell antigens are present (Chaye et al., 1992). It is proposed that tolerance is induced in CRS patients to the immunodominant E1 protein, rather than to all viral proteins as has been reported in the LCMV model.

### **1.3 Rubella Vaccine**

#### **1.3.1. History**

Following the descriptions of the devastating outcome of congenital rubella (Gregg, 1941), a worldwide effort was undertaken to develop measures to prevent fetal RV infection. Initially, gamma globulin was administered to pregnant women recently exposed to rubella (described in Chase, 1984) but it was not until 1962 when the etiological agent of rubella was finally isolated by two independent groups (Weller and Neva, 1962; Parkman et al., 1962) that vaccine development could be initiated. Between 1965 and 1967, several live attenuated RV strains were developed and tested as vaccines. Of these HPV 77/DE 5 and RA 27/3 have been licensed in Europe and North America. Currently the RA 27/3 strain of RV which is passaged on human diploid fibroblasts (Plotkin et al., 1969) is the commonly used vaccine strain in Canada and the USA. This vaccine was not licensed for use in North America until 1979 but has been used in many European countries from 1969 onward. With the development of less virulent vaccine strains the public health offices of North America changed their recommended vaccine scheduling. The USA health policy in 1969 recommended universal vaccination of children at age one year and a one time vaccination effort of children up to age 12 (Hinman et al., 1983). This vaccine policy was in effect until the late 1970s when it became clear that the incidence of CRS

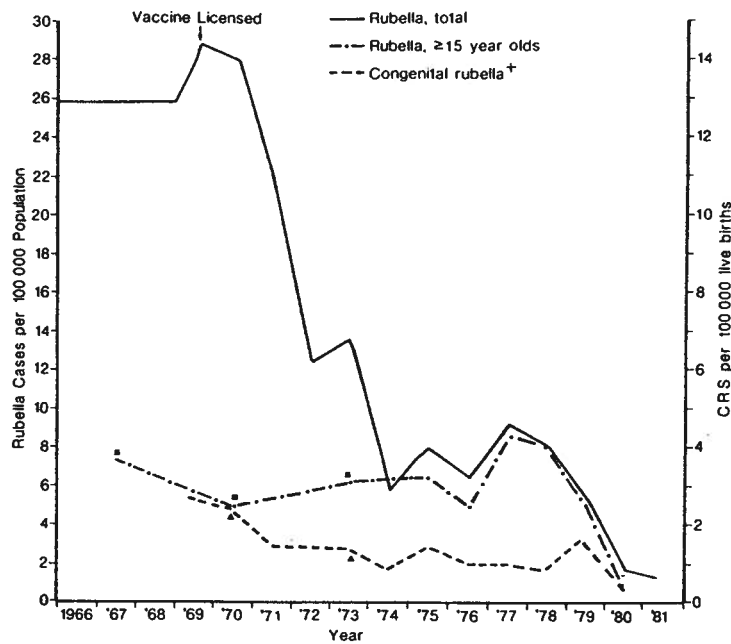


Figure 7: Incidence of reported rubella and CRS cases in the USA, 1966-81 (Hinman et al., 1983).

and reported rubella cases in females of child bearing age had not dropped to levels that were initially expected (Figure 7). In 1978-1979 it then became common practice to immunize females of child bearing age and to advise *post partum* immunization of RV seronegative mothers (Preblud, 1986). This change in vaccine policy led to a significant drop in reported cases of CRS and has remained vaccine policy in both the USA and Canada.

### 1.3.2. Biology and Immunology

The mechanism of RV attenuation has remained unclear although both wild type strains and attenuated vaccine strains have been sequenced (Zheng et al., 1989). Attenuated strains vary minimally in protein sequence, with only 5 amino acid substitutions occurring in the structural polyprotein of the HPV 77 RV vaccine strain compared to wild type isolates. 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., 1989), although other mechanisms of attenuation (eg: host protease dependence) can not be ruled out.

Immunity to vaccine strain virus is believed to be life long (Plotkin et al., 1973). After MMR vaccination at 12 months or older, 95% of children seroconvert by HAI technique and this immunity has shown to be protective for at least 15 years in 90% of vaccinees (Robinson et al., 1982).

### 1.3.3.Side Effects of RV Vaccination

Controversy over the safety of rubella vaccine has only recently become of public concern. In the early seventies, neuropathies (Kilroy et al., 1970) and transient arthritis (Spruance and Smith, 1971) were describe in children receiving the HPV 77/DE 5 strain and led to the withdrawal of this strain and the licensing of the RA 27/3 strain in the USA and Canada (in Howson et al., 1992). Although the RA 27/3 strain has been shown to cause minimal side effects in children (Polk et al., 1982) it is still associated with significant adverse effects in adolescent and adult female vaccinees. Acute arthritis is estimated to occur in 13%-15% of adult RA 27/3 vaccine recipients (Reviewed in: Howson and Fineberg, 1992). Case reports of severe and chronic arthritis have also been reported following the administration of RA 27/3 in adult females (Tingle et al., 1986). In the pre-licensure trials of the RA 27/3 vaccine this strain was determined to be safe for use in children. Subsequently these results were interpreted to hold true for adults as well, leading to the extension of vaccine administration to women of child bearing age without further evaluation of potential side effects (Tingle, 1990). Ongoing double blinded, randomized and placebo-controlled clinical trials of rubella vaccine in adult seronegative women at the BC's Children's Hospital Vaccine Evaluation Centre will provide a more definitive estimate of the incidence of acute and chronic forms of arthritis following RV vaccination.

The pathogenesis of Rubella Associated Arthritis (RAA) has remained unresolved (Ford et al., 1986). Rubella can be isolated from synovial fluid and in peripheral lymphocytes in individuals with RAA, in the years following RV 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 the synovial fluid or the synovial membrane. RAA is most commonly associated with peripheral joints (Sauters and Utsinger, 1978) which are at temperatures of 5#- 6#C below the body's core temperature. This is of significance in light of reports that RV readily establishes persistent infections of synovial cell cultures at 32#C but not at 36#C (Cunningham and Fraser, 1985). Recently others have shown that wild type and vaccine strains of RV replicate to different extents in human synovial cell lines or in human synovial membrane organ cultures (Miki and Chantler, 1992). They showed that strains which have been reported to have a higher incidence of RAA are also characterised by a high degree of synoviotropism. Although it is likely that RV may persist in local tissues it remains unclear if this non-lytic virus is sufficiently virulent to cause direct damage in the joint or whether persistent RV antigen is connected with the initiation of immunopathology. It thus remains an enigma why some individuals mount a response which

leads to rapid viral clearance and uncomplicated outcomes in Rubella while others, especially adolescent and adult females, mount immune responses which may not clear RV in localized organs and lead to the development of acute or even chronic arthritis.

The pathogenesis of infectious arthritis has been summarized as "...a chronic T-lymphocyte - and macrophage dependent response to foreign or autoantigens present in the synovial tissue." (Decker et al., 1984), however the question remains what initially attracts these cells to the synovium. A role of CIC and their deposition in the synovial space has maintained itself as an attractive hypothesis for involvement in the pathogenesis of RAA as well as in the manifestations of other forms of infectious arthritis (Inman et al., 1987). IC are involved in inflammatory responses of CRS patients (Tardieu et al., 1980) and are most likely responsible for the vessel damage which presents as the rubelliform rash in acute Rubella (Heggie and Robbins, 1969). It is feasible that RV antibody and low grade locally persisting viral antigen may result in IC deposition and lead to the initiation of an inflammatory response. Studies regarding the presence of immune complexes have shown that levels of CIC in RAA patients are not significantly increased in comparison to healthy Rubella patients (Singh et al., 1986), however others have shown that CIC containing anti-RV IgG are increased following RV vaccination (Coyle et al., 1982) but this study did not compare anti-RV containing CIC in healthy and RAA patients. A study investigating the levels of CIC containing anti-RV IgG or RV antigen as well as IC in the joint space may clarify the role of IC-mediate injury in RAA.

Reactivity of the virus-specific immunity with self-antigens has been proposed (Lund and Chantler, 1991), describing cross-reactivity of rubella specific epitopes with an unidentified protein derived from synovial epithelium. The hypothesis of molecular mimicry remains of interest and studies showing cross-reactivity of viral specific T - and B-cells may shed further light on the pathogenesis of RAA.

A recent model for the pathogenesis of RA 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. Interestingly the RV capsid molecule shares structural similarities with the well defined *S.aureus* enterotoxin superantigen (Marrack and Kappler, 1990), in that both contain an 8 membered  $\beta$ -pleated barrel as their core structure.

Evaluation of the proposed immunopathological mechanisms in RAA requires a good understanding of the

"normal" adult immune response in order to be able to define differences in the immune responses of RAA patients which may be involved in the induction of the inflammatory processes. Furthermore similarities may exist in the immune repertoire of CRS- and RAA patients as both patients group may develop inefficient immune responses which lead to viral persistence.

#### **1.3.4. Viral Vaccine Efficacy**

The recent controversy in the public media and medical literature (reviewed in: Howson et al., 1992) over RV vaccine and its induction of infrequent debilitating side-effects, has obscured the benefit that this vaccine program has brought to the public. The incidence of CRS in the USA has dropped from 106 cases in 1979 to 20 cases in 1985 with a continuing downward trend, so that CRS is hoped to be on the verge of elimination in North-America (Cochi et al., 1989). In the case of seronegative females, it should be remembered that the incidence of joint-manifestations following RA 27/3 administration is significantly lower than following wild RV infection, to which these vaccinees would have been susceptible (Tingle, personal communication).

#### **1.4. Laboratory Diagnosis of Rubella Virus Infection**

In order to initiate the comparative study on the humoral immune responses to RV and RV proteins in congenitally- and post natally infected individuals, new assays had to be developed and established assays had to be adapted for application to the individual RV antigens. This section will review the most commonly used assays for the detection of RV or RV-specific immunity.

##### **1.4.1. Virus Isolation**

The definitive diagnosis of RV infection is achieved by the isolation of this virus by tissue culture techniques. The virus will infect a broad range of cell lines. Virus samples can be isolated from pharyngeal swabs or nasopharyngeal secretions at the time of onset of the exanthem. In the congenitally infected infant, virus is readily isolated from stool, urine, nasopharynx, blood and cerebrospinal fluid. Virus can also be isolated from blood, synovial and breast milk lymphocytes (Chantler and Tingle, 1982), but these preparations do not yield virus in all cases and are not used for routine clinical diagnosis. Excretions or cell preparations are most commonly taken up in solutions of physiological salt solutions containing 1% BSA or 5-10% fetal calf serum and

transferred immediately onto the cell line of choice for viral propagation. Two commonly used cell lines, African Green Monkey kidney cells (Vero) and Baby Hamster kidney cells (BHK-12) are used for growing high yield virus. The virus causes little cytopathic effect in these cells and virions can be readily isolated from tissue culture supernatants following the second day of infection. Other cell lines such as primary African Green Monkey kidney cells have been used to detect RV by interference with echovirus 11 replication and Rabbit kidney cells (RK-13) are extensively used for RV titre determination as this cell line exhibits a detectable cytopathic effect (Nawa, 1979). With the advent of sensitive molecular probing assays, direct detection of RV genome may replace viral isolation by tissue culture. Reports of RNA hybridization for the detection of RV RNA in chorionic villus samples (Ho-Terry et al., 1986) and the use of PCR technology in direct RV detection (Eggerding et al., 1991) indicates the direction of future virus detection.

Serological testing for antigen specific antibody responses, remains the primary diagnostic means for RV infection. Serological techniques are independent of the narrow time interval when virus can be isolated and therefore can be used retrospectively to confirm and time RV infection. The following paragraphs will introduce the serological techniques which have found application in RV serology and some of the problems associated with their application and interpretation.

#### 1.4.2. Neutralization Assay

The neutralization test (NT) is one of the oldest assays in viral diagnosis and serology, dating back to the early work on vaccinia virus (Sternberg, 1898 reviewed in: Lennette et al., 1989). Sternberg observed that certain sera were capable of rendering vaccinia virus non-infective to a susceptible host, and termed this activity "neutralization". This serologic test has been the "goldstandard" against which other serological techniques have been measured. In rubella serology this assay has been in use since the isolation of this agent and directly measures the individual's capacity to neutralize RV infection *in vitro*. However because NTs are labour intensive they have not found much application in routine diagnosis and are mainly employed in research laboratories for the standardization of new serodiagnostic tools (Steward et al., 1967) or in the characterization of RV specific monoclonal antibodies (Waxham and Wolinsky, 1985b; Brush, 1989). In the case of RV neutralization it was soon realized that serum components other than immunoglobulins had the capacity of neutralizing the virus.

These have been determined to be complement, b-lipoproteins (Clarke and Cassals, 1958) and phospholipids (Mastromarino et al., 1990). Such non-specific inhibitors have to be removed by pretreatment of sera with 57 °C incubation followed by kaolin treatment or by precipitation with manganese chloride/heparin (Kawano and Minamishima, 1987) prior to the determination of neutralization titres.

#### **1.4.3.Hemagglutination Inhibition (HAI) Assay**

Many viruses from varied families are capable of agglutinating erythrocytes from a variety of species. This hemagglutination (HA) phenomenon forms the basis for the serodiagnostic test of antibodies which bind to the virus and thereby inhibit HA (HAI). RV was observed to bind to day-old chick erythrocytes as well as white goose erythrocytes and this interaction can be selectively inhibited by antibodies as well as the non-specific inhibitors described above. The HAI assay had been extensively tested and compared for sensitivity and specificity to the NT (Lennette et al., 1967; Field et al., 1967) and has been found to show a good correlation with NT in human test populations. The standard protocol (U.S. Dept. of Health, Education and Welfare, 1970) recommended pretreatment of sera with manganese chloride/heparin and adult chicken erythrocytes for the removal of non-immunoglobulin inhibitors of HA and has set a HAI titre of 1:8 as the cut-off for protective immunity. The HAI assay will provide results in one day and demands a fraction of the costs of the NT; therefore HAI was quickly adapted by most laboratories as the mainstay of rubella serology from the mid 1960s to 1980s, and provided the reference standard to which novel immunoassays had to be compared (Herrmann, 1985). The molecular mechanism of the HA interaction is not completely understood in that the receptor on the erythrocyte is not defined. The HA activity on the virion on the other hand has been studied extensively. HA activity resides on the E1 protein of RV and is dependent on intact conformation as well as complete glycosylation (Qui and Gillam, 1992). Binding sites for HAI antibodies have been mapped to a 40 amino acid region on E1. These are in close proximity to the defined neutralizing epitopes but do not correlate in all cases (Terry et al., 1988; Waxham and Wolinsky 1985a).

#### **1.4.4.Enzyme-Linked Immunoassays (EIA)**

Enzyme-linked immunoassays have found much application in serodiagnosis since the large scale availability of enzyme-conjugated monoclonal and polyclonal antibodies directed to immunoglobulins first described in 1969



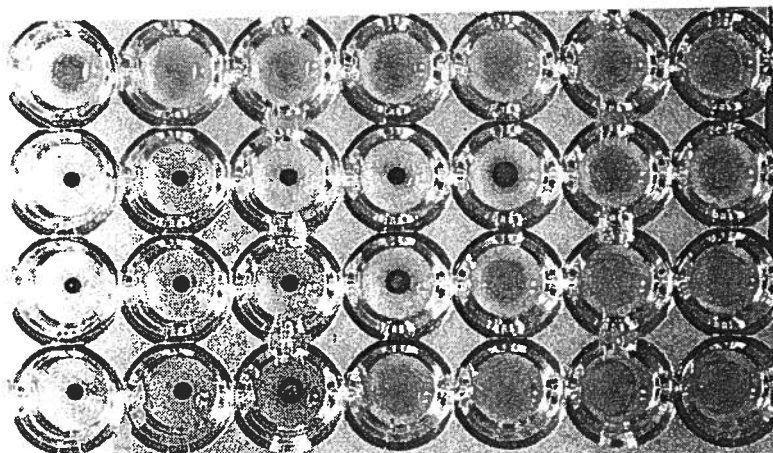


Figure 8. Hemagglutination-Inhibition (HAI) Test. Erythrocyte sedimentation [ A ] correlates to the presence of antibodies which have bound to E1 protein and inhibited the cross-linking of cells by the virion. Hemagglutination [ ⊙ ] means the absence or the diluting out of antibodies, as the virus exerts its agglutination properties. Each row represents a two-fold serum dilution from 1:8 to 1:512. Top row illustrates a negative serum, the remaining three are positive with titres of 1:64, 32 and 16 respectively (From: Lennette et al., 1989).

(Avrameas, 1969; Engvall and Perlmann, 1971). Conjugation procedures and substrate systems have improved dramatically in the last 15 years and have led to the large scale acceptance of this technique. Although fluorescent- and radiolabelled immunoassays find application in specialized fields, the enzyme conjugates, mainly alkaline phosphatase and horseradish peroxidase, have emerged as the most versatile and applicable to immunodetection and diagnosis (Lennette et al., 1989). For RV serology in most clinical and research laboratories, EIAs have replaced the older HAI and NT assays because of the increased sensitivity and economy of the EIA (Steece et al., 1984).

The two following paragraphs will briefly review two applications of RV specific EIA which are central to this thesis: the enzyme-linked immunosorbent assay (ELISA) and Western blot.

#### 1.4.4.1 Enzyme-Linked Immunosorbent Assay (ELISA)

The use of ELISA in RV serodiagnosis was initially described in 1977 (Bidwell et al., 1977). By the early 1980's it had become the main laboratory diagnostic test for rubella. As this test allows the determination of levels of all immunoglobulin classes, it lends itself to automation and can be readily scaled up to screen large populations (Leinikki et al, 1978; Buimovici-Klein, 1980). A world-wide serum reference system has allowed for the uniform and standardized interpretation of ELISA results (WHO, 1971). A good correlation was observed between ELISA seropositivity and positive HAI titres (Shekarchi et al., 1981; Herrmann, 1985; Stokes

et al., 1986) in normal patient populations while others have found this correlation to be poor in CRS patients (Hancock et al., 1986). The commercially available whole RV ELISAs measure antibodies directed mainly to surface RV epitopes of the envelope proteins and do not necessarily measure antibodies which are functionally significant for *in vitro* neutralization or which provide the host with protective immunity. This stands in contrast to functional antibody tests, such as HAI and NT, which measure antibody to a narrow range of epitopes on the E1 protein.

#### 1.4.4.2. Western Blotting

The electrophoretic transfer of proteins onto nitrocellulose from SDS-PAGE gels and the detection of protein by enzyme-conjugated antibody was first described in 1979 (Towbin et al., 1979). Although Western blotting most commonly employs enzyme-conjugated detection antibodies (immunoblot) the use of fluorescing antibodies have come into use over the last years. In the case of RV serology, Western blotting allows the further dissection of the humoral immune response by determining the protein specificity of immunoglobulins. However disadvantages inherent to this technique (ie: protein denaturation, long processing time) have given it few applications in large scale serology, with the exception of AIDS serology (Steckelberg and Cockerill, 1988), so that this technique has remained a tool largely used in research laboratories.

Serological techniques for the analysis of Rubella immunity have evolved as a consequence of the demand for assays capable of rapidly, repetitively and cost-effectively handling large numbers of samples. Today, ELISAs have filled this demand and provide a sensitive and specific test for the presence of anti-RV immunoglobulins. These assays are therefore of use in the diagnosis of previous virus exposure - but do not provide a final answer as to whether the detected immune response will protect an individual from reinfection. This problem is illustrated well by CRS patients who test strongly positive on RV ELISA but remain susceptible to reinfection (Cooper et al., 1971).

#### 1.4.5. Affinity Assays

The above discussed RV serology assays have been developed to measure RV- or RV protein specificity of

immunoglobulin classes, their quantitation and to some extent their biological function but provide limited information about the affinity of these immunoglobulins. The high affinity of the immunoglobulin molecule to its antigen is an important functional parameter and is ultimately involved with antigen-specificity as well as the biological activity of the molecule (Table 4). As the terms affinity and avidity will be used extensively in the text and have often been used interchangeably in the literature, the brief definitions below will help in the clarification of these terms. The term "Affinity" describes the quantitative interaction of hapten and antibody. The Law of Mass Action can be applied to this interaction and the stability of the antibody:hapten complex can be expressed and experimentally defined as the association equilibrium constant,  $K$ . For an accurate experimental determination of the  $K$ , both antigen and antibody should be present in pure and homogeneous form. The determination of  $K$  is therefore most precise in a system involving monoclonal antibodies interacting with monovalent or haptenic antigens (Steward and Steensgaard, 1983). The term avidity or "functional affinity" is used to qualitatively describe the strength of antigen:antibody interaction. Although directly dependent on the affinity of the antigen binding site with the antigen, it is strongly influenced by antigen valence and immunoglobulin class. This can be best explained by a set of hypothetical IgG and IgM molecules with identical antigen binding sites for a multivalent antigen. Both molecules have identical affinities however the IgM molecule will bind more avidly as it can combine with more sites on the antigen.

It has been realized for many years that the affinity of antibodies progressively increases following immunization and this phenomenon has been termed affinity maturation. The mechanism by which such an increase of the average affinity occurs in response to immunologic challenge has received much attention and several underlying molecular mechanisms have found acceptance: somatic mutations (Milstein, 1987), antigenic selection (Allen et al., 1987) and a unifying hypothesis, combining the latter two and proposing hypermutation of the IgG V region on a DNA level during the  $G_0$  phase prior to B-cell progression (Manser, 1990).

The affinity maturation of anti-RV IgG responses following primary rubella infection occurs over a three to four month period (Hedman and Rousseau, 1989), with antibody affinity increasing by an estimated 100 fold. This range of increase in affinity is supported by studies in rabbits using DNP-lysine antigen, indicating that IgG from the acute response rose in affinity from  $5 \times 10^5$  to  $10^8 \text{ lM}^{-1}$  in the months following antigenic challenge (Eisen and Siskind, 1964). The well defined increase in the affinity of anti-RV IgG has been recently been exploited to determine the time point when RV infection occurred (Hedman et al., 1989). This is of significant

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Reaction	Reference
Passive hemagglutination	(Levine and Levytska, 1967)
Complement fixation	(Fauci et al., 1970)
Hemolysis	(Warner and Ovary, 1970)
Immune elimination of antigen	(Alpers et al., 1972)
Virus neutralization	(Blank et al., 1972)
Damage to DNP-sensitized liposomes	(Six et al., 1973)
Protection against bacterial infections	(Ahlstedt et al., 1974)
Blocking antibodies	(Adkinson et al., 1979)
Hemolysis typing	(Hedman et al., 1989)

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Table 4: Biological Reactions in which High Affinity Antibodies are Superior to Low Affinity Antibodies.

diagnostic importance in determining intrauterine exposure if recent RV infection during early pregnancy is suspected as the determination of affinity will provide a good estimate if Rubella occurred before or after conception.

Antibody affinity considerations are also of importance in the study of immunological tolerance. The LCMV mouse model of tolerance was initially described to show complete immunological tolerance following neonatal virus inoculation (Traub, 1938), but was later shown to be a form of split tolerance. Cytotoxic responses were lacking in these animals (explaining persistent virus replication) but IgG responses to all three viral structural proteins were present. In most animals LCMV-specific antibodies were found as CIC or IC deposited in the reticuloendothelial system or in the kidneys. These antibodies were of low affinity in comparison to those found in adult responding mice and had no *in vitro* neutralizing activity. It remains unclear if the decrease in antibody affinity during immunological tolerance is due to preferential deletion of high affinity clones or if the lack of Th cell results in inefficient stimulation of B-cell clones in the germinal centres. However recent evidence indicates

that high affinity responders to self or toleragen are selectively purged (Nossal, 1991). Predominance of low affinity responses to a replicating antigen is therefore an indication that split immunological tolerance has occurred.

A wide range of methods are available for the determination of antibody affinity. Equilibrium dialysis and ammonium sulphate precipitation have found much application in studies using monoclonal antibodies but are of little use for the determination of functional affinities of sera to complex mixtures of antigens such as the Rubella virion. Two methods will be introduced here which will be extensively described in the body of the thesis.

#### 1.4.5.1. Chaotropic Elution Assays

Antigen-antibody interactions can be disrupted by exposure to pH extremes, chaotropic agents and detergents. Studies on immune complexes described that low affinity antibodies could be readily dissociated by chaotropic agents, whereas high affinity interactions were resistant to these conditions and could only be dissociated by conditions leading to the denaturation of both antigen and antibodies (Hogben et al., 1986). Protein denaturants could therefore be used to selectively disrupt the binding of low affinity antibodies by choosing the reagent concentration and the exposure time of the immune complex to the denaturant. Urea acts as a denaturant by interfering with hydrophobic interactions of polypeptide chains (Kamoun, 1988) and exposure of immune complexes for short periods to 8M urea, or other denaturants such as 4M isothiocyanate, leads to selective elution of low affinity antibodies from solid phase bound immune complexes. This can provide a measurement of the average affinity of serum antibodies by calculating the amount of total immunoglobulin which have resisted the chaotropic elution (elution ratio [ER(%)]). This ratio, also referred to as the affinity index, is believed to be related to the average affinities of the circulating immunoglobulins directed to the antigen system, as these techniques have been successful in demonstrating affinity maturation in response to a variety of antigens: RV and RV vaccine (Thomas and Morgan-Capner, 1988; Mauracher et al., 1992a), Toxoplasmosis (Hedman et al., 1989), Respiratory syncytial virus (Meurman et al., 1992) and Varicella zoster virus (Tilley and Junker, 1992).

It is still argued whether the chaotropic elution ELISA indirectly measures affinity or the avidity of immunoglobulins. In the titles of papers and abstracts which use this method both terms are used, at times

interchangeably. As chaotropic agents most likely directly interfere with the epitope-antibody binding site it would seem reasonable to suggest that this method measures antibody affinity. To resolve this issue the interaction of low and high affinity anti-dinitrophenol (DNP) monoclonal antibodies with variably substituted DNP-albumin could be investigated by chaotropic elution ELISA. If this assay can selectively differentiate between high and low affinity antibodies, independent of DNP concentration on the albumin molecules, then it could become possible to conclude that the chaotropic elution assay indirectly allows for the assessment of antibody affinity.

#### 1.4.5.2. Inhibition ELISA

In the ELISA technique, affinity of a serum can be estimated by the slope of the titration curve, especially if non-repeating antigens are used (Steward and Lew, 1985). Low affinity monoclonal antibodies need to be present in far higher concentration to produce similar absorbance values as high affinity antibody. Similar observations have also been made in inhibition ELISA, where low affinity monoclonal antibodies require higher concentrations of liquid phase inhibitor to decrease solid phase binding (Rath et al., 1988). This principle was adapted in a One-Well Inhibition ELISA where a constant amount of antibody was added to wells with constant amounts of coated antigen. The amount of liquid phase inhibitor was variable and the amount of inhibitor required to bring about a 50% decrease in absorbance ( $I_{50}$ ) was found to correlate well with the average affinity of sera (Steward et al., 1991). These authors used polyclonal sera directed to an 18 aa peptide representing the neutralizing domain of the foot-and-mouth disease virus and were able to verify the affinity values obtained by the inhibition ELISA with those obtained by the ammonium sulphate precipitation technique.

### 1.5. Thesis Rationale and Thesis Objectives

Rubella virus is a pathogen which provides the rare opportunity to study the influence of intrauterine viral infections on human immunity. The study of CRS patients as a model for the induction of immunological tolerance is of advantage, as RV biology, pathology and molecular genetics are well established and clinical specimens of both pre- and postnatally infected individuals were readily available in our laboratory. This thesis tested the hypothesis that intrauterine infection of the human fetus in the first trimester of gestation with Rubella virus leads to development of immunological tolerance. A study of the tolerization of the human

immune system with RV will help in the understanding which mechanisms for immunological self/non-self recognition are operational in humans. Furthermore, the induction of immunological tolerance may be an important mechanism for viral persistence. Persistence of RV in CRS patients has been well documented. However, RV persistence may also play a role in the pathogenesis of chronic inflammatory disorders such as RAA. The identification of immune responses which may be inefficient in clearing viral infections may therefore be of relevance in both the congenitally-infected patient and patients developing chronic rubella associated arthritis.

The objectives of the thesis are discussed in point form below and also reflect the titles under which the individual sections of the results and discussion chapter are presented. The development of reagents and techniques, needed for the comprehensive evaluation of RV-specific humoral responses, were initially established. Once these assays had been established it was possible to first define the normal adult response to RV infection followed by the analysis of antibody responses to RV in CRS patients.

**I) Preparations and Characterization of RV for Use in Solid Phase Immunoassays.** In order to compare the antibody responses of CRS and control patients, new virus specific immunoassays had to be developed and standardized. It was therefore necessary to produce a large and uniform preparation of RV which would provide a large enough stock to be used for all further experiments. This virus stock was defined for its RV titre, antigenicity and purity. Whole RV ELISA methods were optimized using this preparation and this RV preparation was also used in the evaluation of suitable methodologies of anti-RV IgG affinity assays.

**II) RV Protein Specific Immunoassays.** The objective of the methodological development was to have techniques available which could measure the following parameters of the RV-specific antibody response: 1) Quantitation of IgG to whole RV and RV proteins E1, E2 and C, 2) Measurement of relative affinities of IgG directed to whole RV and RV proteins E1, E2 and C, 3) Measurement of biological activity of sera by HAI assay and 4) Determination of specificity of antibodies to linear and topographic epitopes of the viral proteins.

The development of new techniques was necessary in order to initiate the comprehensive study of patients infected with RV in the pre- and postnatal period. These included methods for evaluating the specificity and affinity of antibodies to individual RV proteins. Immunoprecipitation, Western blotting and ELISA using

separated RV proteins were evaluated as techniques suitable for the detection of RV protein-specific immunoglobulins and needed to be capable of measuring both antibody quantity and affinity. It had been reported that both E1 and E2 proteins contained mainly conformationally dependent epitopes and it was necessary to show that purified RV antigens retained their native structure, before using these proteins in comparative immunoassays.

**III) RV-Specific Antibody Responses in Postnatally Infected Patients.** In order to determine if a state of RV-specific tolerance existed in the CRS patients it was first necessary to characterize the humoral immune response of healthy patients to RV. Although antibody responses to RV in adults have been evaluated for more than four decades a comprehensive study of biological activity (HAI), whole virus and virus protein specificity, antibody affinity and linear/topographic epitope specificity of RV-specific antibody responses has never been undertaken. Once the RV-specific antibody response had been defined in healthy responders it was possible to analyze the responses of CRS patients. Differences in the responses of these patient groups may reveal defects in the immune repertoire which would suggest a state of immunological tolerance in CRS patients.

**IV) RV-Specific Antibody Responses in Congenitally Infected Patients.** To test the hypothesis that intrauterine exposure to RV had established immunological tolerance, the RV-specific antibody responses of CRS patients had to be analyzed and compared to those of the control population. If differences in the humoral response were detected, a model of tolerance could be proposed which could accommodate serological findings and current data on cellular responses in this patient group.



## **2. MATERIALS AND METHODS**

### **2.1. Virus Preparation, Titration and Concentration**

#### **2.1.1. Bulk Preparation of Rubella Virus (M-33 Strain)**

Rubella Virus, M-33 strain (VR-315, ATCC, Rockville MA, USA) (Parkman et al. 1962) was grown by infecting 80% confluent monolayers of Vero Cells (CRL 1586, ATCC) as follows. Vero cells were grown in complete MEM containing 1% Glutamine (30 mg/ml), 100 units/ml and 0.1 mg/ml of Penicillin and Streptomycin respectively to prevent bacterial contamination (all from Gibco, Grand Island NY, USA), 20 mM Hepes (Sigma, St. Louis MO, USA) and 2% fetal calf serum (HyClone Laboratories, Logan UT, USA) using T-175 Falcon tissue culture flasks (Becton-Dickinson, Lincoln Park NJ, USA) at 36 °C in 5% CO<sub>2</sub>. Supernatant was aspirated and cells were washed once with 10 ml PBS at 36 °C. RV (at an m.o.i. of 10) was then added in 30 ml of medium without fetal calf serum. Virus was left to absorb at 36 °C for 2 hours. 120 ml of complete medium was then added and cells were returned to the incubator for 3 days. The primary virus harvest at three days following infection was used as the starting material for further viral purification. The technical help and the many innovations which Ms. S. Farmer introduced to the growing of RV is acknowledged and greatly appreciated.

#### **2.1.2. Rubella Virus Titration on RK-13 Cells**

RK-13 cells (CCL 37, ATCC) were seeded into 96-well Falcon plates, at 0.2 ml per well, at a concentration of 20,000 cells/ml, using M199 Medium, 1% penicillin/streptomycin and 2% fetal calf serum (suppliers as above). At 48 hours, medium was removed and the wells were washed with warm PBS. Tissue culture supernatants containing RV were diluted in 10 fold dilution series and 50 µl loaded in triplicate onto the confluent RK-13 cells followed by a one hour incubation 36 °C. Following virus absorption, 150 µl of complete medium was added to each well and infectious foci were scored after 3-4 days.

#### **2.1.3. Concentration of Rubella Virus Tissue Culture Supernatant**

Tissue culture supernatant was clarified by centrifugation on a Silencer H-103 N centrifuge at 3000 rpm ( $G_{av} = 1000$ ) for 15 minutes. Pooled preparations were then brought to 0.5 M NaCl and 10% w/v polyethylene

glycol 20,000 (BDH, Poole, England) while stirring slowly at 4 °C for 2 hours. The suspension was then precipitated by centrifugation in a Sorvall GSA rotor in a RC-5B Sorvall Centrifuge (DuPont & Co, Newton CT, USA) at 8000 rpm ( $G_{av}=7000$ ) for thirty minutes. The virus pellet was resuspended in TE buffer (10 mM Tris, 5 mM EDTA, pH 7.4)

## **2.2. Preparative SDS-PAGE**

### **2.2.1. Electrophoresis**

RV proteins were separated on 1.5 mm thick slab gels using the Protean II mini-gel system (BioRad, Richmond CA, USA). The discontinuous buffer system according to Laemmli (Laemmli, 1970) was used. The polyacrylamide separating gels (10%/0.25% Acrylamide/Bis, 0.375M Tris-HCl, 0.05% Ammonium Persulphate, 0.05% Temed, pH 8.8) were overlaid with stacking gels (4%/0.1% Acrylamide/Bis, 0.125 M Tris-HCl, .05% Ammonium Persulphate, .05% Temed, pH 6.8) and a custom-made preparative teflon comb used for well formation. In order to counteract "smiling" of the dye front, the comb was cut with the reference wells exceeding the preparative well by 1.5 mm in length. In all cases, gels were poured and left to polymerize over night in the dark at 4 °C.

Sample buffer for preparative PAGE was devoid of reducing agents, contained less SDS than commonly used and was prepared monthly as double strength stock (0.25 M Tris-HCl, pH 6.8, 0.5% SDS, 20% Glycerol, .02% Bromophenol Blue). 50  $\mu$ l of RV preparation was diluted in 300  $\mu$ l dH<sub>2</sub>O followed by the addition of 350  $\mu$ l of Sample buffer. Tubes were incubated in a 60°C waterbath for 3 minutes and loaded into the preparative wells. The reference wells received 50  $\mu$ l of pre-stained molecular markers (Sigma, St. Louis MO, USA). Electrophoresis was performed at 40 mAmps per gel, constant current, until the dye-front reached the bottom of the slab. The location of RV protein bands in the slab gel was detected by excising a thin lane of gel containing half the lane of pre-stained MW markers and the edge of the preparative lane containing RV, followed by transfer to nitrocellulose and immunoblotting. The nitrocellulose strips were developed using a human anti-RV serum containing IgG to E1, E2 and C (See immunoblotting section for methods). These bands were then used as a guide in the excision of gel strips containing antigen.

### **2.2.2.Electroelution**

Gel strips 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  $\mu$ m filter. Electroeluates of each protein were collected, frozen at -70 °C until enough of each protein was prepared to provide sufficient material for completing the anticipated patient study. Each preparation was assayed by immunoblot and the preparations of each viral protein, showing no contamination with other RV structural proteins, were pooled. The C antigen pool showed residual contamination with E1 and was further purified using Triton X-114 extraction (see below).

### **2.2.3.Detergent Extraction**

Capsid preparations were treated with KCl for SDS removal as follows. 100  $\mu$ l of 0.4 M KCl were added to 1 ml of C preparation at room temperature. The preparation of the C antigen pool was then put onto ice and precipitation of the potassium lauryl sulphate salt allowed to equilibrate for 30 minutes. Tubes were then centrifuged for 1 minute at 10,000rpm on a bench-top centrifuge and the SDS-free C preparation transferred to new tubes and left on ice. Triton X-114 (Sigma, St. Louis MO, USA) was added to a final concentration of 0.5% and the tubes rotated at 4#C for 1 hour and then brought to room temperature. Triton X-114 reaches its clouding point at 17 °C, above which it can be removed from an aqueous solution by centrifugation. The detergent free C preparation was then aliquoted and stored at -70 °C.

### **2.2.4.Yield Determination of Electroelution**

L-[<sup>35</sup>S]methionine labelled RV (see below) was loaded into two wells of reducing 9% SDS-PAGE slab gels with each well containing antigen being flanked by pre-stained MW markers. Gels were run until the BPB dye front reached the bottom of the gel. The region of the lanes containing labelled virus located between the MW the 58- and 26 kD markers was excised, fragmented and added to 2 ml scintillant (ScintiVerse E, Fisher Scientific, Fair Lawn, NJ) with the addition of 0.4 ml of a 1% SDS solution. The other lane was electroeluted as described above and the total volume of 0.4 ml of electroeluate was added to an additional scintillation vial. Vials were kept overnight, in the dark, and counted the following day on a Beckman LS6800 Scintillation Counter. Yields were determined by comparing the counts obtained from the electroeluate and the fragmented gel strip.

### **2.3. Metabolic Radiolabelling of Rubella Virus**

Vero cells were grown in T-175 flasks to a confluence of 80% and infected with RV (M 33 strain) as described above. After adsorption of virus 30 ml of medium was aspirated and 150 ml of fresh complete MEM (as defined above) was added. After 24 hours the medium was changed with complete MEM lacking L-methionine. Following a 2 hour incubation this medium was aspirated and 150 of fresh medium added (1:1 of complete MEM and MEM lacking L-methionine with the addition of 18.5 MBq of L-[<sup>35</sup>S]methionine) (Amersham, Arlington Heights IL, USA). After a further 24 hour incubation, virus was harvested and concentrated by ultracentrifugation. Tissue culture supernatant was centrifuged at 27,000 rpm ( $G_{av}=90,000$ ) for 2 hours, in polyallomer tubes, each holding 38.5 ml TCS. Pellets were washed once with 10 ml ice cold PBS and spun for 30 minutes at 27,000 rpm. The supernatant was discarded and pellets taken up in 385  $\mu$ l TBS.

### **2.4. Serum Separation**

Separation of IgG, IgA and IgM from human serum was performed using anion exchange chromatography. Sera were centrifuged on a bench-top centrifuge for 10 minutes at 10'000rpm to remove particulate material and chylomicrons. Samples were then diluted 1:2 in running buffer and filtered through 0.22  $\mu$ m Millipore filters. Solutions used in chromatography were as follows:

Running buffer (Buffer A):	20 mM Tris-HCl, pH 8.3, 5 mM $\text{NaN}_3$
High salt buffer (Buffer B):	20 mM Tris-HCl, pH 7.1, 500 mM NaCl, 5 mM $\text{NaN}_3$
Column rinse buffer 1	200 mM NaCl, 1% Triton X-100
Column rinse buffer 2	1 M Acetic Acid
Dialysis buffer	50 mM Ammonium acetate, 10 mM NaCl, pH 7.2

All buffers were filtered through 0.22  $\mu$ m Millex-GV filters (Millipore, Bedford MA, USA) and stored in the cold room. As chromatography was performed at room temperature, buffers were equilibrated to ambient temperature and degassed before use. Separation was performed on a Mono Q HR5/5 (Pharmacia, Uppsala, Sweden) column attached to a LKB 2150 HPLC system (LKB, Bromma, Sweden). Sample (200  $\mu$ l) was loaded onto the column and proteins were eluted by a step-gradient illustrated in Figure 18. Following each increase of Buffer B, eluted proteins were pooled. The three pools collected, contained separated serum immunoglobulin classes in the following order: IgG in pool 1, IgA in pool 2 and IgM in pool 3. Each pool was dialysed over

night at 4°C and lyophilized. Ammonium sulphate was used as salt in the dialysis buffer in order to allow the removal of this volatile salt during lyophilization. Samples were taken up in 200 µl of dH<sub>2</sub>O before analysis.

## **2.5. Gel Staining**

### **2.5.1. Colloidal Coomassie Stain**

Coomassie Brilliant Blue G-250 (Sigma, St. Louis, MO) was prepared according to Neuhoff et al., (1988) as follows: 4 grams of Coomassie Brilliant Blue G-250 was dissolved in 250 ml of hot 7.5% acetic acid. Ammonium sulphate (44 grams) was added slowly and the solution cooled to 4°C. The recrystallized dye was washed with cold 7.5% acetic acid, 18% ammonium sulphate solution and dried. The staining solution was obtained by dissolving 0.5 g of the recrystallized G-250 in 10 ml of water which is added to 500 ml of a 0.45 M ammonium sulphate, 2% phosphoric acid solution. Before staining, gels were fixed in 10% acetic acid, 1% trichloroacetic acid and then agitated overnight in the colloidal stain working solution. Gels were briefly rinsed in 20% methanol, 10% acetic acid and dried.

### **2.5.2. Silver Stain**

Silver staining was performed with a commercially available kit (Bio-Rad, Richmond, CA). All glass plates were cleaned with Chromerge prior to gel casting and deionized water was used in all reagents.

## **2.6. Solid Phase Immunoassays**

### **2.6.1. Western Blotting**

For gel electrophoresis separation of RV proteins for immunoblotting either reducing or non-reducing sample buffer was used. Buffers used were as follows:

Sample buffer 2X (Reducing):            0.1 M Tris-HCl, pH 6.8, 20% Glycerol, 0.5% SDS, 3% Mercaptoethanol,  
0.01% BPB

Sample buffer 2X (Non-Reducing):    0.1 M Tris-HCl, pH 6.8, 20% Glycerol, 0.5% SDS, 0.01% BPB

Stacking Gel:                                4% Acrylamide, 0.1% Bis, 0.125 M Tris-HCl, pH 6.8, 0.05% Temed and  
APS

Separating Gel:                            9% Acrylamide, 0.2% Bis, 0.375 M Tris-HCl, pH 8.8, 0.05% Temed and

## APS

Running buffer:	200 mM Glycine, 25 mM Tris, 0.1 % SDS
Transfer buffer:	200 mM Glycine, 25 mM Tris, 0.1 % SDS, 20 % Methanol
Immunoblot Sample Buffer:	PBS, pH 7.3, 3% skim milk powder, 0.02% Tween 20
Immunoblot Conjugate Buffer:	Same as sample buffer but without Tween 20
Substrate buffer:	50 mM Tris-HCl, pH 9.6, 140 mM NaCl, 5 mM MgCl <sub>2</sub> , 0.3 mg/ml BCIP, 0.3 mg/ml NBT

SDS-PAGE was performed on Mini-Protean II apparatuses gels using a preparative 0.75 mm comb to form wells. RV preparation (20  $\mu$ l) was diluted with 300  $\mu$ l using either non-reducing or reducing sample buffer, heated at 60 °C for 3 minutes and loaded. Separation was performed at 30 mAmps constant current run until the BPB dye front reached the bottom of the gel. Proteins were then electrophoretically transferred to nitrocellulose for 30 minutes at 30 mAmps constant current using Transfer buffer. Nitrocellulose sheets were then blocked in immunoblot sample buffer for one hour. Sheets were then cut into 4mm wide strips and added to test tubes containing 300  $\mu$ l of the appropriate serum sample dilution in immunoblot sample buffer. Strips were incubated over night at 4 °C, washed in three changes of PBS-Tween with a last wash in Conjugate buffer. Strips were added to test tubes containing 300  $\mu$ l Conjugate buffer with 1  $\mu$ g/ml alkaline phosphatase-conjugated Goat IgG anti-Human IgG c-chain. Following a 2 hour incubation and a further wash, strips were developed in Substrate buffer. Quantitative analysis of immunoblot strips was performed by densitometry using a Model 620 Video Densitometer (BioRad, Richmond CA, USA). Using the reflectance mode, the apparatus integrated the intensity of each band and quantitated signal strength as OD x mm.

### 2.6.2. Immunoprecipitation

The conventional protein A-sepharose immunoprecipitation technique was used, with modifications described by others (Loo et al., 1986). Protein A-sepharose suspension (Pharmacia, Uppsala, Sweden) was washed in Sample buffer (20 mM Tris-HCl, 150 mM NaCl, 1% TX-100, pH 7.3) and resuspended in a minimal volume of Sample buffer. Patient serum (30  $\mu$ l) was diluted in 200  $\mu$ l of Sample buffer and added to 50  $\mu$ l washed bead slurry, the resulting suspension was incubated at 4 °C for 4 hours on a rotator. Beads were washed 3 times and <sup>35</sup>S-methionine-labelled RV was added, in a volume of 200  $\mu$ l Lysis buffer (20 mM Tris-HCl, 150 NaCl, 1%

sodium dioxcholate, 1% TX-100, 0.1% SDS, 5 mM EGTA, 1 mM EDTA) and the mixture incubated over night at 4°C. Beads were washed 4 times and then boiled for 4 minutes in reducing SDS-PAGE Sample buffer to release antibody and bound RV antigen, spun at 10,000rpm for 1 minute and loaded onto 10% SDS-PAGE gels and separated as described above. Following electrophoresis, gels were soaked in Amplify (Amersham, Arlington Heights, IL) for 5 minutes, dried and autoradiographed using X-OMAR, AR diagnostic film (Eastman Kodak, Rochester, NY).

### 2.6.3. Enzyme Linked Immunosorbent Assays (ELISA)

ELISA was used to quantitate immunoglobulins directed to whole RV or separated RV structural proteins. All ELISAs were performed in 96-well, flat-bottom, microtitre plates using either: Immulon-2 (Dynatech, Chantilly, VA) or Falcon 3915 (Becton Dickinson Labware, Lincoln Park, NJ). ELISA buffers are listed below:

Coating buffers a) Carbonate (pH 9.6): 15 mM  $\text{Na}_2\text{CO}_3$ , 35 mM  $\text{NaHCO}_3$ , 0.02% Azide, filtered.

b) PBS (pH 7.3): 40 mM NaCl, 16 mM  $\text{Na}_2\text{H}_2\text{PO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , 3 mM KCl, 0.02% Azide

Blocking buffer (bb): PBS, 2% Normal Goat serum, 0.02% Tween-20, filtered (0.22  $\mu\text{m}$ ).

Sample buffer (sb): Same as Blocking buffer.

Heat Denatured Sample buffer ( $\text{sb}_b$ ): Boil 25 ml of SB for 5 minutes, then immediately mix with 25 ml of ice cold SB and transfer onto ice (Mauracher et al., 1991b).

Wash buffer (wb): PBS, 0.1% Normal Goat serum, 0.02% Tween-20.

Conjugate buffer (cb): PBS, 2% Normal Goat Serum

Substrate buffer (subb): 1 M diethanolamine, 1 mM MgCl, pH 9.6, 2 mg/ml p-nitrophenyl phosphate disodium

#### 2.6.3.1. Whole Virus ELISA

Purified RV was coated at a dilution of 1:3000 in PBS Coating buffer onto Immulon-2 plates by overnight incubation at 4°C overnight using 100  $\mu\text{l}$ /well. Plates were warmed to RT for 1 hour, flicked and blocked with sb for one hour at RT with 200  $\mu\text{l}$  buffer per well. Serum samples were diluted in  $\text{sb}_b$  and serial two-fold dilutions were added in triplicate at 100  $\mu\text{l}$ /well. Following incubation at 36°C for 2 hours the plates were

washed 3 times in wb then alkaline phosphatase-conjugated goat anti-human IgG( c-chain) antibody (Kirkgaard and Perry Laboratories, Gaithersburg, MD, USA), diluted to a final concentration of 1 lg/ml in cb, was added at 100 ll/well. Following a further 2 hour incubation at 36#C, the plates were washed 4 times and developed by adding 170 ll/well subb, followed by incubation at 36#C. Each plate contained five duplicate dilutions of a standard serum, whose concentration of anti-RV IgG was previously determined to be 943 IU/ml, by titration versus the WHO reference serum (WHO, 1971). Standart serum was diluted serially in two-fold dilution starting at 1:1000. The end-point of development was reached when the 1:1000 dilution of the standard curve reached a net absorbance of 1.0 AU at 405nm. Plates were analysed on a microplate reader (Model 3550, Bio-Rad) using a wavelength of 405 nm. Using the five point standard curve immunoglobulins in the samples were quantified and assigned IU/ml of anti-RV IgG using quadratic regression (Microplate Manager , software, Bio-Rad).

#### **2.6.3.2.RV Protein ELISA**

Electrophoretically purified RV structural proteins, E1, E2 and C, were coated onto Immulon-2 plates using PBS buffer at 4#C overnight. Stock preparations of each protein were prepared, divided into aliquots and stored at -70#C. Optimal coating concentrations varied for each viral protein preparation: E1 was coated at 1:60, E2 at 1:80 and C at 1:40 using PBS Coating buffer. ELISA was performed as described above. The standard serum (943 IU/ml) was analyzed on non-reducing immunoblot and the relative amounts of IgG directed to each viral protein were determined by densitometry. The percentages of protein specific IgGs were expressed as arbitrary units (AU) in realation to the 943 IU/ml of whole virus specific antibody. Accordingly this standard contained: 424 AU/ml anti-E1, 405 AU/ml anti-E2 and 114 AU/ml anti-C.

### **2.7. Affinity Assays**

#### **2.7.1. Chaotropic Elution ELISA**

ELISA buffers and general protocol were followed as described above and has been described previously for both whole RV as well as for separated RV protein (Mauracher et al., 1992a). Serum samples were diluted in sb<sub>b</sub> and incubated in duplicate dilution series in RV antigen coated microtitre plates: one series to be exposed to Elution buffer (eb) (8 M urea, 150 mM NaCl, 20 mM Tris-HCl, 0.02% Tween-20, pH 9.6) and the control



series to be exposed to wb. After a 2 hour sample incubation step at 36°C, plates were washed twice with wb, then one dilution series was exposed for 4 minutes to 200 µl/well of eb while the control dilution series was treated with the an equivalent amount of wb. Following elution, the plates were washed twice and routine ELISA protocol was resumed. The end-point of the assay was reached when the least dilution of the standard curve reached 1 absorbance unit. Elution ratios [ER(%)] were determined for each serum sample by selecting the dilutions which fell between absorbance units of 0.7 and 1.5 and were calculated according to the following formula:

$$ER(\%) = [(A405 \text{ Urea eluted} - A405 \text{ Background}) \div (A405 \text{ Control} - A405 \text{ Background})] \times 100$$

### 2.7.2. One Well Inhibition ELISA

This assay was adapted from the whole RV ELISA described above and was only employed using whole viral antigen preparations. A similar assay was previously described for use with peptide antigens (Rath et al., 1988). Microtitre plates were coated with a constant concentration of whole RV and blocked. A dilution series of liquid phase RV antigen was then added to the plates followed by the addition of a constant dilution of serum. Constant serum concentrations were determined by choosing the concentration of serum which fell into middle of the linear region of the titration curve on whole RV IgG ELISA. The wells which received the highest concentration of liquid phase antigen will result in the lowest absorbance reading, following the development of the plates, as antibody binding to the solid phase will have been prevented by the competing liquid phase antigen. The concentration of liquid phase antigen needed to bring about 50% inhibition of antibody binding ( $I_{0.5}$ ) was related to antibody affinity, with low affinity antibodies requiring more liquid phase antigen to bring about  $I_{0.5}$  (Andersson, 1970). Whole RV was coated onto Falcon plates in carbonate coating buffer at a dilution of 1:3000 and sera titred out from dilutions of 1:10 to 1:50,000 in sb. A dilution which yielded 0.5 absorbance units after one hour incubation was chosen as the serum concentration to be used in the inhibition assay. In case of FPLC purified IgG fractions, the lyophilizate was taken up in 100 µl sb and then further diluted as serum samples. RV preparation was diluted from 1:5 through 1:5000 in 5 ml test tubes using ELISA sb, the final dilution contained no virus and was taken as the infinitely diluted virus. 50 µl of each dilution was added to each well in triplicate, using Falcon plates which had been coated overnight with whole RV (1:3000) and blocked with

sb for 1 hour at RT. A double strength dilution of the serum sample was then added to each well at 50  $\mu$ l/well and incubated for 2 hours at 36°C. Routine ELISA protocol was employed in all the following steps. The dilution of liquid phase inhibitor to bring about a reduction of 50% net absorbance was determined by fitting a best fit line through the linear portion of the inhibition curve.

## **2.8. Hemagglutination Inhibition Assay**

RV Gilchrist strain (Whittaker MA Bioproducts, Walkersville, MD, USA) was used as hemagglutinin. HA titres of this preparation were determined for each virus lot by serially diluting the HA antigen in two fold dilution starting at 1:4. The last dilution yielding complete agglutination of a total of 100  $\mu$ l of a 0.125% solution of 1 day old chick erythrocytes (PML Microbiologics, Tualatin, OR) was assigned the concentration of 1HA unit. Following HA determination the viral antigen was aliquoted and stored at -70°C. All serum samples (200  $\mu$ l) were pre-treated for 15 minutes with 200  $\mu$ l of  $\text{MnCl}_2$ /heparin solution (0.5 M  $\text{MnCl}_2$ , 2500 IU/ml Porcine heparin) at 4°C. Next, 200  $\mu$ l of a 50% chicken erythrocyte (PML Microbiologics, Tualatin, OR) solution in HSAG buffer (25 mM HEPES, 140 mM NaCl, 1 mM  $\text{CaCl}_2$ , 1% BSA, 0.25% Gelatin, pH 6.5) was added and incubated at 4°C for 1 hour. An additional 600  $\mu$ l of HSAG buffer was then added and the tubes centrifuged for 10 minutes at 1000 x G and the supernatant (now at a serum dilution of 1:8) was collected. Pre-treated serum (50  $\mu$ l) was serially diluted two fold in "U"-shaped polyvinylserocluster plates (Costar, Cambridge, MA) and 25  $\mu$ l RV hemagglutinin, containing 4 HA units, was added to each well. After 1 hour of incubation at 4°C, 50  $\mu$ l of a 0.25% solution of 1 day old chick erythrocytes was added and the plates interpreted for HAI titre following 3 hours and again after an overnight incubation at 4°C.

## **2.9. Biological Function of RV Proteins**

### **2.9.1. Solubility Shift of C Protein at Acidic pH**

Tissue culture supernatant (TCS) containing L-[ $^{35}\text{S}$ ]-methionine labelled RV was extracted in 1% TX-114 (Bordier, 1981) (Calbiochem, San Diego, CA), replacing Tris-HCl buffers with McIlvane's citrate-phosphate buffer (McIlvane, 1962) in order to accommodate detergent extraction over pH range 4.0 to 9.0 (Figure 9). Extraction was performed with cold solutions by rotating tubes end-over-end at 4°C for 1 hour. This solution was then overlaid onto a sucrose cushion (6% sucrose, 0.02% TX-114, with pH adjusted accordingly) and the tube was

allowed to warm to 36°C. Tubes were then centrifuged for 1 minute on a benchtop centrifuge, and the aqueous phase removed. Detergent pellets were washed once in McIlvane's buffer and then resuspended in water to the original volumes.

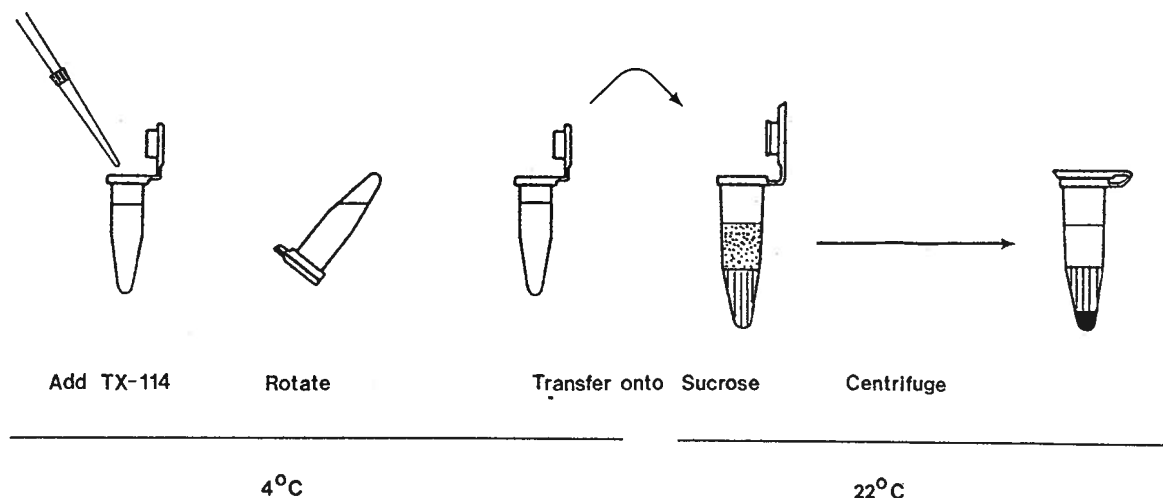


Figure 9. Triton X-114 extraction of RV from Tissue Culture Supernatant. Ice cold TCS is made to 1% TX-114, and extraction performed by end-over-end rotation at 4°C. TCS is then overlaid onto a 6% sucrose cushion (||) and the tube warmed to RT. The TCS turns opaque as TX-114 reaches clouding point (⦿). Following centrifugation TX-114 is concentrated as a pellet (■) below the sucrose cushion and the TCS is devoid of detergent. Hydrophobic proteins are in the pellet, hydrophilic proteins remain in the TCS.

#### 2.9.1.1.Ultracentrifugation

TCS containing high titres of RV was treated at either pH 7.0 or 5.0 with or without 1% TX-114 and with 1% SDS. The aqueous supernatants from the TX-114 extractions and the SDS and non-detergent treated TCS were then placed on 22% sucrose cushions and centrifuged for 3 hours at 39,000rpm in a SW41 Ti rotor (Beckman, Palo Alto, CA). The pellet was resuspended in dH<sub>2</sub>O and analyzed by Western blot or by PCR.

#### 2.9.1.2.Polymerase Chain Reaction for Detection of RV RNA

An aliquot of 50  $\mu$ l from the resuspended pellets from ultracentrifuged TCS was solubilized in 4 M guanidinium

isothiocyanate, phenol/chloroform extracted and RNA precipitated in the presence of tRNA (Maniatis et al., 1982). Complementary DNA was synthesized using M-MLV reverse transcriptase (BRL, Gaithersburg, MD). One tenth of the volume was added to the PCR mix, containing 10  $\mu$ M of each forward and reverse primer shown below and 1 U Taq polymerase (Bio/Can Scientific, Mississauga, ONT) (Saiki et al., 1988). The primers used were both 24-mers which amplified a 287 bp fragment from the E1 gene of RV: 5'-TTGAACTTCAGCC-CCAAGGGGCCC-3' and 5'-TCCCCGGTTTGCCAACGCCACTCC-3'. Thermal cycles of 95°C for 45 sec, 68°C for 15 sec and 72°C for 30 sec, were performed on a Perkin-Elmer-Cetus thermal cycler (Mauracher et al., 1991a). The work of Mr. R.Shukin in the development and design of this protocol is acknowledged.

### **2.9.2.Hemagglutination Activity of Purified E1 Protein**

An aliquot of E1 and C protein preparation was treated with KCl to remove SDS, as described above, and employed to competitively bind HAI antibodies (C protein was used as the negative control). Pooled RV seropositive human serum with an HAI titre of 1:256 was used as the source of IgG. Five rows of the serocluster plates received 5 wells of 25  $\mu$ l each of serum dilutions of 1:40, 80, 160 and 320. To each row of serum dilutions, a dilution series of E1 (or C) protein was added, starting with 25  $\mu$ l of E1 preparation, 12.5, 6.25, 3.1 and 0  $\mu$ l of E1. Each well now contained serum with E1 or C protein and incubated at 4°C for 1 hour. Each well then received 25  $\mu$ l of RV containing 4 HA units and received 25  $\mu$ l of a 0.5% CRBC solution after a further 1 hour incubation at 4°C. Negative controls included cells incubated with either E1 or C protein but no serum, CRBC with serum and no HA antigen and CRBC in buffer only.

### **2.10. Patient Sera**

Blood samples were collected by venipuncture and sera were stored at -70°C until needed. CRS patients were diagnosed clinically and were shown to be positive for cord blood anti-RV IgM and/or by RV isolation during the neonatal period. Time of intrauterine infection was established for all CRS patients on the basis of maternal history and by serological confirmation. The age of patients at the time of serum collection was 1 to 34 years. One CRS patient in the study was also diagnosed with chronic progressive rubella panencephalitis. Control patients were adults and were either mothers of CRS patients used in the study or individuals who were infected during the 1984/85 rubella epidemic in British Columbia.

### **2.11. Statistical Methods**

Statistical analysis using the unpaired T-test was used for most population comparisons and distributions tested for normality using the Martinez and Iglewicz test. Level of confidence was set at  $\alpha=0.05$  for all analyses. Populations showing non-normal distribution were analyzed using the Mann-Whitney U test.

Analysis of variance (ANOVA) was used when multiple comparisons of populations were performed. One-Way ANOVA was used with level of confidence set at  $\alpha=0.05$ . Means were separately analyzed with the Fisher's LSD post-hoc test.

Means and standard deviations (SD) were determined using descriptive statistic on the software package. All error bars in the figures of this thesis are  $\sim 1$  SD.

All statistical calculations were performed on NCSS 5.01 software (J.L.Hintze, 1987). The help of Mrs. R.Milner and Mr. M.McKinnon from the Statistical Research Support Unit, Research Centre, in the statistical design is greatly appreciated.

### 3. RESULTS AND DISCUSSION

#### 3.1. Section I: Virus Preparation and Use in Solid Phase Immunity and Affinity Assays.

At the start of the project preparations of RV had to be established which were uniform, well characterized and would be of sufficient quantity to provide antigen for both phases of the experimental part of the thesis: methodology development and patient studies. This was important to eliminate RV antigen preparation as a variable in the comparative studies of the immune responses of pre- and postnatally RV infected patients.

##### 3.1.1. Rubella Virus Preparation

The RV stock, which was used for all direct antigen assays and for the preparation of purified RV proteins E1, E2 and C was obtained from a pool of 24 liters of tissue culture supernatant (TCS), and purified as one batch. The virus was obtained from ATCC (M-33 strain, ATCC VR-553) and further experiments verified the stock to contain infectious RV. This wild type RV isolate was used as antigen in all serological assays, as responses to wild type infection (both in post- and prenatally infected individuals) were analyzed.

##### 3.1.1.1. Virus Titration

The original TCS contained RV at a concentration of  $2.7 \times 10^6$  pfu/ml, as determined by virus titration on RK-13 cells (Nawa, 1979). The morphology of the infectious foci (Fig. 11) on RK-13 cells were characteristic for the cytopathic effect caused by RV in this cell line (Nawa, 1979).

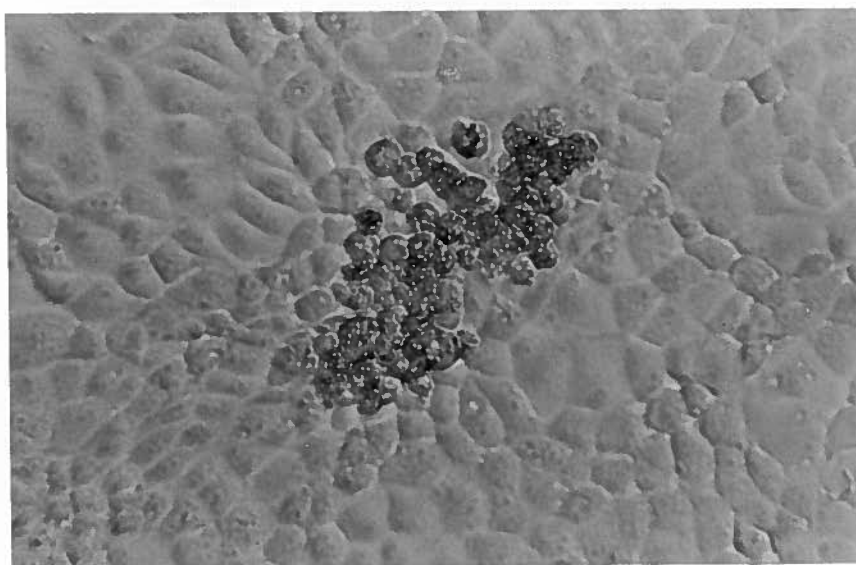
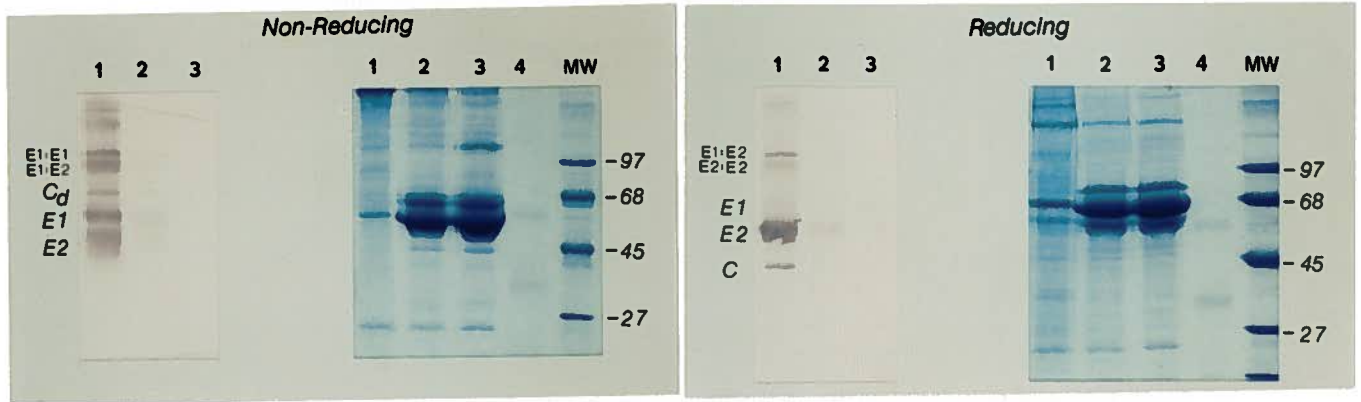


Figure 10. Micrograph of RV Infectious Focus on RK-13 cell Monolayers. Phase contrast microscopy (500 x) of a RK 13 cell monolayer 3 days after *in vitro* infection.

Figure 11A shows whole protein stain and Western blots of the RV concentrate (lane 1), the starting TCS (lane 2) and TCS obtained from mock-infected Vero cells. The final concentrated viral preparation contained  $2 \times 10^{10}$  pfu/ml and had a 150 fold increase in antigenic activity per gram protein. Protein bands for E1, E2 and C were

**A**



**B**

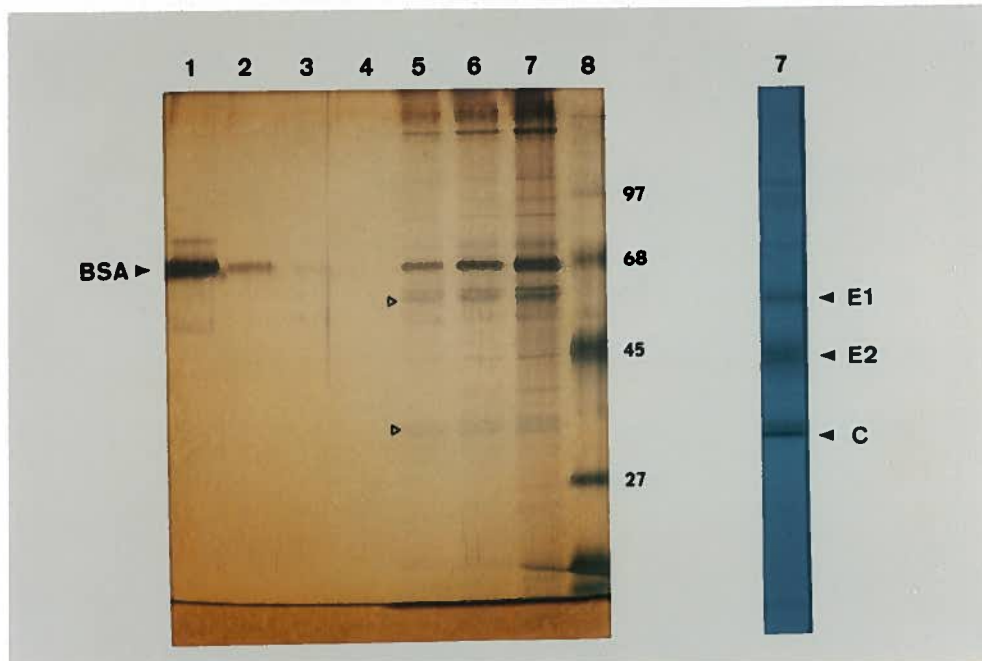


Figure 11. A) Western Blot and Colloidal Coomassie Blue Stain of RV Preparations. 0.5  $\mu$ l RV concentrate (1), 10  $\mu$ l starting TCS (2) or mock-infected Vero cell TCS (3). Prestained MW markers (4). MW markers are indicated on the right side of the gel. Western blots were developed with anti-RV reference standard human serum, anti-human IgG (c-chain) alkaline phosphatase-conjugate and BCIP/NBT stain. Relative positions of RV proteins are indicated on the left side. E1, E2 and C refer to monomeric forms of RV structural proteins,  $C_d$  dimeric capsid. Dimeric forms of the envelope proteins are indicated as E1:E1 (homodimer) and E1:E2 (heterodimer).

B) Silver Stain and Autoradiograph of concentrated RV Separated by Reducing SDS-PAGE. Lanes 1 through 4 contain a BSA titration (20, 5, 1 and 0.2 ng/band). RV preparations, contained 1/10 volume of  $^{35}$ S-methionine labelled RV, were loaded in doubling dilutions in lane 7, 6 and 5 (10, 5 and 2.5  $\mu$ l of RV concentrate respectively). Lane 8 contains MW markers. The corresponding autoradiograph of lane 7 is shown to the right with relative positions of RV proteins indicated. Arrow heads (▷) in the gel indicate possible E1 and C bands.

not clearly identifiable by either colloidal Coomassie or silver staining. The detection limit for protein in the silver stained gel in figure 11B lies between 1 - .2 ng BSA per band. If the faint bands (Y) in lane 5 are interpreted to represent the E1 and C protein, then the final protein concentration of RV antigen can be estimated to lie in the order of 11g/ml. The relative molecular masses of the RV structural proteins, as determined by reducing SDS-PAGE were: E1 (58 kD), E2 (47-42 kD) and C (33 kD). Under non-reducing conditions, the capsid protein was detectable in its dimeric form ( $C_d$ ) at 68 kD and E1 travelled at a lower apparent molecular mass of 55 kD, in comparison to its position on gels run under reducing conditions where its apparent mass was estimated to be at 58 kD (Baron et al., 1991, Mauracher et al., 1992a).

### **3.1.2 Whole Rubella Virus ELISA**

Although basic conditions for whole RV ELISA had been established (Bidwell et al., 1977; Tingle et al., 1989) and numerous commercial suppliers have marketed reliable anti-RV IgG ELISAs (Dimech et al., 1992), a series of modifications to the whole RV ELISA protocol were developed. These improved both sensitivity and specificity of the RV ELISA.

#### **3.1.2.1. Coating Conditions and Antigen Treatment**

Whole RV was coated onto 96-well ELISA plates (Falcon 3915 or Immulon-2) diluted at 1:100 - 1:25,000 in PBS (pH 7.3) followed by blocking in NGS-containing Sample buffer. The addition of Triton X-100 to the whole RV preparation improved signal strength (Fig. 12). Whole RV was made to a final concentration of either 2%, 0.5% or 0.2% TX-100 and compared to RV, without any detergent added, in a titration of antigen using constant concentration of detection antibody. Whole RV, in the absence of detergent, showed a shallow titration curve with a saturated region at dilutions stronger than 1:500. A marked increase in signal was observed between dilutions of 1:200 and 1:6400 for viral preparations which had been treated with detergent. A strong prozone was observed in the preparations containing 0.5% and 2% TX-100 suggesting that detergent may compete for hydrophobic binding sites on the polystyrene plastic surface. The increase of the signal in the detergent-treated virus may result from disaggregation of the virus or to the disruption of the viral envelope leading to increased exposure of the capsid as well as E2 protein.



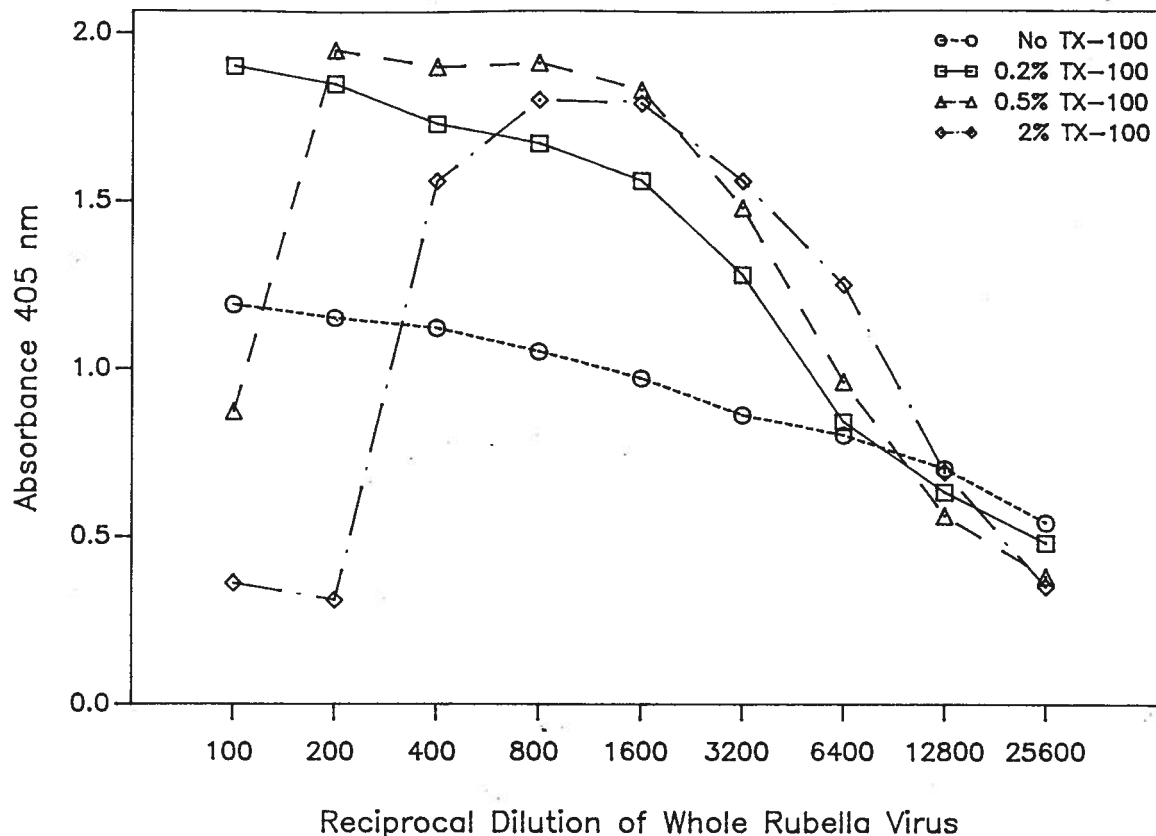


Figure 12. Titration of RV preparations Treated with Triton X-100. Virus was brought to a final concentration of 0.2% (□), 0.5% (△) or 2% (◇) TX-100 using a 20% stock solution. As a control an equivalent amount of PBS was added to the preparation which received no detergent (○).

### 3.1.2.2. Heat Denatured Sample Buffer

During the course of these investigations it was frequently observed that sera from certain patients had high backgrounds on RV IgG ELISA. Such high background could result in false positive RV serology. Increased backgrounds were especially common in infants (Dr. L.A. Mitchell, Dept. of Pathology, UBC, personal communication), who had RV vaccine as well as several other vaccinations during the same period. It was observed that much of the non-specific background in such patients could be removed by pre-adsorption of serum on NGS blocked microtitre plates (Fig. 13). This additional step was time consuming and costly and it

was found that it could be replaced by including heat-denatured protein in the Sample buffer (Fig. 13) (Mauracher et al., 1991b). Briefly, SB was boiled for 5 min. and then mixed with an equal volume of ice cold SB. The resulting buffer, termed "SB<sub>b</sub>", for boiled sample buffer, contained heat denatured and aggregated proteins which remained in suspension. Table 5 shows the quantitation of serum IgG directed to whole RV as well as the individual structural proteins: E1, E2 and C. Two vaccinees, 2 and 3 showed no difference in their quantitative level of anti-RV IgG, expressed in IU/ml when either SB or SB<sub>b</sub> was used as a diluent. These observations indicate that sample buffer containing the heat denatured protein did not reduce background levels by masking RV-specific signals. A value of 20 IU/ml of anti-RV IgG was determined in the pre-immunization serum of patient 1 using SB. While this value was in the range of positive immunity (ie.,  $\geq 15$  IU/ml), an equivalent dilution prepared in SB<sub>b</sub> reduced RV-specific IgG levels to 0 IU/ml. RV seronegativity was also confirmed by determination of a HAI titre of  $< 1:8$ . These observations suggest that SB<sub>b</sub> can aid in the reduction of non-specific binding, without interfering with RV antigen-specific binding.

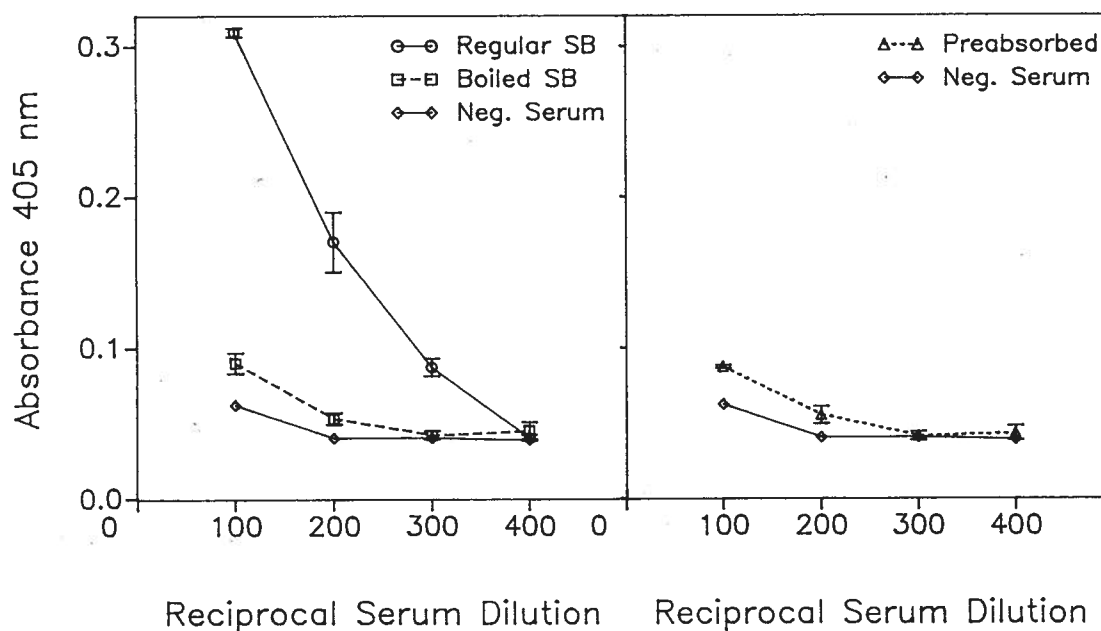


Figure 13. Determination of RV-Specific IgG by ELISA. A single patient's serum sample, taken prior to RA 27/3 immunization, was serially diluted in regular sample buffer (○) or in SB<sub>b</sub> (□). An equivalent dilution series in regular sample buffer was preadsorbed on NGS-blocked microplates (△). Negative control (◇).

Proteins may be structurally altered by binding to plastic surfaces (Van Regenmortel, 1990). Also, it has been shown that immunoglobulins which do not bind to liquid phase antigen will recognize new epitopes (cryptotopes) revealed by structural deformation of protein occurring during solid phase binding (Friguet et al., 1984). Similarly, new epitopes could potentially be created by the juxtapositioning of monomeric proteins onto solid phase (neotopes). It is possible that such cryptotopes and neotopes are exposed or created during heat denaturation and aggregation respectively. The decrease of non-specific binding, described above could be attributed to the competitive removal of antibodies reactive to denatured NGS constituents in the liquid phase of the diluent. Immunoglobulins reactive to such common blocking agents as BSA and normal animal sera have been previously reported (Levinson and Goldman, 1988) and are thought to have arisen as a result of activation of the immune system with dietary antigens or by vaccine contaminants.

Vaccinee	Weeks post-vaccine	IgG rubella ELISA (IU/ml)							
		Regular sample buffer				Boiled sample buffer			
		E1	E2	C	Whole RV	E1	E2	C	Whole RV
1	0	4	5	8	20	0	0	0	0
	6	21	64	28	62	7	21	10	24
	54	51	49	16	156	20	20	4	52
2	1	0	0	1	0	0	0	0	0
	6	10	8	4	28	12	8	6	30
	64	103	70	10	306	108	75	12	321
3	0	0	0	1	0	0	0	1	0
	6	19	26	4	60	20	22	3	64
	54	58	46	6	180	60	42	7	198

Table 5. Rubella IgG ELISA Responses to Whole RV and RV Structural Proteins. The level of IgG directed to viral antigen was determined using the two sample buffers SB and SB<sub>b</sub> in three individuals at sequential time intervals following immunization with RA 27/3 Rubella vaccine. (From: Mauracher et al., 1991b)

### 3.1.3. Affinity Assays

For the measurement of affinity, the ideal experimental situation requires that both antibody and antigen be present in pure form (Steward and Steensgaard, 1983), such as monoclonal antibody reacting with haptenic antigen. Well established techniques, such as equilibrium dialysis (Eisen and Karush, 1949) and ammonium sulphate precipitation (Steward and Petty, 1972) have found application in such antibody-antigen studies. However if the affinity of IgG responses from sera are to be estimated in an antigen system which contains multiple proteins, then the traditional techniques for affinity measurement are not applicable (Hedman et al.,

1988). Two techniques which had been previously reported for their ability to measure average affinities of IgG from serum (Steward et al., 1991; Inouye et al., 1984) were investigated for their applicability in the RV antigen system. Experiments were performed to establish the ability of these techniques to demonstrate affinity maturation of anti-RV serum IgG following primary RV infection in humans.

### 3.1.3.1. One-Well Inhibition ELISA

This ability of this technique to measure relative affinities of sera to RV was evaluated using patient sera collected at sequential time intervals after onset of natural Rubella infection to determine if the affinity maturation of the IgG response in the first 3 months following infection could be demonstrated. Prior to these experiments it was established that the concentration of liquid phase antigen required to bring about a 50% reduction of the signal obtained with no competitor antigen ( $I_{0.5}$ ) was independent of the constant antibody

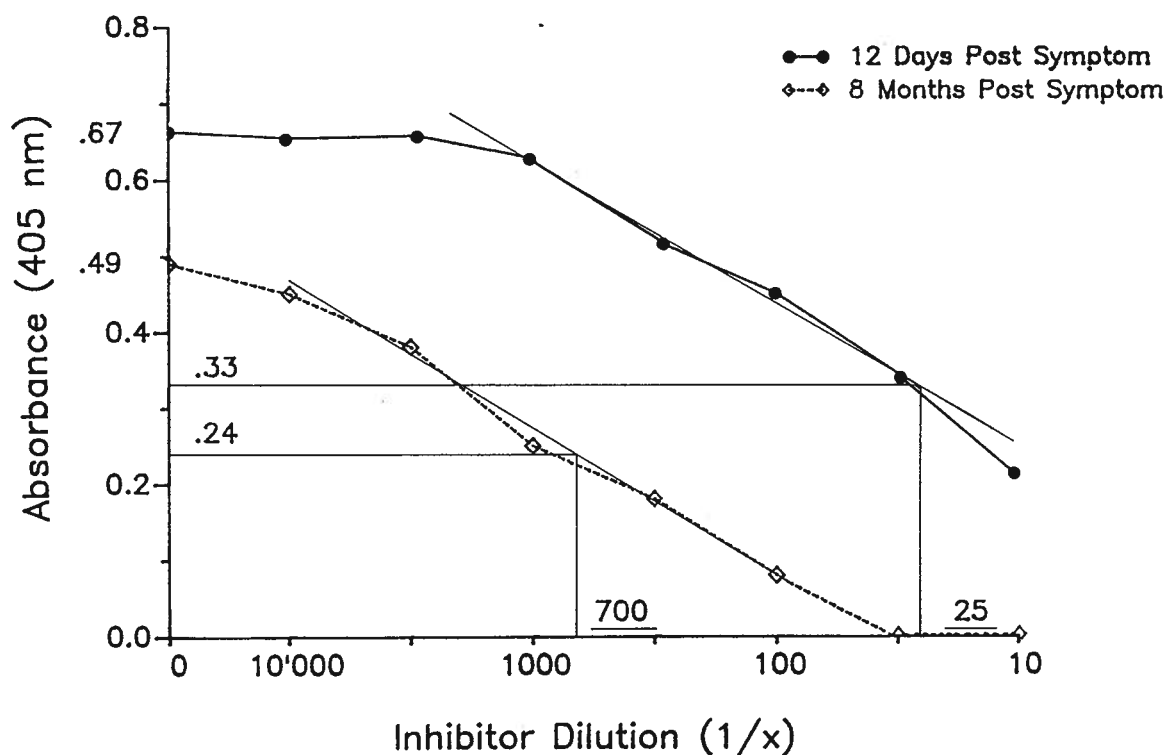


Figure 14: Determination of  $I_{0.5}$  in Acute and Convalescent Sera. Serum samples were obtained at 12 days (acute) and at 8 months (convalescent) following diagnosis of Rubella rash. The specific absorbance of the serum in the absence of competing antigen is shown on the left side of the y-axis (0.49 and 0.67 for the convalescent and acute sample respectively), with the 50% absorbance values indicated on the right hand side of the axis. The  $I_{0.5}$  values were extrapolated from the best fit line through the linear region of the titration curve and are indicated as underlined values on top of the x-axis.

concentration chosen, as long these antibody concentrations were on the linear region of the RV ELISA titration curve. For all sera tested the  $I_{0.5}$  were identical at serum dilutions of 1:250 to 1:1000. It was however required to plot RV ELISA titration curves for each serum in order to establish the linear range dilutions. Figure 14 illustrates the determination of the  $I_{0.5}$  for an acute (12 days) and convalescent (8 months) serum sample following the onset of a rubelliform rash in an adult patient.

The  $I_{0.5}$  values were determined in three separate experiments for paired (acute and convalescent) serum samples from 3 patients. The results shown in Figure 15 reveal that this technique could demonstrate affinity maturation of anti-RV IgG in the months following Rubella, although patient numbers were not of sufficient size to determine statistical significance.

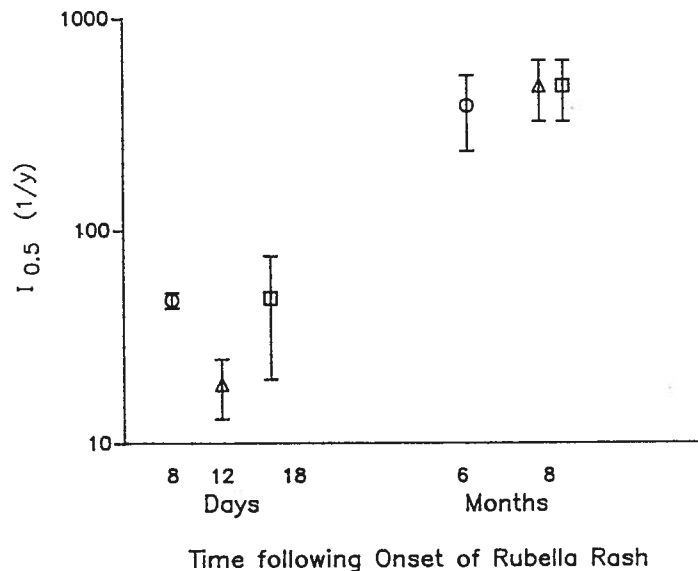


Figure 15. Affinity Maturation of Anti-RV IgG Measured by Inhibition ELISA. The  $I_{0.5}$  values were measured in three individual patients (O, Δ, □) at 8, 12 and 18 days or 6 and 8 months following the onset of a rubelliform rash. Each patient sample was tested three times. Error bars indicate ~ SD.

### 3.1.3.28M Urea Elution ELISA

Low affinity antibodies are easily eluted from their antigen by briefly exposing the antibody-antigen complex to denaturants such as 8 M urea, whereas high affinity antibodies are resistant to such treatment. This differential sensitivity of low and high affinity antibodies to chaotropic agents provides the basis for the 8 M urea elution ELISA. It was initially established that a 4 minute exposure of antibodies complexed to solid phase-bound RV to 8 M urea, provided the optimal conditions for differentiating the low affinity anti-RV IgG in acute sera from high affinity anti-RV IgG in convalescent sera (Fig. 16). The antigenicity of solid phase bound RV was not adversely affected by exposure to 8 M urea, nor was antigen eluted from the microtitre plate by such treatment. This was established by exposing RV-coated and blocked microtitre plates to a 4 minute wash of 8 M urea prior to the addition of a 1:1000 dilution of anti-RV standard serum (Fig. 17). This simple technique allowed the rapid determination of relative affinities of anti-RV IgG in human sera and was capable of demonstrating the affinity maturation of these antibodies in the months following Rubella infection.

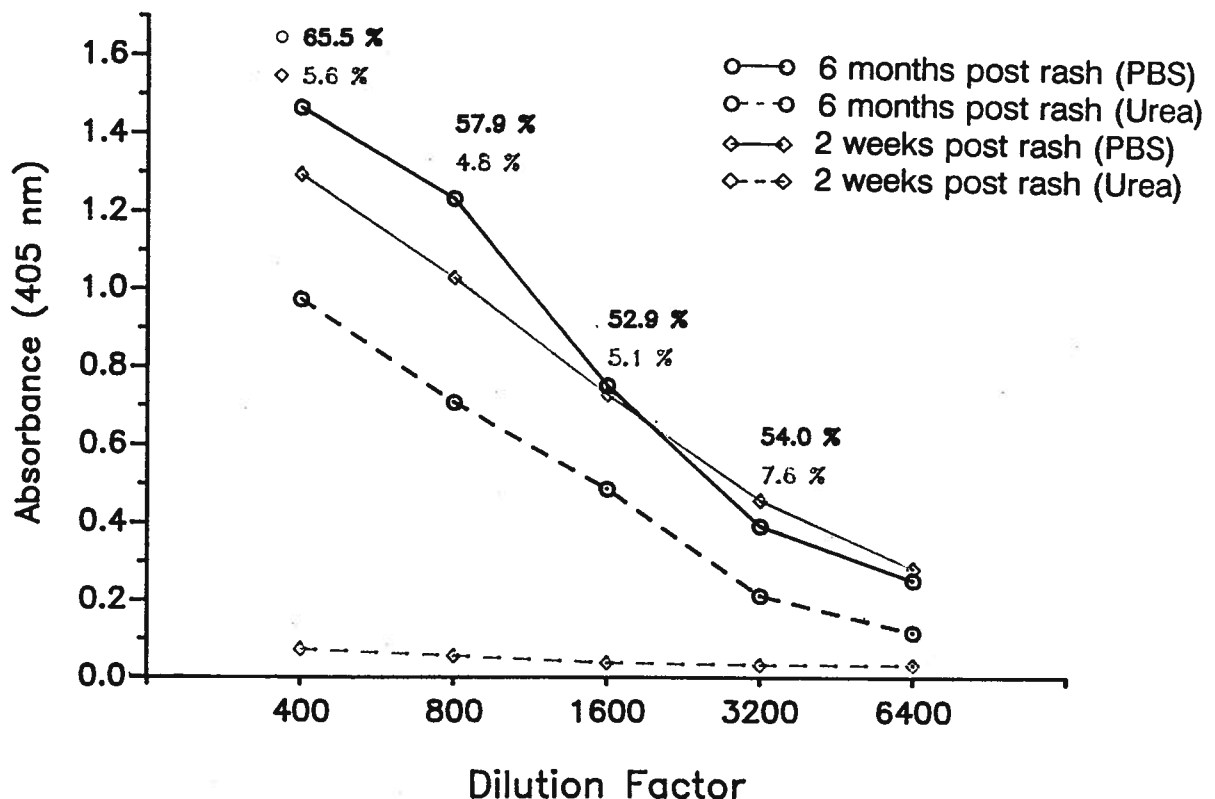


Figure 16. 8M Urea Elution ELISA for Determination of Antibody Affinity. Paired serum samples from a patient taken at 2 weeks ( $\diamond$ ) and 6 months ( $\circ$ ) following the onset of a Rubella rash. The dilution series washed in WB only are indicated by solid lines, the 8 M urea washed dilution series is indicated as a dashed line. Above each point the calculated elution ratio [ER(%)] is given, showing that ER(%) remains relatively constant over a wide absorbance range.

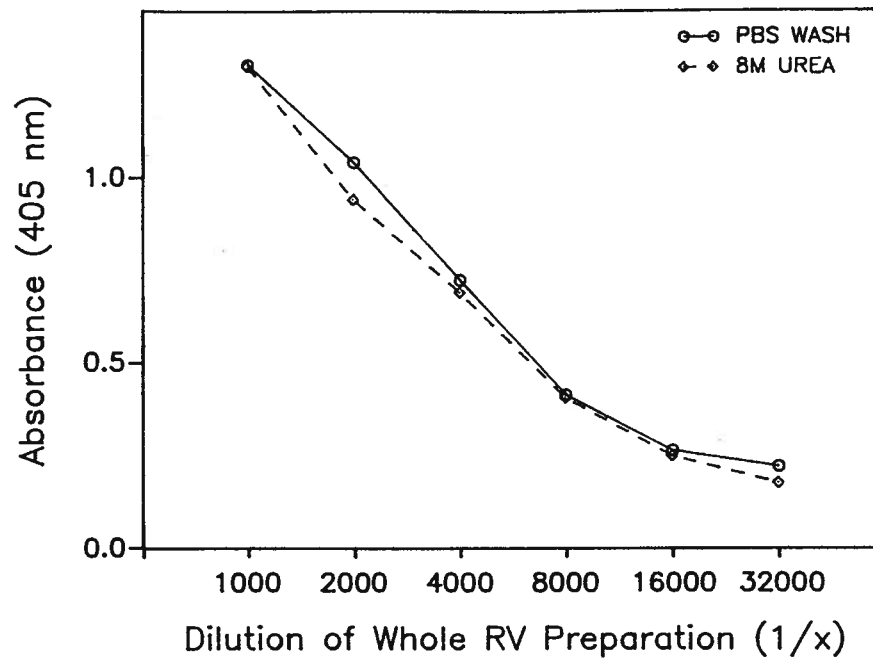


Figure 17. Treatment of Solid Phase Bound Rubella Virus with 8M Urea. Whole RV was coated in two-fold dilutions onto microtitre plates and then either washed for 4 minutes with 8 M urea wb (◇) or with wb (○), in order to determine whether 8M urea affected the antigenicity of RV. Standard ELISA protocol was then followed using a 1:1000 constant dilution of anti-RV standard serum to detect RV antigen.

### 3.1.3.3. Preparation of IgG Fractions from Human Sera

Both techniques described above were capable of measuring low affinity anti-RV responses in the first weeks following Rubella. Although the primary response is initially characterized by a low affinity IgG response, it is also typical to detect high levels of antigen-specific IgM in the first weeks following infection. Hence, it was important to determine if the presence of IgM in RV antisera influenced affinity measurements of anti-RV IgG. For that purpose, IgG fractions were prepared by separating this immunoglobulin class from IgA and IgM using FPLC anion-exchange chromatography. The FPLC system allowed rapid and repetitive runs of 100  $\mu$ l serum. The elution profile of 33%, 48% and 70% buffer B, allowed the best separation of three immunoglobulin classes G, A and M (Fig. 18A). The overall yield of IgG was 60%, with the yield of IgG in the purified IgG pool being 50%. Losses of protein may have occurred during filtration of sera, losses of protein in peak trails which were not included in the pooled fractions or during dialysis. The dialysed Ig fractions were lyophilized and stored at -70°C. IgG fractions and sera were compared for three patients, each containing an acute and a convalescent sample, using the One-Well Elution ELISA and the 8 M Urea Elution ELISA. It was shown that the presence of RV specific IgM could adversely influence the measurement of IgG affinity when using the One-Well

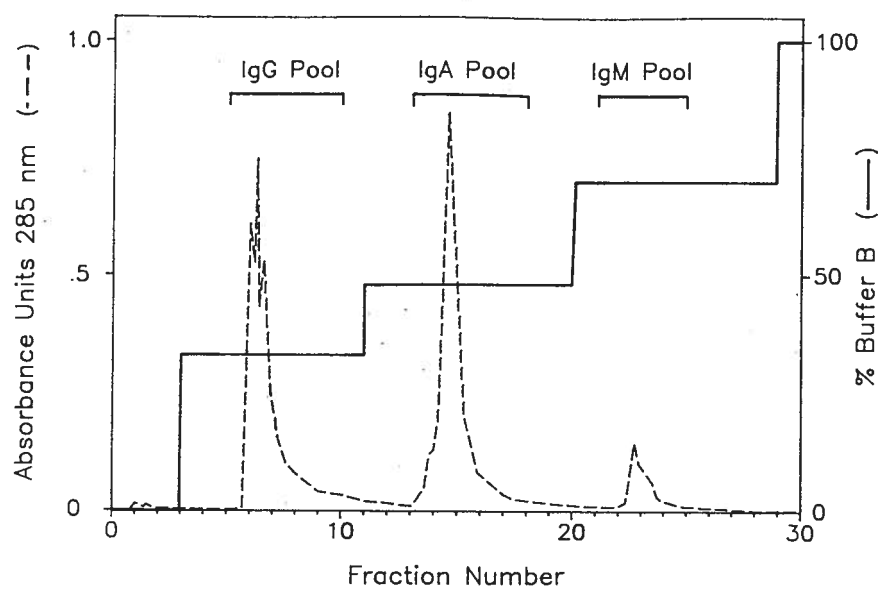
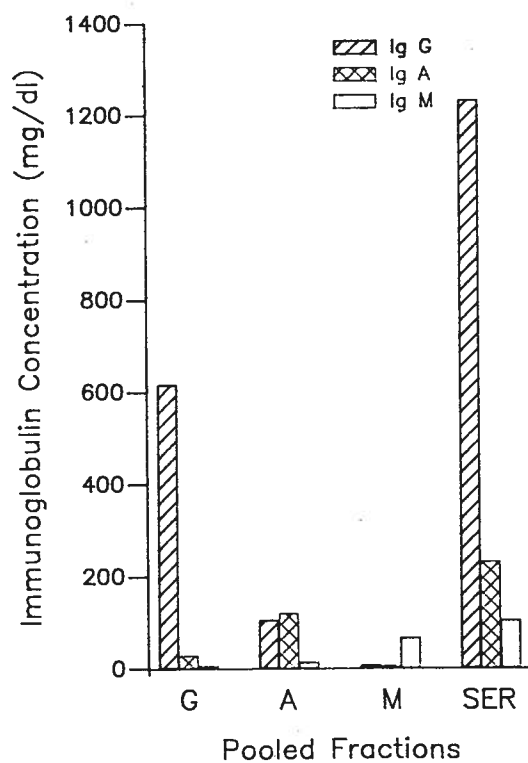
**A****B**

Figure 18. A: Elution Profile of Serum Separation. Each fraction contained 1 ml and a step gradient was run as indicated by the solid line. Eluted protein is indicated by the dashed line. Three pools were collected, each containing 5 mls. B: Quantitation of immunoglobulins in each of the pooled fractions (G, A or M) and serum (SER).



Inhibition ELISA (Table 6). It is likely that RV-specific IgM binds liquid phase antigen resulting in the increased requirement of liquid phase antigen to compete out IgG binding. This results in a shift to the right of the inhibition titration curve and in an overestimation of the  $I_{0.5}$ . In contrast, the affinity measurements using

	ER(%)		$I_{50}$	
	Serum	IgG Fraction	Serum	IgG Fraction
Patient 1 acute	8	5	7	50
convalescent	53	48	390	400
Patient 2 acute	1	2	20	63
convalescent	52	47	398	410
Patient 3 acute	12	18	50	60
convalescent	70	71	450	450

Table 6. Relative Affinity of Anti-RV IgG in Serum or Purified IgG. Serum samples from three patients were collected during the acute and convalescent period following Rubella. IgG fraction, prepared by ion-exchange chromatography, and whole sera were compared for their relative anti-RV IgG affinity measured by using both 8M Urea Elution ELISA (ER(%)) and the One-Well Inhibition ELISA ( $I_{0.5}$ ).

the Elution ELISA were not affected by the presence of RV-specific IgM. The overall amount of RV-specific IgG binding to the solid surface may well be decreased by competing IgM, this however should not affect the ratio of low and high affinity antibodies, as measurements of ER(%) were performed in antigen excess (ie: in the linear region of the titration curve).

#### Comparison of the One-Well-Inhibition ELISA and Elution ELISA

The inhibition ELISA showed two main disadvantages over the Elution ELISA:

- 1) The presence of anti-RV IgM interfered with the determination of affinity values when using the inhibition assay. The valence of IgM may give this molecule an advantage over IgG for binding antigen in the fluid phase. The reason why this interference of IgM with affinity measurement of IgG is observed remains somewhat of a puzzle. Other groups using a similar assay with peptide antigen, have not observed any

difficulty with IgM in acute sera (Dr. M.W.Steward. personal communication).

- 2) The amount of antigen required for the liquid phase inhibitor exceeds the requirement of antigen by the Elution ELISA by 100 to 1000 fold. This would have provided a significant obstacle for the use of purified RV proteins.

Based on the above describe observations, it was decided to proceed with the Elution ELISA as the assay of choice, which would be employed to study the relative affinities of sera to whole virus as well as to the separated RV structural proteins E1, E2 and C.

### **3.2. Section II: Protein Specific Immunity: Antibody Quantitation and Affinity to Separated RV E1, E2 and C.**

Rubella virus contains three structural proteins which are immunogenic in humans. However it is the E1-specific antibodies which have hemagglutination inhibition activity and the majority of the *in vitro* neutralization activities. In mouse monoclonal antibody studies 4 E1-specific epitopes have been defined which have both *in vitro* neutralizing (in absence of complement)- and hemagglutination inhibition activity. A further 2 epitopes have been described to have hemagglutination inhibition activity only. On the other hand only 1 epitope has been defined on E2 which can neutralize RV replication *in vitro* (Green and Dorsett, 1986). It is therefore believed that the response to the envelope protein E1 is paramount in protective humoral immunity. As whole RV assays, such as those described above, do not differentiate between protein specific responses, it is necessary to develop protein specific immunoassays to quantitate the antibody responses directed to individual viral proteins. Assays such as immunoprecipitation (Katow and Sugiura, 1985), purified RV protein ELISA (Mauracher et al., 1992a) and Western blotting (Zhang et al., 1992) have allowed the investigation of protein-specific immune responses following Rubella infections. This section will discuss the development and some applications of the latter two techniques referenced above.

#### **3.2.1. Western Blotting**

##### **3.2.1.1. Reducing versus Non-Reducing Conditions**

Figure 19 illustrates a Western blot and autoradiograph of gel separating viral proteins under reducing ( + 2-ME) in the left lane and non-reducing conditions ( - 2-ME) in the right lane. This SDS-PAGE separation was unorthodox in that both wells were positioned side by side to each other and the diffusing of reducing agent during the stacking run into the right lane, which contains non-reducing sample buffer, illustrates the decrease of both E1 and E2 antigenicity in the presence of reducing agents. Capsid, under non-reducing conditions travels as a dimer ( $C_d$ ). Its antigenicity is not stronger in the absence of reducing agents, and  $C_d$  is poorly visible in the Western blot in Figure 19. The autoradiograph of the Western blot shows that the increase in signal strength observed in the immunoblots is due solely to antigenicity and not to quantity differences. The apparent molecular mass of E1 was observed to increase by about 4 kD upon reduction. This suggests that native E1 is held in a compact tertiary conformation. E1 contains an average of 5 mole % cysteine residues (Clarke et al.,

1987) which likely contribute to the highly folded conformation of this protein. Although E2 contains a similar distribution of cysteines, its apparent MW is not affected by reducing conditions. The C protein travelled as a dimer under non-reducing conditions, and the transition from the 66 kD dimer to the 33 kD monomer can be seen in the autoradiograph in Figure 20A. As the addition of reducing agents selectively affected the antigenicity

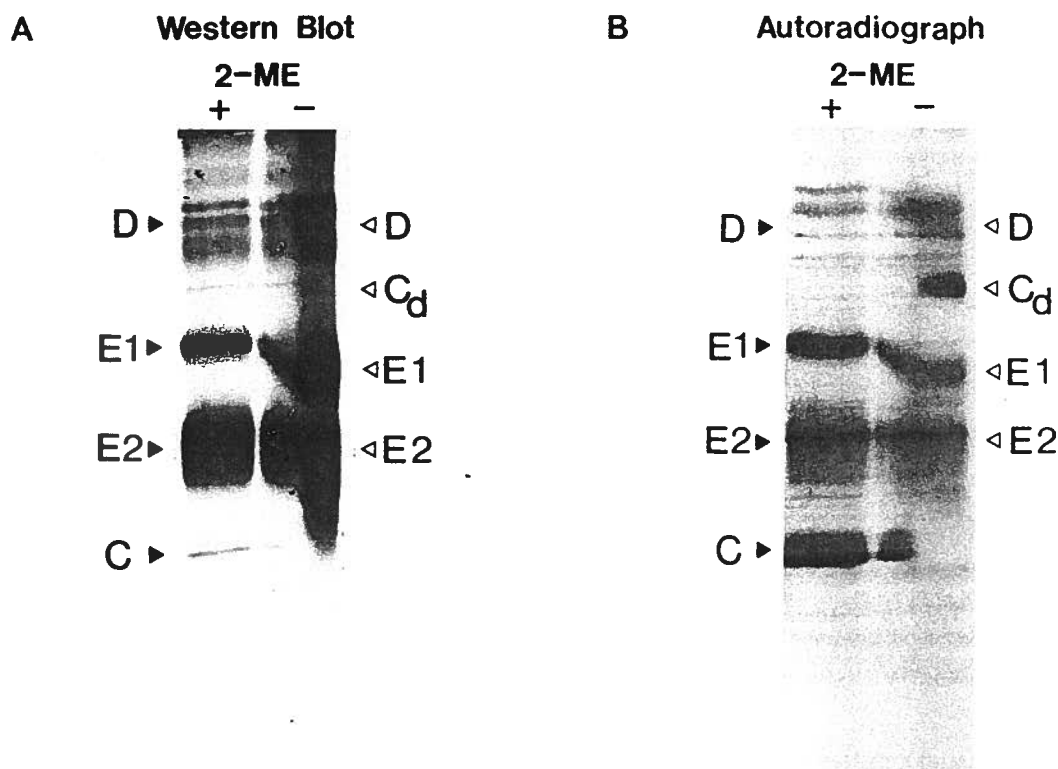


Figure 19. Western Blot and Autoradiograph of Reduced and Non-Reduced Preparations of RV. A: Western blot developed with polyclonal human anti-RV serum as primary antibody. Both lanes contain equal amounts of L-[<sup>35</sup>S]methionine labelled RV, run in the presence (+ 2-ME) or absence (- 2-ME) of 2-mercaptoethanol. Reduced ( ) and non-reduced ( ) viral structural proteins are indicated at the margin: (D) homodimeric and heterodimeric forms of E1 and E2, (C<sub>d</sub>) capsid dimer, as well as the monomeric forms of E1, E2 and C. B: Autoradiograph of the Western blot illustrated in A. (Mauracher et al., 1992a)

of E1 and to a lesser extent that of E2. Western blot protocols were put into practice which excluded the use of reducing agents. It became apparent that non-reducing blots were about between 1 - 2 log units more sensitive than those performed under reducing conditions and evidence by others indicates that the non-reducing blots may show equal sensitivity as RV IgG ELISA (Dr.L.A.Mitchell and Ms.M.Ho, Dept. of Pathology and Pediatrics, UBC, personal communication). Before the development of the immunoblotting technique for the detection of RV specific IgG, immunoprecipitation of radiolabelled RV was the assay of choice for the detection

of protein-specific IgG, and had been used in two studies to determine the protein specific responses in rubella responders (Katow and Sugiura, 1985; de Mazancourt et al, 1986). If the immunoblot technique was to replace the accepted methodology for the measurement of protein specific responses it had to be demonstrated that immunoblot provided similar response profiles as observed with immunoprecipitation.

### 3.2.1.2. Comparison of Immunoprecipitation and Western Blot under Non-Reducing Conditions

Six patient sera were compared for their RV protein-specific IgG responses using immunoprecipitation and immunoblot assays performed under non-reducing conditions (Fig. 20). The results show that immunoprecipitation overestimates the amount of anti-E2 IgG present in serum. Lane 2 contains a serum with no E2 specific antibody as determined by the immunoblot technique. This may be due to two factors. If L-[<sup>35</sup>S]-methionine is used as a metabolic label then differential distributions of this amino acid in the RV proteins has to be taken into account. E1 contains only 3 methionine residues whereas E2 and C contain 7 and 6 respectively (Clarke

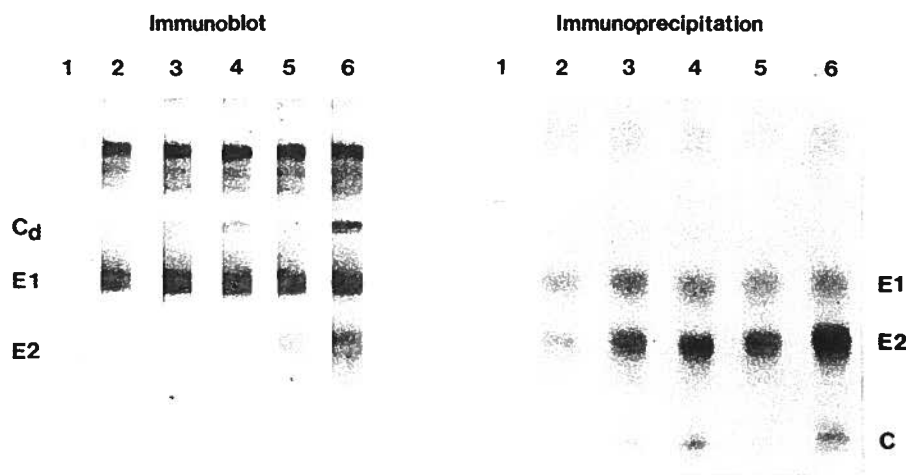


Figure 20. Comparison of Anti-RV IgG Western Blot and Immunoprecipitation Assays. Six serum samples from one negative control and 5 RV serology positive patients were subjected to comparative analysis by Immunoblot or Immunoprecipitation. Lane 1: negative control; lanes 2-4 responders to wild RV infection; lanes 5 and 6: CRS patients.

et al., 1987). This problem could be overcome by replacing methionine with tritiated amino acids in order to ensure equal labelling of RV proteins. The problem of co-precipitation of E1 and E2 was more difficult to solve. Monoclonal mouse IgGs directed against E1 often precipitate E2 protein (Brush, 1989) as dimerized

forms of the envelope protein are resilient to dissociation even in the presence of ionic detergents. The discrepancy between immunoprecipitation and Western blot techniques with respect to detection of E2 signals may therefore be due to co-precipitation in the immunoprecipitation assay. The two techniques correlate well in respect to E1- and C-specific signals.

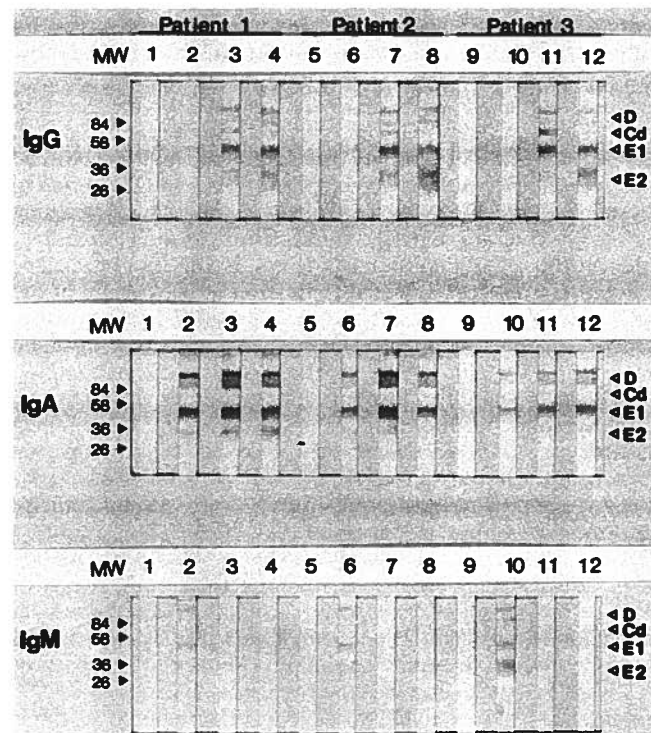


Figure 21. Immunoblot Analysis of RV-Specific IgG, IgM and IgA antibodies at intervals following wild Rubella Infection. Western blots of RV proteins separated by non-reducing SDS-PAGE were developed with representative sera obtained at the following time intervals after the diagnosis of a rubelliform rash: lanes 1, 5 and 9: 0-6 days (early acute phase); lanes 2, 6 and 10: 7-30 days (acute phase); lanes 3, 7 and 11: 1-11 months (convalescent phase); lanes 4, 8 and 12: 1-3 years (late convalescent).

Western blotting provided a good alternative to immunoprecipitation and showed considerable advantages: a) independence from radiolabelled reagents; b) technical difficulties such as unequal protein labelling and co-precipitation are avoided; c) rapid results ; d) more amenable to the processing of large samples and e) allowing the determination of RV protein-specific IgM and IgA antibodies in addition to IgG.

The immunoblot technique was sensitive enough to detect IgA and IgM directed to the individual RV proteins (Fig. 21). An analysis of three patients, recovering from wild type RV infection shows the rapid increase of RV-specific IgM in the first months of RV infection, followed by its decline within a year following infection. Both

IgG and IgA responses are maintained in the first three years following primary rubella. The kinetics of protein-specific responses of IgG and IgA are discussed further in section III.

### 3.2.1.3. Western Blot Affinity Assay

The principle of chaotropic elution of low affinity antibodies was applied to Western blots (Fig. 22). Patient sera were applied in duplicate to nitrocellulose strips containing blotted RV proteins, separated by reducing SDS-

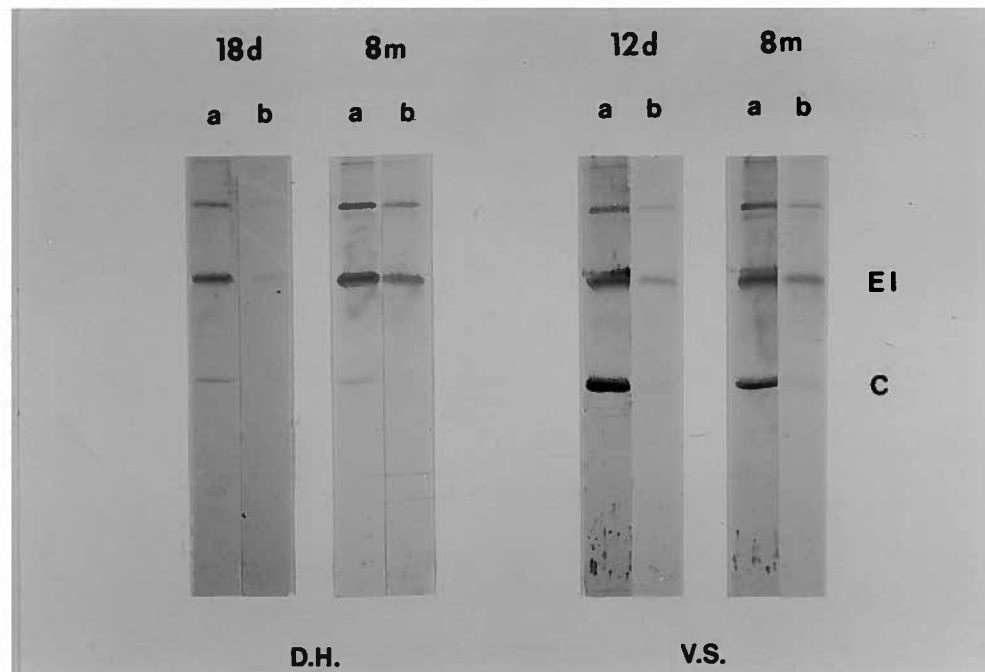


Figure 22. 8M Urea Elution of Western Blot. Paired serum samples from Patient A and B, taken during the acute (18 or 12 days) and convalescent phase (8 months) following natural Rubella infection. Strips labelled "a" represent nitrocellulose strips washed in PBS buffer following serum incubation. Strips "b" show blots incubated with the same sera as in lanes "a", but were washed for 3 minutes in 8M urea elution buffer. Position of E1 and C is indicated on the right side. Anti-C IgG was readily eluted by urea, both in acute and convalescent samples whereas anti-E1 IgG resisted urea wash in the convalescent samples, indicating a differentially higher affinity of anti-E1 IgG in the convalescent sample.

PAGE, one strip was washed in PBS, the other to be washed for 3 minutes in elution buffer, as described above for affinity ELISA. As was observed with whole RV ELISA, signal strength was not effected when blots were washed with urea prior to the addition of antibody. The paired, acute and convalescent, serum samples obtained from patients following Rubella showed antibodies reactivity to E1 and C, but not to E2. The E1-specific IgG matured in its affinity and became resistant to elution in the 8 month samples. In contrast the anti-C IgG was not resistant to urea elution in either the 18, 12 day or 8 month samples, suggesting that these antibodies did

not undergo affinity maturation.

These results indicated that the affinities of antibodies directed to the individual viral proteins may not be equally distributed. Differential affinities of antibodies to proteins of a viral pathogen had not been reported previously and the possibility that one protein was immunodominant and was giving rise to both higher levels as well as higher affinity antibodies was investigated further (Section III).

Most investigators which have analyzed the specificity of anti-RV antibodies have employed either immunoprecipitation (Katow and Sugiura, 1985; de Mazancourt et al., 1986) or immunoblotting under reducing conditions (Partanen et al., 1985; Cusi et al., 1989). With the new observations reported here, concerning sensitivity and applicability of immunoblotting performed under non-reducing conditions to the detection of Ig of all classes (Mauracher et al., 1992; Zhang et al., 1992) this technique may find application in serology during the early post-conception period to verify RV infection. The ability to determine specificity of IgA for individual RV proteins may aid in the understanding of the role of both serum and mucosal IgA in the protection against viral reinfection and provide a technique to further our understanding of the role of persisting serum IgA as an indicator of persistent RV infection (Morris et al., 1985).

Some disadvantages remain inherent to the Western blot technique. The technique is more time consuming and costlier than ELISA and the quantitation of peaks by densitometry can introduce bias in the interpretation of protein specific signals. It became desirable to obtain RV proteins E1, E2 and C in pure form and to utilize these proteins in the ELISA protocol.

### **3.2.2. Separation of RV Proteins, Their Use in Solid Phase Immunoassays**

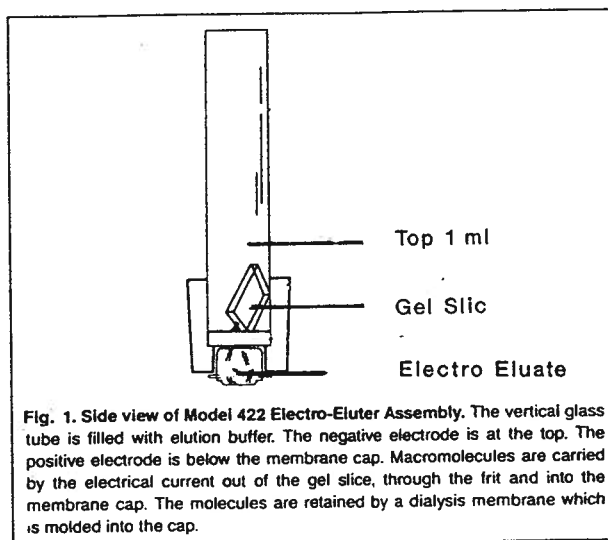
Many strategies for the separation of viral proteins have been described. The properties of E1, E2 and C, such as size, charge or solubility, can be used to purify these proteins. The starting material for the separation of the RV proteins was the highly concentrated virus stock described earlier in section I, which was highly antigenic and also immunogenic in animals. The goal of the protein purification strategy was not necessarily to arrive at preparations which showed high levels of purity for each protein, but to arrive at preparations which were pure in respect to other RV proteins. Hence the term "RV protein separation" will be used rather than "protein purification".

Although the envelope proteins E1 and E2 can be readily separated from the capsid protein using detergent



extraction (Mauracher et al., 1991a) (Fig. 29), the separation of E1 from E2 was more difficult. Both envelope proteins have similar solubilities and have overlapping pI ranges due to glycosylation with complex sialylated N-linked sugars (Lundström et al., 1990). More importantly they form heterodimers which are resistant to all but the most severe denaturing conditions. As described above, non-reducing immunoblots showed similar immunoreactivities as immunoprecipitations and supported the concept that SDS-PAGE under non-reducing conditions and low heat may yield separated RV structural proteins with antigenic properties similar to that of native

**A**



**B**

Viral Protein	PRE Gel Slice	Electro Eluate	Top 1 ml	POST Gel Slice	Yield %
<i>C</i>	1737	1960	120	76	112
<i>E1</i>	939	923	65	62	98
<i>E2</i>	2067	2025	108	72	98

Figure 23. Yield of Radiolabelled RV Proteins after Electroelution. A: Bio-Rad model 422 electro-eluter assembly. The vertical glass tube is filled with elution buffer. The negative electrode is at the top, the positive electrode is below the membrane cap. RV macromolecules are carried by the electrical current out of the gel slice, through the frit and into the membrane cap (400 lI). The molecules are retained by a dialysis membrane (MW cutoff 12-15 kD) which is moulded into the cap. (From: Bio-Rad, manual 87-01330387, 1987) B: Yield of radiolabelled RV proteins by electroelution. Counts per minute (cpm) were determined in gel slices before and after electroelution (PRE and POST), "Top 1 ml" indicated a 1 ml sample of elution buffer taken from above the frit.

protein. Using preparative SDS-PAGE, followed by electroelution provided a feasible strategy for RV protein separation (Mauracher et al., 1992a).

#### 3.2.2.1. Feasibility of Electroelution; Yield and Purity of RV Proteins.

The yield of this separation technique was initially established as follows: L-[<sup>35</sup>S]methionine labelled RV was separated on 10% acrylamide slab gels and the relative positions of the viral proteins were determined by an overnight autoradiograph of one gel lane. The RV proteins E1, E2 and C were excised from the gel, with one-half being electroeluted and the other half stored for the determination of radioactivity prior to elution. Samples of electroeluted proteins in the membrane cap, the electroeluted gel slices above the frit and elution buffer in the glass tube were solubilized in the scintillant and counted for radioactivity and was compared to the counts in the gel slices which were not electroeluted. Gel slices and electroeluate were added to 1 ml of scintillant and left in the dark over night. Counts per minute from pre- and post-eluted gel fragments, electroeluate and elution buffer as well as yield determinations are shown in Figure 23. The yield of protein was 90 - 100%. This was

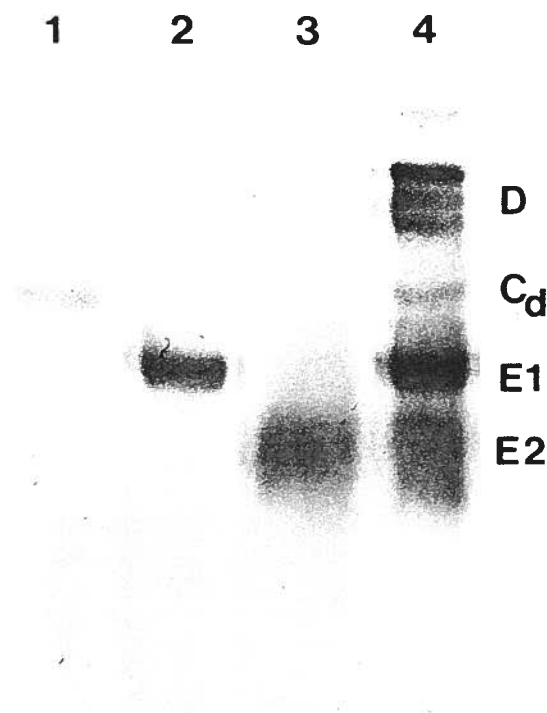


Figure 24. Western Blot of Separated RV proteins. Preparations of capsid dimer (Cd), E1 and E2 proteins were run on non-reducing SDS-PAGE in lanes 1, 2 and 3 respectively. Purified whole RV was loaded in lane 4, all viral structural proteins were visualized including the dimerized species of E1 and E2 (D). (From Mauracher et al., 1992a)

also confirmed by electroeluting known amounts of BSA and determining protein concentrations following electroelution (data not shown).

The monomeric species of the RV envelope proteins, E1 and E2, and the C dimer differ in mass by about 10 kD each. This difference in MW is sufficient to separate these proteins on 10% SDS-PAGE slab gels. Gels were run until the 27 kD pre-stained MW marker reached the gel front, then the proteins were excised using the pre-stained MW markers as guides and electroeluted. Each electroeluate was checked for purity by Western blot and the preparations shown to be free of the other two respective viral proteins were pooled. Figure 24 illustrates a Western blot of the stock preparations of separated RV E1, E2 and C<sub>d</sub>, each antigen preparation is pure in respect to viral protein contamination.

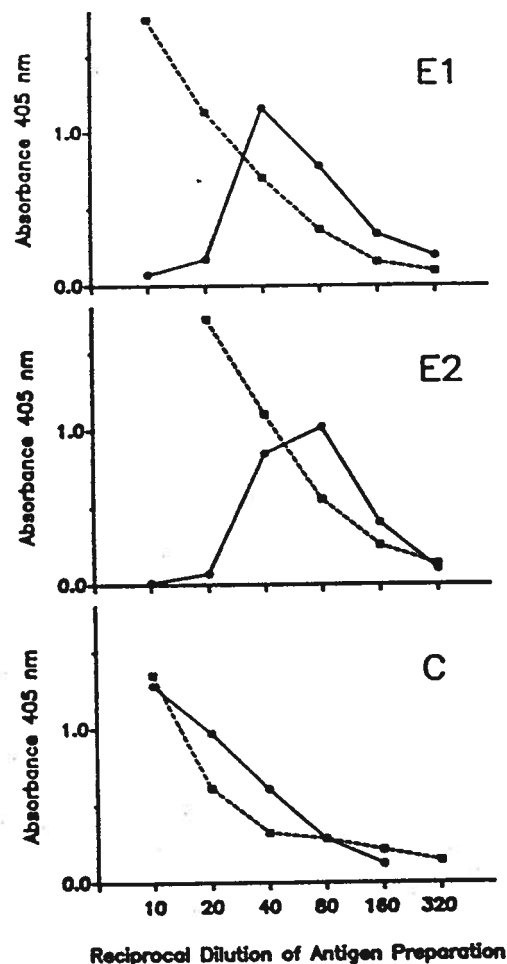


Figure 25. Comparison of Optimal Coating Concentration for E1, E2 and C<sub>d</sub>. Anti-RV protein IgG ELISAs were performed with a constant concentration of anti-RV reference serum on microtitre plates coated with serial dilutions of E1, E2 or C<sub>d</sub> containing 0.1% SDS (●) and following removal of SDS by KCl precipitation (○). (From: Mauracher et al., 1992a)

#### 3.2.2.2. Maximization of Protein Binding

Polystyrene, in the format of 96-well microtitre plates, was chosen as the solid phase for the adsorption of the purified RV proteins. Different plates supplied by Nunc, Costar, Falcon and Dynatech were tested for their abilities to bind RV proteins under various coating conditions: PBS, phosphate buffer pH 7.4, carbonate buffered saline pH 9.4, carbonate buffer pH 9.4, distilled water and drying protein onto solid phase in distilled water. Immulon-2 plates in combination with PBS as the coating buffer were found to be optimal for the coating of each RV protein. Each protein preparation had an optimal coating concentration (Fig. 25) and the dilution yielding peak absorbances were established to be: 1:40, 1:80 and 1:20 for E1, E2 and C<sub>d</sub> respectively. The titration of the E1 and E2 preparation exhibited decreased absorbance values at concentrations of SDS of 0.01% and 0.005%, whereas C<sub>d</sub> was not affected by the presence of SDS. When SDS was removed from the antigen preparations by KCl precipitation prior to coating, the marked prozone seen at dilutions of 1:10 and 1:20 was not observed. However, a loss of up to 50% of the protein was realized during KCl precipitation. The decrease of E1 and E2 antigen signals at low dilutions may be due to the competition of SDS with protein for hydrophobic binding sites on the plate or actual electrostatic repulsion of SDS-protein complexes from the charged polystyrene surface.

#### 3.2.2.3. Quantitation of anti-E1, -E2 and -C IgG by ELISA

IgG directed to the individual RV proteins was determined in a similar manner to that directed to whole RV. The laboratory standard serum containing 943 IU/ml of anti-RV IgG was analyzed on non-reducing blots and the ratios of IgG directed to each protein were determined. The 943 IU/ml of anti-RV activity were then assigned to anti-E1, -E2 and -C IgGs according to the percentage determined by Western blot. Figure 26 shows a Western blot of the standard serum with its densitometric trace. The dimeric forms of the envelope proteins, E1 dimer (E1<sub>d</sub>) and E1-E2 heterodimer make up about 35% of the total signal on the blot, and were included in the calculations. The slowest travelling dimer band was assumed to be E1<sub>d</sub>. A second dimer band, below the E1<sub>d</sub>, often stains more weakly, and was presumed to be the E1-E2 heterodimer. The signal from this band is attributed to both anti-E1 and -E2 IgG and was divided according to the ratio of E1/E2 monomer specific IgG. E2 homodimers has thus far not been described. It is not known if distinct epitopes exist for the dimeric E1 or E2 forms, but all the described E1- and E2-specific mouse monoclonal antibodies show reactivities to both

monomeric and dimeric forms of E1 or E2 (J.Wolinsky, University of Texas, personal communication).

The standard serum was blotted and scanned in four separate experiments and the serum was assigned the following concentrations of RV protein specific antibodies, expressed in arbitrary units per millilitre of serum (AU/ml): E1 (456 AU/ml), E2 (390 AU/ml) and C (97 AU/ml). Five dilutions of the standard serum were included on all plates coated with either detergent solubilized RV or electrophoretically separated E1, E2 or Cd. The concentration of protein-specific IgG could then be determined by performing quadratic regression on the absorbance values obtained for the dilutions used for the construction the standard curve.

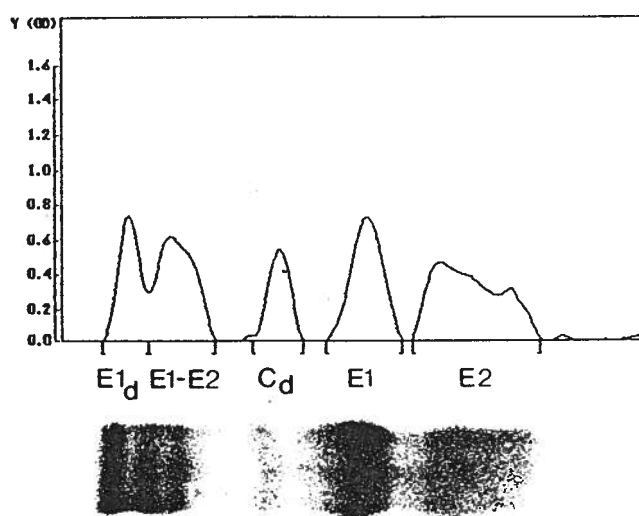


Figure 26. Western Blot and Densitometric Scan of Anti-RV Standard Serum. 48.4% of total signal strength was attributed to anti-E1 IgG (13.9% from E1<sub>d</sub>, 9.6% from E1-E2 and 24.9% from E1); 41.4% due to anti-E2 IgG (11.5% from E1-E2 and 29.9% from E2) and 10.2% due to anti-C IgG (10.2 from C<sub>d</sub>). The E1-E2 heterodimer can be seen as a doublet on the blot, but is poorly resolved, as a peak with a shoulder, on the densitometric scan.

### 3.2.3. Antigenicity and Biological Activity of Separated RV Structural Proteins

As described above it was established that the antigenicity of the E1 and E2 proteins were sensitive to denaturation by reducing agents. It was important to determine the effects of SDS and heat, the main denaturants involved in non-reducing SDS-PAGE, on the antigenicity of the separated RV proteins. Antibody quantitation and affinity measurements were dependent on intact proteins, which had retained their epitopes during the purification protocol.

Initial results indicated that denaturation by low concentrations of SDS (0.25%) was reversible but that boiling

of RV in non-reducing sample buffer lead to irreversible denaturation leading to a loss of antigenicity as measured by ELISA (Fig. 27). Whole RV preparations were diluted 1:10 in a 0.25% solution of SDS and then either boiled for 3 minutes or left at RT. As a control, a preparation was diluted in PBS only. All preparations were then serially diluted from 1:10 to 1:1280. The control titration curve is non-linear at dilutions of 1:10 and 1:160 and becomes linear beyond that. The virus, treated with SDS without heating, showed an equal end-point titre to the control preparation, indicating that denaturation by SDS was reversible. In the saturated region of

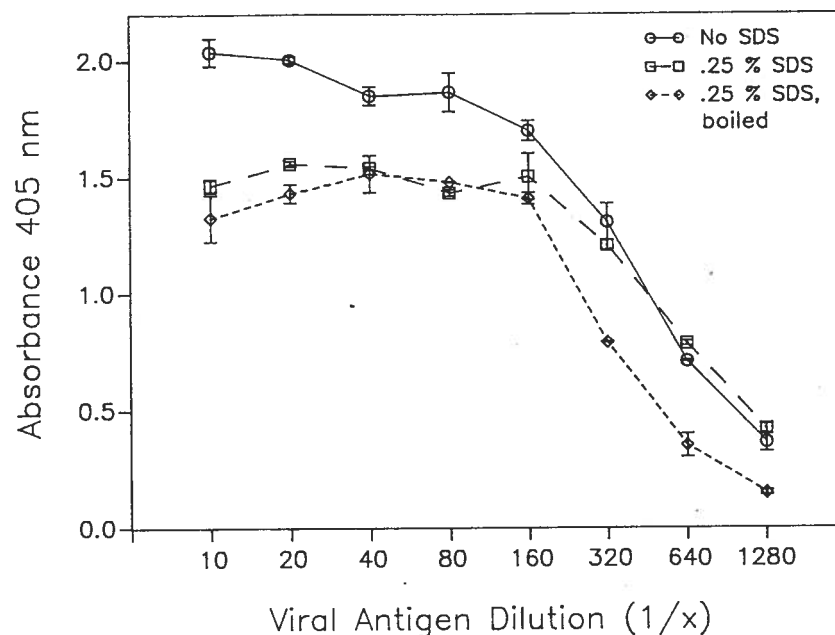


Figure 27. Effect of SDS and Temperature on the Antigenicity of Whole RV. RV preparation were either not treated with SDS (O) or treated with SDS (0.25%) and subsequently held at either 60°C (□) or boiled (◇) for 3 minutes. Each RV treatment group was coated in serial dilution onto microtitre plates and its antigenicity was tested by RV IgG-specific ELISA, using a constant dilution of human anti-RV reference serum (From: Mauracher et al., 1992a).

the titration curve a noted depression of absorbance was observed indicating that SDS at concentrations above .001% interfered with binding of protein to the solid phase. Heat denaturation in presence of SDS led to irreversible loss of antigenicity indicated by a lower end-point titre of this preparation. This treatment reaches the same plateau as the unboiled SDS-treated preparation in the saturated region of the titration curve, again suggesting that the loss of absorbance is due to binding inhibition rather than denaturation. Although the above

experiment indicated that denaturation of RV proteins due to the employed SDS-PAGE conditions (0.25% SDS, 60#C, non-reducing conditions) were minimal, the effect of separation on the biological activities of E1 and C protein were further investigated to determine if the purified RV proteins had retained their antigenic integrity.

Biological activity of a protein is generally dependent on its structure. It was therefore reasoned that if separated viral proteins had maintained their biological functions, then their structure and also their structurally dependent epitopes had remained intact throughout the purification procedure. Both E1 and C protein preparations were analyzed for some of their biological activities.

### **3.2.3.1. Function of E1**

The E1 protein of RV contains the majority of the currently identified neutralization domains (Waxham and Wolinsky, 1985b) as well as the region involved in the agglutination of chick and goose erythrocytes. Both these activities are sensitive to denaturation (Ho-Terry et al., 1985; Qui and Gillam, 1992). This protein is not only thought to be involved in attachment of the virus to the target cell, but is also involved in low pH induced virus-endosome membrane fusion, through low pH (< 5.5) activation of fusogenic properties (Katow and Sugiura, 1988). Separated E1 protein preparations were analyzed for their HA- and HAI antibody binding activity. The separated E1 protein by itself was not capable of hemagglutinating chick red blood cells (CRBC) (Dr. S. Gillam, Dept. of Pathology, UBC, personal communication). This was not surprising as CRBC agglutination requires whole virion or cross-linked E1. The HA activity of the electrophoretically separated E1 was measured indirectly by its capability to compete for HAI antibodies with native Rubella virions (Fig. 28). The E1 preparation was capable of competing for HAI antibodies in human serum. E1 preparations at a volume of 25 and 12.5  $\mu$ l, were capable of binding sufficient antibody in the 1:160 diluted antiserum to allow virus to cross-link CRBC and form a mat. At volumes of 6.25  $\mu$ l or less of competing E1, sufficient HAI IgG was left to inhibit CRBC crosslinking. The equivalent series using C preparations showed that the competition for HAI antibodies was exclusively due to E1. As HAI antibodies recognize structural domains on E1 (Ho-Terry et al., 1985), it was possible to conclude that the separated E1 had maintained its native structure during purification procedures.

An alternate approach for directly measuring intact HA activity of E1 preparations would have been to let the KCl treated E1 preparation interact with CRBC followed by the exposure of such sensitized erythrocytes

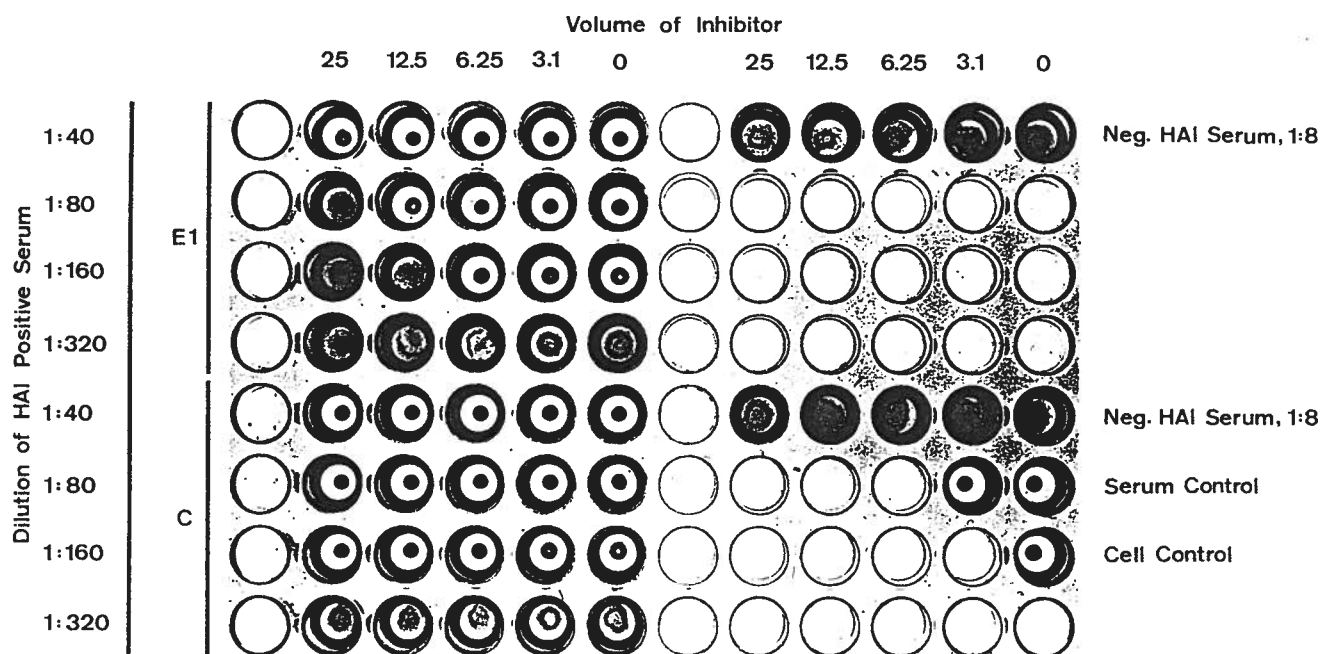


Figure 28. HAI Antibody Competition Assay. E1 and C preparations were used to compete for HAI antibodies with whole rubella virions. Four dilutions of the anti-RV standard serum (1:40, 1:80, 1:160, and 1:320) with a HAI titre of 1:160 were added to 5 wells per row. To each row, containing constant serum dilutions, varying amounts of either E1 or C preparations were added (25, 12.5, 6.2, 3.1 or 0  $\mu$ l). Each well was then brought to a final volume of 50  $\mu$ l. After a 1 hour incubation at 4°C each well received 25  $\mu$ l of 4 HA units of RV. Following 30 minutes incubation each well was loaded with 25  $\mu$ l of a 0.02% solution of CRBC. Negative controls included the use of RV negative serum at 1:40 with both E1 and C antigen. Serum controls tested both negative and positive serum for endogenous HA activity. Cell control tested for button formation in the absence of both serum and HA antigen.

to HAI positive antisera in the presence of complement. Lysis of the cells would have indicated binding of E1 onto the erythrocytes, thereby proving biologic activity of the HA domain of E1.

### 3.2.3.2. Function of C protein

The biological activities of the RV C and E2 proteins are less well defined. Although E2 protein was recently described to contain both N- and O-linked oligosaccharides (Lundström et al., 1991) and is proposed to be an absolute requirement for the transport of E1 to the cell surface, followed by virion assembly (Hobman, 1989),



a clear biological function of E2 in the assembled virion has not been established. Hence, it was not possible to analyze the biological function of purified E2 preparation. Similarly the capsid protein of RV has no described biological function in the assembled virion, other than the described morphological feature of the nucleocapsid. By inference, from the work on the related alphaviruses, SFV and SV, it is proposed that the C protein initially binds to the 5' end of the full length + sense RNA (Weiss et al., 1989) to initiate nucleocapsid assembly, leading to the budding of the assembled nucleocapsid from the plasma or trans-Golgi membrane (Hobman, 1989).

During experimentation on the detergent extraction of RV envelope proteins, using TX-114, it was noted that the capsid protein, which under physiological pH conditions is not soluble in detergent, became detergent-soluble at pH 4.5 (Fig. 29A). This change in solubility properties was examined further using detergent extractions of

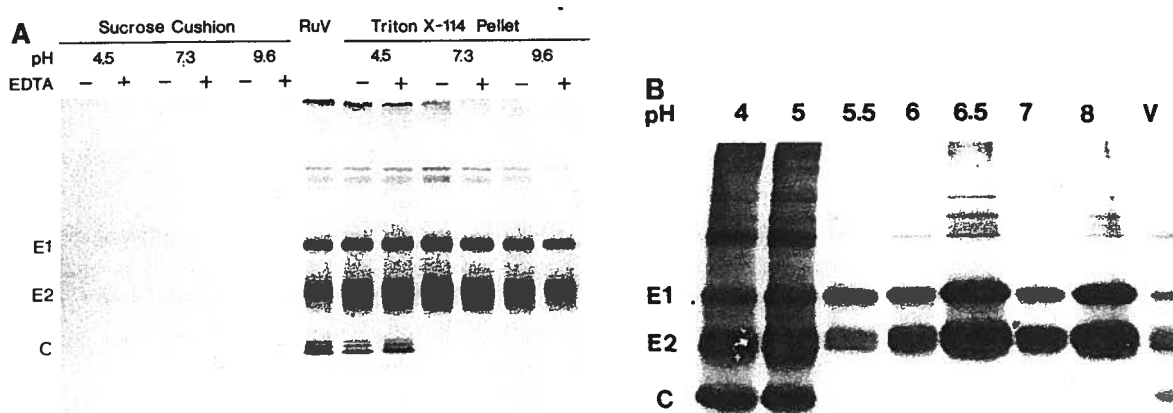


Figure 29. Autoradiographs of TX-114 Extracted RV. L-[<sup>35</sup>S]methionine-labelled RV in TCS was detergent extracted and separated by reducing SDS-PAGE. A: Extraction performed at pH 4.5, 7.3 and 9.6, in the presence and absence of 10 mM EDTA, showing the detergent pellets and the corresponding 6% sucrose cushion. The lane marked RuV contains untreated rubella virus. B: Detergent pellets from TX-114 extraction performed at pH 4-9 are shown in the first seven lanes from the left. Lane V contains untreated rubella virus. (From: Mauracher et al., 1991a)

TCS containing radiolabelled RV performed at narrow pH intervals (Fig. 29B). C protein underwent a solubility shift from being hydrophilic to hydrophobic below pH 5.5. RV proteins are not acid precipitated at pH 4.5, nor does Triton X-114 change its properties within the pH range used in these experiments. The appearance of the

capsid band in the detergent phase below pH 5.5 can be explained by a pH-induced structural change of this protein, either causing an intrinsic change in its solubility properties or allowing it to interact more strongly and co-solubilize with the lipid soluble E1. To rule out the latter, TX-114 extractions were performed on cell lysates of insect cells expressing the cloned RV capsid gene, independent of E1, E2 or the RV RNA (baculovirus expressed C protein was generously supplied by Dr.S.Gillam, Dept. of Pathology, UBC). This recombinant capsid protein also underwent the solubility shift described for the wild-type capsid, indicating that the proposed solubility shift was an intrinsic feature of the C protein (Mauracher et al., 1991a). To establish that the observed capsid solubility shift also represented a property of the intact nucleocapsid, the effect of TX-114 extraction and SDS treatment at pH 5 and 7 on the sedimentation of the virion and nucleocapsid through 22% sucrose was determined (Fig. 30A). The relative concentration of virion pelleting through the sucrose cushion following treatment at pH 5 or 7 in the absence of detergent was compared to the equivalent volume of TCS loaded directly onto the gel. The pronounced "smile" and lowered apparent MW is caused by the displacement of E1 by large amounts of BSA present in the TCS. TCS treated with 1% SDS at both neutral and acidic pH resulted in the disassembly of both the virion and the capsid into individual structural proteins, which remain buoyant in 22% sucrose, resulting in the absence of viral proteins in the pellet. Intact capsid can be differentiated from disassembled capsid by its ability to pellet through the 22% sucrose cushion. Extraction of TCS with 1% TX-114 at neutral pH did not interfere with the capsid structure as C protein was detected in the pellet of the ultracentrifuged aqueous supernatant of the pH 7 extraction. If the TCS was acidified following the addition of TX-114, capsid was removed by its solubility in the detergent phase, resulting in the absence of a detectable C protein in the pellet upon ultracentrifugation. Viral RNA was detected in the pellets of all treatment groups (Fig 30B). The naked 40S genome of RV pellets through 22% sucrose (Oker-Blom et al., 1984). The presence of RV genome in the pellet following TX-114 extraction at low pH indicates that RNA had been released into the aqueous supernatant following the solubilization of the proteinaceous structure of the nucleocapsid into the detergent phase.

Recent observations made on the cytopathic vacuoles of SFV infected cells (Froshauer et al., 1988) have shown that the virus-encoded RNA polymerase remains associated with the limiting membrane of these vacuoles and that ribosome-RNA-capsid complexes form upon fusion of the viral envelope. This would be consistent with a proposed acid-induced intra-endosomal uncoating strategy, where the solubility shift of the capsid protein at

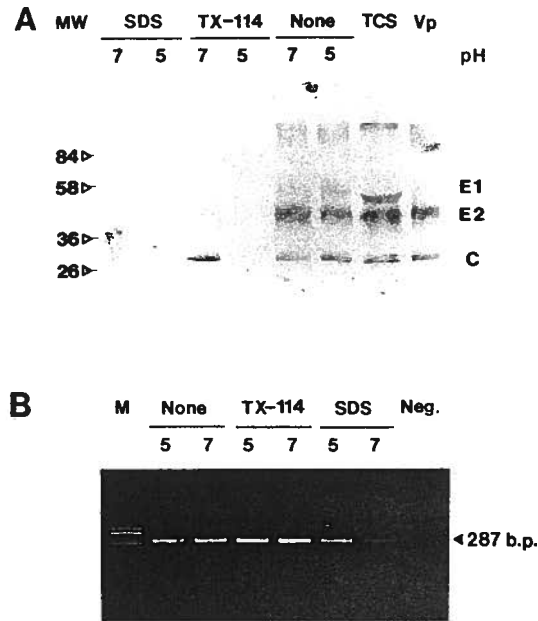


Figure 30. Ultracentrifugation of RV Following Detergent Extraction A: Western blot analysis of resuspended pellets following ultracentrifugation. TCS containing  $10^5$  pfu/ml of RV was treated with 1% SDS at pH 5 or 7 (SDS, pH 5 or 7) or with no detergent at both pH 5 or 7 (None, pH 5 or 7)., overlaid onto 22% sucrose and centrifuged at 39'000 rpm. Alternatively TCS was brought to a final concentration of 1% TX-114 and then acidified or left at neutral pH. Following extraction, the aqueous supernatant was overlaid onto sucrose cushion and centrifuged (TX-114, pH 5 or 7). The lane labelled Vp contains 0.1  $\mu$ l purified RV, lane TCS contains the equivalent amount of RV that was loaded onto the gel for each of the above described treatment groups. Molecular weights ( $\times 1000$ ) are indicated on the left margin, and the identity of the RV proteins on the right. B: Visualization of RV-specific PCR products. cDNA synthesized from the resuspended pellets of the six treatment groups described above was amplified by PCR using E1-specific primers delineating a 287-bp fragment. Lane labelled M contains *HaeIII* digest of pUC 19. Lane labelled Neg. contains a negative control for contaminating DNA sequences. (From: Mauracher et al., 1991a)

pH 5.5 leads to the disassembly of the capsid within the endosome and the release of the viral RNA (Fig. 31). This concomitant shift in structure and properties of both E1 and C below pH 5.5 suggests that the solubility shift of the capsid plays a role in capsid uncoating. It has been questioned whether the acidification of the endosome may necessarily lead to the exposure of the nucleocapsid to an acidic environment (Kielian and Helenius, 1986). These authors reasoned that the viral membrane remains impermeable to  $H^+$  ions and that the endosomal milieu would, therefore, not affect nucleocapsid integrity. Experiments using ion flux measurements across artificial bilayers upon SFV membrane fusion have shown that ion permeability of the viral membrane occurs only after injurious events such as sonication, freeze-thawing or storage of the virus at RT (Young et al., 1983). These observations support the hypothesis that the intact viral membrane of freshly

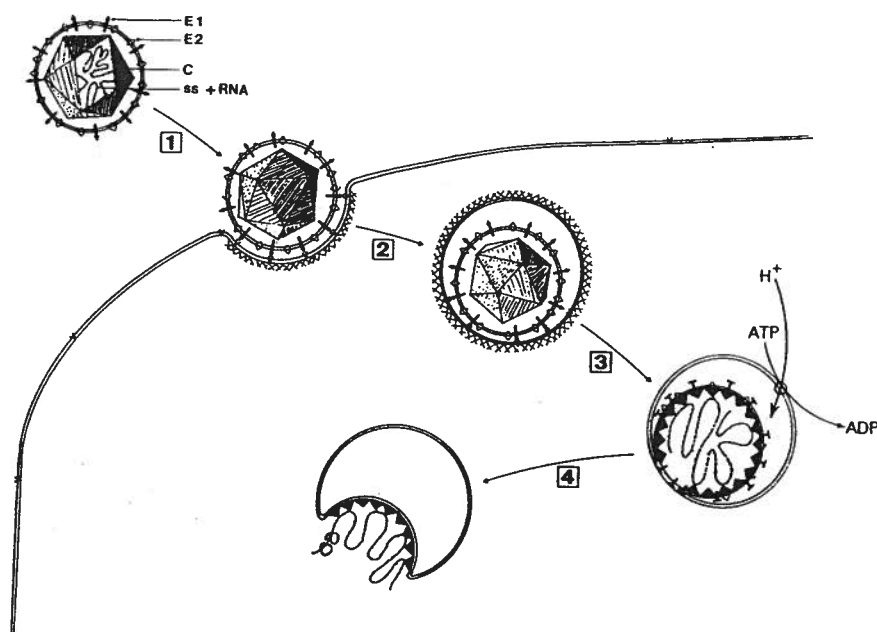


Figure 31. Proposed Model for the Entry of RV into the Host Cell. Following attachment to the cell surface receptor (1) the virus is taken up via the receptor and internalized into a coated vesicle (2). The low pH in the endosome induces the E1 protein to become fusogenic (Katow and Sugiura, 1988) and facilitates the fusion between virus and lysosome membranes. In parallel, the low pH induces a solubility shift of the capsid, allowing it to associate with membrane, causing intra-endosomal uncoating (3). Upon membrane fusion, viral RNA is released into the cytoplasm. (From: Mauracher et al., 1991a)

harvested virions remains impermeable to small ions. However this group concludes that endocytosis in itself can compromise membrane integrity. Recent evidence suggests that the spike proteins in SFV act as an ion channel upon activation by the low pH environment of the endosome (Kempf et al., 1988). The current hypothesis on the entry of togaviridae suggests that within the endosome a low pH-induced conformational change of the viral spike proteins takes place resulting in a) a proton influx into the virion, b) the low pH-induced solubility shift of the nucleocapsid leading to the uncoating of the viral RNA and c) the fusion of viral and endosomal membrane (Mauracher et al., 1991a; Schlegel and Kempf, 1992).

The investigation of the solubility properties has led to the formulation of a novel biological property for the capsid and has led to a more complete understanding of the events occurring during early events in viral replication. The property of togavirus capsid proteins to undergo a structural shift leading to solubility changes and the unique  $\beta$ -barrel structure common to many RNA virus capsid proteins (Fuller and Argos, 1987) makes this event in viral replication a good target for drug intervention. Rhinovirus (picornaviridae) employs a similar

entry strategy to the togaviridae, in that the low pH of the endosome leads to the uncoating of the nucleocapsid (McKinlay et al., 1986). This event can be specifically inhibited by WIN 51711, a drug which tightly associates with the capsid and causes the protein shell to become less flexible, thereby preventing uncoating of RNA (Badger et al., 1988). It would be of interest to screen similar compounds for their ability to interfere with the solubility shift of the RV C protein in an effort to identify a chemotherapeutic route to interfere with the replication of RV and other togaviruses.

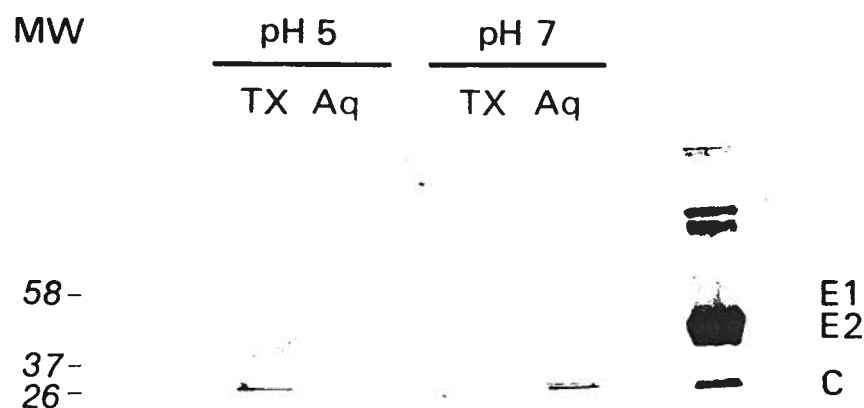


Figure 32. Detergent Extraction of Separated C protein. Preparations of separated C protein were treated with 50 mM KCl to remove SDS and then adjusted to pH 7 and 5 with 1 N HCl. Preparations were then extracted in 1% TX-114, and both the detergent phase (TX) and the aqueous phase (Aq) separated on 10% SDS-PAGE under reducing conditions. Lane RV contains whole RV, all other lanes contain pre-stained MW markers, indicated on the left margin.

The description of a function of C protein, that is structurally dependent, allowed the analysis of the electroeluted C protein. The C preparation was extracted in TX-114 in both acidic and neutral pH, following the removal of SDS by KCl precipitation (Fig. 32). C protein retained its characteristic biological function and

became detergent soluble under acidic pH, suggesting that its structure was not irreversibly altered during non-reducing SDS-PAGE.

#### **3.2.4.Summary**

Both Western blotting under non-reducing conditions and RV protein ELISA can be used to study the response of all immunoglobulin classes to the viral proteins. ELISA has the advantage of being faster, requiring fewer reagents and also providing easier interpretation of quantitative antibody measurement. For the quantitation and the assignment of units of protein-specific antibodies, care has to be taken to use both serum samples and the standard serum at dilutions in the linear region of the titration curve. Software programs which are capable of performing quadratic regressions and can transform absorbance data are of great value (ie: BioRad's Microplate Manager<sup>®</sup>), especially if they can analyze data directly from the microplate reader.

Much of the work in the development of protein-specific ELISA was concerned with the determination of the optimal conditions for binding proteins to polystyrene microtitre plates. Although coating buffers were of importance in maximizing signal to background ratios, the crucial variable was found to be the type of microtitre plate used in the ELISA. The presence of non-ionic detergent (ie: Tween-20), at concentrations lower than 0.05%, in the sample buffer was also critical in obtaining good signals, particularly when using E1 and E2 protein preparations. It is possible that the non-ionic detergent replaces the protein bound SDS and allows the re-naturation of the protein on the plate surface.

Of interest was the development of what initially appeared to be an insignificant observation, namely the pH-induced solubility shift of RV C protein. One of the values of the academic environment is that it allows an individual to pursue an interesting phenomena on an intuitive basis. In the case of C protein the work lead to an additional study which culminated in the proposal of a new uncoating mechanism for RNA virus. Ultimately these extra studies became an important part in this thesis, by establishing that RV C maintained at least one of its biochemical properties following purification.

### **3.3. Section III: RV-Specific Humoral Immune Responses in Adults Following Rubella**

With the establishment of RV-specific antibody techniques it became possible to perform comparative analysis of the immune responses of patient populations following infection with RV. The first subject population studied was composed of adults who had a confirmed clinical and serological diagnosis of Rubella. These subjects will frequently be referred to as the "normal" or "control" study group. Subjects were randomly chosen from the study sample inventory list and were not selected on the basis of their clinical histories or the outcome of RV infection.

It is necessary to define the normal RV-specific antibody response in an adult population in order to be able to compare and determine differences in groups developing pathological conditions following RV infection, such as CRS or RAA. If such group differences in the immune response are detected, it may become possible to determine correlations with the pathogenesis or the etiology of the disorder. It is also important to establish the range in normal immune responses to RV as defined in adults using current, as well as newly developed, laboratory techniques.

#### **3.3.1. Response to Whole Virus and Separated RV Proteins**

The levels of IgG in a group of 15 adults who had confirmed clinical rubella at least six months prior to obtaining the serum sample, were determined using ELISA employing whole RV or individual RV proteins. The average value of IgG directed to whole RV was 202 IU/ml  $\sim$  65 (X  $\sim$  SD), which was in accordance with previously reported anti-RV IgG levels (Dimechi et al., 1992). Currently RV-specific IgG levels  $\geq$  15 IU/ml are considered protective (WHO, 1971). Hence all individuals tested in this group are considered immune to RV re-infection. The response to E1 was dominant (208 AU/ml  $\sim$  115) and significantly ( $p=0.005$ ) higher than the measured responses to E2 (56 AU/ml  $\sim$  16) or C (33 AU/ml  $\sim$  18) (Fig. 33).

#### **3.3.2. Biological Activity of Sera**

Patient sera shown to be RV positive by the above described ELISAs were also analyzed by HAI assay. The median titre for this population was 1:32, with values of  $<1:8$  being interpreted as negative (U.S. Dept. of

Health, Education and Welfare, 1970). The comparison of serological data obtained by traditional HAI

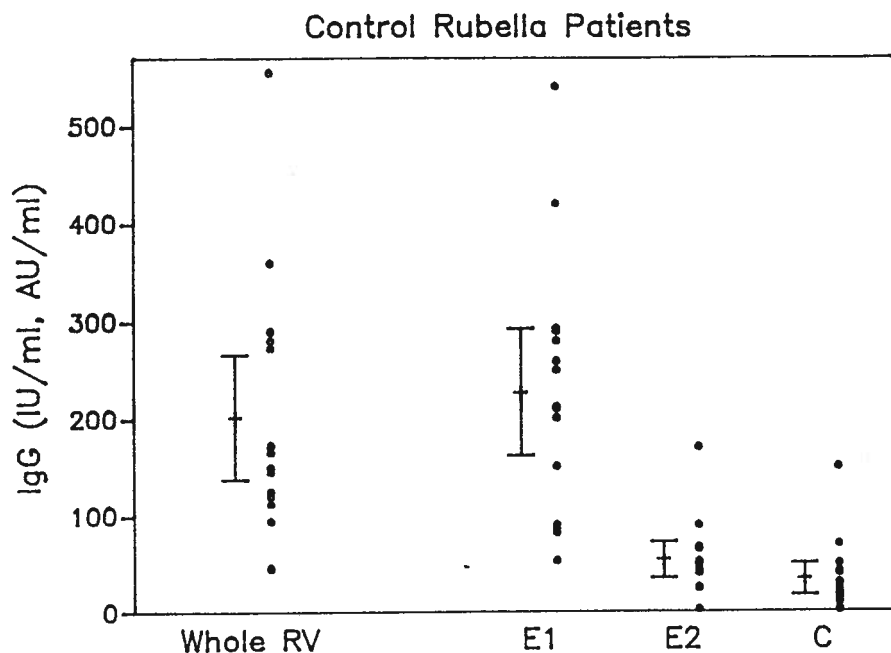


Figure 33: Quantitation of IgG to Whole RV and RV Proteins. Levels of IgG measured by whole RV ELISA are expressed in IU/ml, whereas levels of IgG to separated RV proteins are expressed in AU/ml. The horizontal bars indicate the mean, error bars indicate  $\sim 1SD$ . Values for individual patients are indicated by dots.

techniques and by solid phase immunoassay were in agreement. Similar experiments, performed on a larger scale (Herrmann, 1985) showed a good correlation between HAI and ELISA titres, leading ultimately to the substitution of HAI by EIA. Although these two approaches for the detection of RV-specific antibodies give similar results in this patient population, the data collected during this study show that the presence of high levels of anti-RV or anti-E1 immunoglobulins does not necessarily correlate with the presence of biologically active antibodies detected by HAI (see Section IV).

### 3.3.3.Reactivity to Linear and Topographic Epitopes

As described above, E1 and E2 were sensitive to denaturation by reducing agents. By performing Western blots it was possible to determine the distribution of reactivities to continuous and discontinuous (topographic)



epitopes among anti-E1, -E2 and -C IgG (Table 7).

E1	10% $\pm$ 4%
E2	46% $\pm$ 41%
C	125% $\pm$ 83%

Table 7: Percentage of Signal Remaining on Western Blot under Reducing Conditions. 14 convalescent patient sera were sampled on reducing and non-reducing blot, and analyzed by densitometry. The OD<sub>mm</sub> for each protein was determined and ratios of protein specific signal strength determined for reducing/non-reducing conditions. The mean ratio  $\pm$  one standard deviation for these 14 patients is shown.

Conclusions from the data shown in Table 7 were that in normal adults responding to RV infection, the E1 specific response was mainly directed to topographic epitopes with only 10% of the antibodies recognizing linear epitopes. A similar predominance of E1 specific antibodies to topographic epitopes has been reported in mice injected with whole RV (Wolinsky et al., 1991). Antibodies directed to E2 were equally distributed between specificities for continuous and discontinuous epitopes, however individual variability was observed. Antibodies directed to the C protein appeared to recognize both reduced and non-reduced protein. In many cases immunoblot bands were stronger under reducing-conditions, suggesting that linear epitopes may remain inaccessible to antibodies when capsid is in its dimeric form.

#### 3.3.4. Kinetics of the IgG Response

Using the Western blotting techniques, the kinetics of IgG responses directed to RV structural proteins was studied over the first years following RV infection (Fig. 34). The response to E1 was observed to be rapid and could be detected within the first several weeks following the clinical diagnosis of Rubella. The response to this protein reached maximal levels in the months following infection and remains relatively constant in the years following infection, with a slight drop-off observed in the second year following infection. The responses to C<sub>d</sub> were observed to be weaker and transient, with levels being maximal at three months. Responses to the E2 protein differed from E1- and C-specific responses, in that they exhibited a slow onset, with weak responses first observed at three months- and plateauing occurring by 12 months post Rubella.

Why the response to the RV E2 protein exhibits a slow onset remains speculative. The virus is cleared from the blood stream within days of the onset of the rubelliform rash and is not detectable in biological fluids

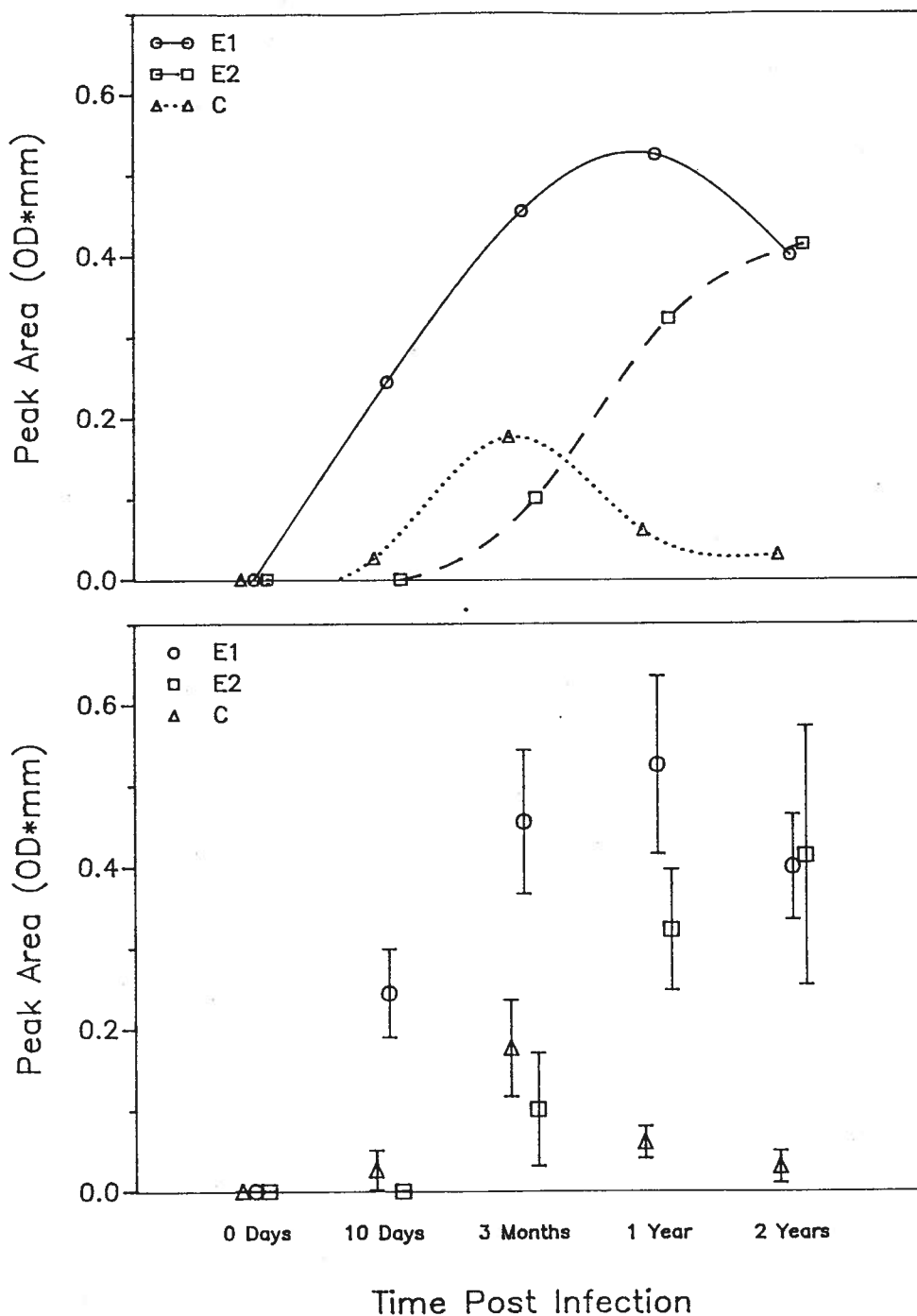


Figure 34. The Kinetics of the IgG Response to RV Structural Proteins. 9 subjects were followed throughout the first two years following Rubella. Serum samples at consecutive time intervals were analyzed by non-reducing Western blot. Each time point represents the average density of the protein-specific bands for 9 patients  $\sim$  SD. The top figure shows the mean value for each protein at the given time interval, with a fitted curve drawn through each point. The bottom figure shows the SD for each point, representing measurements on nine patients.

shortly after the onset of the IgG response; it therefore is difficult to account for a continued B-cell response to E2, long after the virus has been cleared. It is possible that this viral protein may be retained in lymph nodes, as described for other protein antigens (Szakal et al., 1989), or that replicating virions persist. This however does not explain why only the E2 response is specifically stimulated in the months following infection. More intriguing is the hypothesis that continued stimulation of E2 clones is driven by cross-reactivity to self determinants. Reactivities of E1- and E2-specific T cells have been reported to be involved in autoimmune disease in animal models (Yoon et al., 1991) and amino acid sequence homologies have been reported for E2 and the human proteolipid protein of myelin (Wolinsky, 1990). Direct evidence for crossreactivity of E2 specific antibodies to self constituents has remained elusive. Recent evidence has demonstrated that RV specific antisera crossreact with components of human synovial cells (Lund and Chantler, 1991). Therefore it would be of interest to investigate if the slow increase of anti-E2 IgG is associated with synovial tissue cross- reactivity. The analysis of clonal distribution of E2-specific B cells may support this hypothesis, as it would be expected that fewer clones would be detected in the late response if self-constituents were indeed responsible for the ongoing E2-specific antibody response.

### **3.3.5. Relative Affinity of IgG directed to RV and RV Proteins**

Sequential serum samples from seven individuals undergoing serologically confirmed primary RV infection were collected at time periods of 10-20 days, 60-90 days, 360 days and 720 days following the onset of a rubelliform rash. Relative affinities of these sera were determined at each time point for IgG directed to whole RV and the individual RV structural proteins. Mean IgG ER(%) values ( $\sim 1$  SD) are shown in Figure 35. Measurement of IgG anti-E1 affinity in acute phase sera showed initial low ER(%) values of 25% ( $\sim 4\%$ ) with subsequent development of intermediate affinity values of 52% ( $\sim 11\%$ ) at 3 months post-rash and final maturation to high affinity levels of 85% ( $\sim 7\%$ ) and 87% ( $\sim 3\%$ ) at 1 and 2 year intervals, respectively. In contrast, IgG anti-E2 and anti-C responses exhibited minimal increases over the two year post-infection follow-up period. In contrast relative affinity of anti-E2 and anti-C did not increase significantly ( $p > 0.3$ ) over the two year period investigated, with ER(%) values measured at 20 ( $\sim 8\%$ ) in the acute phase and 31% ( $\sim 8\%$ ) after 2 years post infection for E2 and 21 ( $\sim 10\%$ ) to 36% ( $\sim 8\%$ ) for C respectively. Measurements of IgG affinity to whole RV corresponded to the results obtained with purified E1 protein, with ER(%) values increasing from 23% ( $\sim 4\%$ ) in the initial

10 days post-infection, to 52% (~ 11%) at 3 months, to significantly ( $p < 0.0001$ ) elevated levels of 85% (~ 7%) and 87% (~ 3%) at 1 and 2 years following Rubella. Striking differences were noted in the maturation of IgG affinity responses to individual RV structural proteins. Whereas IgG affinity to the E1 envelope protein paralleled results obtained with whole RV, IgG responses to the E2 and C protein underwent minimal affinity maturation, remaining at low levels throughout the 2 year post-infection period. The affinity response detected in the anti-E1 IgG is supportive evidence that this protein is important in eliciting both neutralizing antibody responses (Waxham and Wolinsky, 1985a) and may represent the main target for the cell-mediated immunity also (Chaye et al., 1992). The basis for the immunodominance of E1 remains unclear but may reflect the ability of antigen-processing cells to present the individual viral proteins to the human immune system (Johansson et

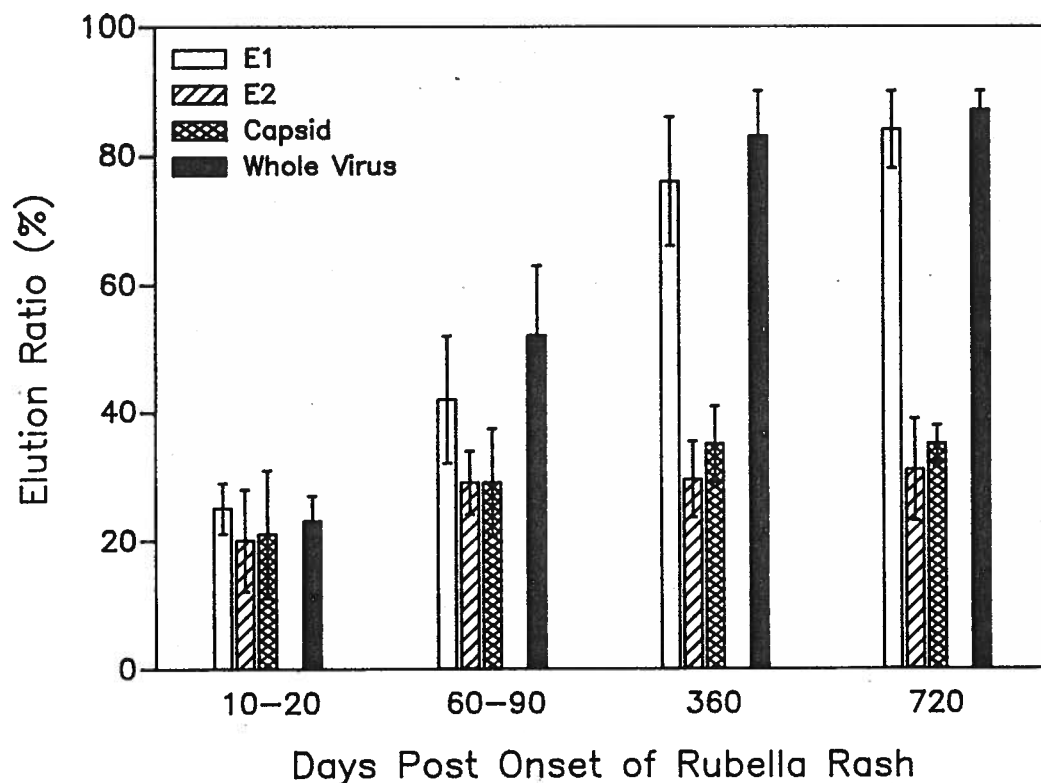


Figure 35. Differential IgG Affinity Maturation to the Structural Proteins of RV. Elution ratios of IgG directed to each of the viral antigens E1, E2 and C, as well as whole RV, were determined at sequential time intervals following diagnosis of clinical rubella in seven subjects. Each column represents the average ER(%) determination  $\pm$  1 SD for the seven patients. (From: Mauracher et al., 1992a)

al., 1987). The high level of glycosylation of E2 with N- and O-linked sugars, both containing sialic acid (Lundström et al., 1991), may be associated with increased resistance to proteolytic cleavage and subsequent reduced binding to the MHC class II complex (Sharon and Lis, 1982). In addition, both E2 and C may be less physically accessible within the intact virion. Capsid is an internal viral protein, while E2 may be masked by carbohydrate shielding (Klenk, 1990) and by virtue of its physical relationship with the E1 protein in the formation of surface spikes (Ho-Terry and Cohen, 1984). As it has been reported that B-cells specific for internal or shielded viral proteins receive less effective help from virus-specific Th cells (Scherle and Gerhard, 1989), it is possible that a lowered affinity of the E2 and C protein is due to decreased antigen-specific stimulation and Th support.

Alternatively, the low affinity response to E2 and C could be explained by protein denaturation and subsequent loss of antigenicity during purification procedures or during binding to the polyvinyl solid phase (Friguet et al., 1984). This possibility seems unlikely as it would be expected that E1, being most susceptible to denaturants, would most easily be affected by alterations of high affinity binding sites. Furthermore it was shown in a previous section that by a variety of techniques denaturation of the separated E1 and C proteins was minimal.

### **3.3.6 Summary and Conclusions**

The findings on the humoral responses to RV in adults are summarized below:

- L E1-Specific Response: the antibody response to this protein is immunodominant and significantly ( $p=0.005$ ) higher than antibody responses to E2 or C. The kinetics of the anti-E1 response is characterized by a rapid onset in the first several weeks following infection and is maintained in the years following Rubella. The affinity of these antibodies is high. Anti-E1 IgG reacts mainly to topographic epitopes (90%), with only 10% being reactive to linear epitopes. Both IgA and IgM are reactive to this protein.
- L E2-Specific Response: the antibody response to E2 is significantly lower than the E1-specific responses. Kinetic studies showed that the onset of these antibodies is slow, reaching maximal levels in the first year following Rubella. The IgG affinity remains low, anti-E2 IgG is reactive to both topographic and linear epitopes.
- L C-Specific Response: IgG responses against this protein are weakest and are transient. Peak reactivity occurs

in the months following Rubella and falls off in the years following infection. Similar to E2-specific responses the affinity of IgG directed to C remains low. Epitopes on this protein are not sensitive to denaturation and it appears that the majority of anti-C IgG is reactive to linear epitopes.

### 3.3.6.1 IgA Responses to RV Proteins

The Western blotting technique had as its primary advantage an increased specificity over immunoprecipitation and the ability to analyze the IgG-, IgA- and IgM-specific responses to RV structural proteins (see Fig. 22). In the investigation of the kinetics of the IgA responses of 33 male and 67 female adults over the years following RV infection it was observed that only females produced IgA antibodies specific to RV E2 (Fig. 36) (Mitchell et al., 1992). No significant differences between male and female were detected in the protein-specific responses of either IgG or IgM. The clinical implications of differences in the recognition of E2 by serum IgA between males and females remains unclear. It was speculated above that E2 may have possible cross-reactive epitopes with self-determinants and this taken together with the well-known increased incidence of RAA in females may suggest an involvement of the serological responses of E2-specific IgA in the initiation of joint inflammation following Rubella or RV immunization.

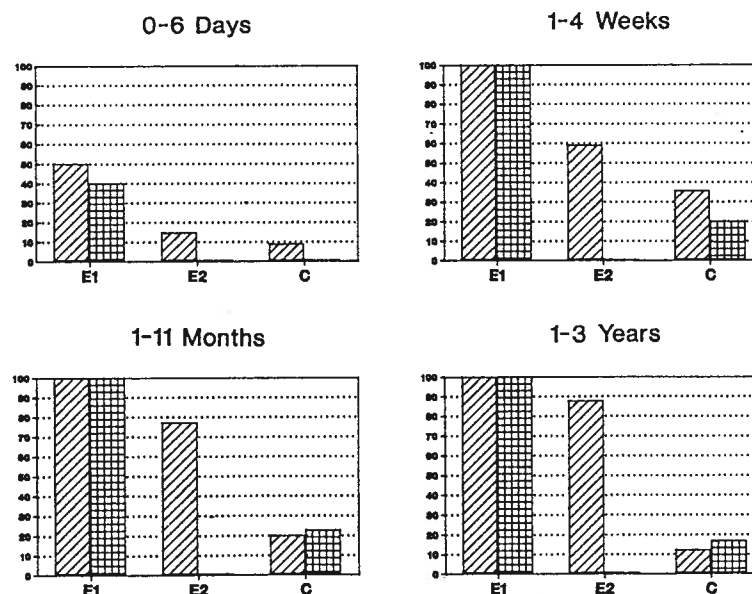


Figure 36. Kinetics of the Appearance of RV Protein-Specific IgA as Determined by Western Blot. The kinetics of appearance of E1-, E2- or C-specific IgA were determined in males (checkers) and females (hatched) patients. Sera were collected sequentially from each patient during the early acute (0-6 days post onset of rash), acute (1-4 weeks), early convalescent (1-11 months) and late convalescent (1-3 years) phase of rubella infection. Specificities were determined by class-specific immunoblot assays. Results shown are the relative percentages of female or male patients at each interval. (From: Mitchell et al., 1992a)

### 3.3.6.2.RV Protein Specific LymphoproliferativeResponses

Following the characterization of antibody responses to the individual proteins of RV it became possible to compare these to lymphoproliferative responses. The proteins obtained by preparative SDS-PAGE were of insufficient concentration when used by others in stimulation assays with human lymphocytes (Dr. D.K.Ford, Arthritis Society, Vancouver; Dr.D.Ou, Dept. of Pathology, UBC). A collaborative study was therefore initiated (Ms.H.H.Chaye and Dr.S.Gillam, Dept. of Pathology, UBC) to compare both B and T cell responses in 14 randomly chosen adults, without previous knowledge of their immune status to RV. IgG responses were analyzed by protein-specific ELISA and T cell responses were determined by using RV E1, E2, and C obtained from a vaccinia virus expression system in lymphoproliferative assays (Chaye et al., 1992). In the latter assay stimulation indices (SI) greater than three were considered significant. The comparison of the B- and T cell responses in these patients is illustrated in Figure 37.

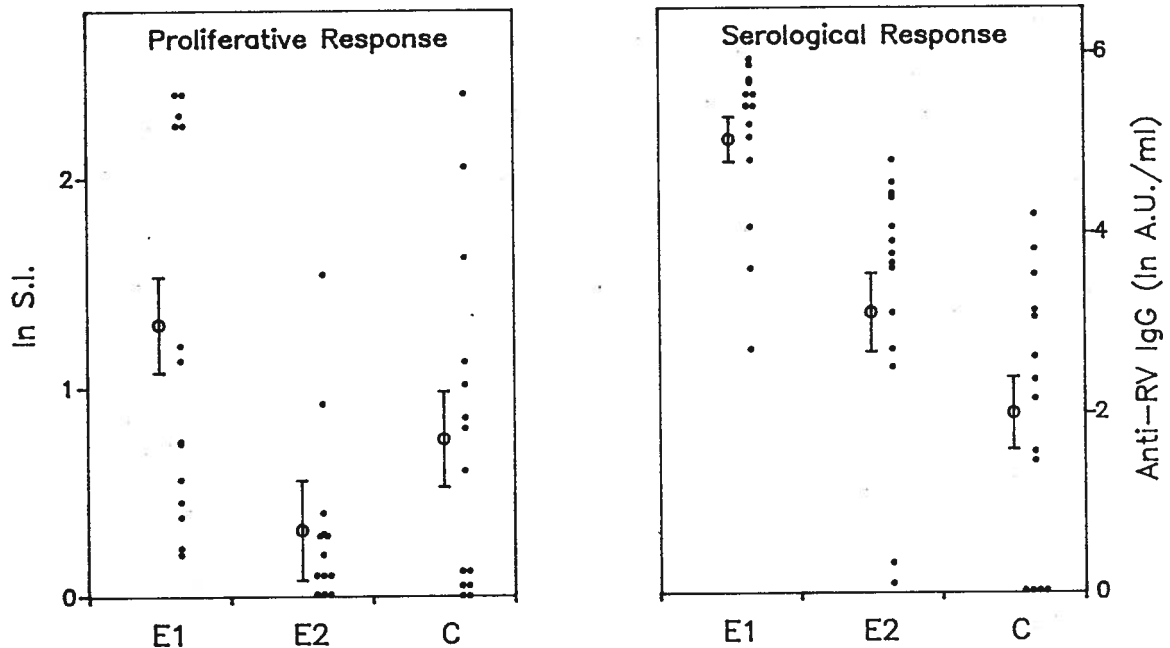


Figure 37: Comparison of Proliferative and IgG Responses to RV Structural Proteins. Values of stimulation indices (SI) for the proliferative responses and A.U./ml for the protein specific IgG responses are on a natural log scale. Error bars indicate ~ the standard error. Proteins used in the serological study were obtained by preparative SDS-PAGE from whole virus, whereas proteins used in the lymphoproliferative assays was derived from cloning RV protein genes into vaccinia virus expression vectors. (From: Chaye et al., submitted)

The comparison of the RV-specific serological and lymphoproliferative responses in this population showed similarities in their protein specificities. The response to E1 was immunodominant in both responses over the reactivity to E2- and C-specific responses, again suggesting that the response to the E1 protein plays a paramount role in the induction of protective immunity.

These data suggest that the E1 protein may be the best candidate for developing subunit vaccines for RV, as it gives rise to both a vigorous antibody and lymphoproliferative response. Investigation of the precise regions of E1 that are involved with neutralization and hemagglutination have been reported to lie between aa residues 244 and 300 of the E1 protein (Terry et al., 1988). Synthetic peptides constructed from this and other region's primary sequence are currently being investigated as potential B- and T cell recognition sites and may lead to the assignment of dominant T-cell epitopes as well as the sites of continuous B-cell epitopes on E1. This approach may ultimately identify small regions from the E1 protein which can be used to induce a protective immune response to RV, thereby avoiding RV epitopes which may be involved with adverse vaccine side effects.

Observations concerning the sensitivity of the E1 protein to denaturation by both detergents and reducing agents have revealed that, by far, the majority of the E1 and E2 epitopes are topographic and structurally-dependent. The use of native antigen will therefore be an important consideration in the development of sensitive immunoassays using bio-engineered RV proteins. The development of expression systems allowing the large scale production of pure, correctly folded and glycosylated RV E1 is currently under investigation at many centres. The use of baculovirus as an expression vector for the production of cloned RV E1 and E2 in *Spodoptera frugiperda* cells has been reported (Oker-Blom et al., 1989; Seto et al., 1991) and is proposed to provide a more economical alternative to the use of whole RV virus in immunodiagnostic assays (Seppänen et al., 1991).



### **3.4. Section IV: RV-Specific Humoral Immune Responses in CRS Patients**

Congenital Rubella Syndrome (CRS) patients and their immune status have been investigated since the initial description of this syndrome in 1941 (Gregg, 1941). Although this manifestation of RV infection has been dramatically reduced in the developed world by the implementation of a successful vaccination programme, and has therefore lost much of its clinical urgency, the disease remains of interest as a model for viral persistence and tolerance induction. The infection of the human fetus with RV early in gestation, clinically presenting as CRS, leads to long term persistence of the virus in these individuals. Virus persistence is thought to result from RV-specific tolerance although little direct evidence has been presented to support such a mechanism for continued viral replication. It has remained a paradox why CRS patients mount a humoral and cellular response yet remain incapable of clearing the virus. To explain viral persistence, the affinity of anti-RV IgG (Fitzgerald et al., 1988), HAI activity (Cooper et al., 1971) or humoral and cellular responses to the individual RV structural proteins have been investigated (Katow and Sugiura, 1985; de Mazancourt et al., 1986; Chaye et al., 1992), in an effort to define a partial tolerant state to RV arising from either selective anergy of neutralizing IgG, clonal deletion of T-effector cells, lowered concentrations of E1 specific antibodies or the inability to produce a high affinity antibody response. RV-specific antibody responses of CRS patients were compared to those of normal adult control patients, described in Section III. The observed differences in how CRS and Control patients responded to RV support the hypothesis that intrauterine RV exposure has led to virus-specific tolerance in CRS patients.

#### **3.4.1. RV and RV-protein Specific ELISA**

CRS patients (n=15) and adult Rubella control patients (n=15) were compared for their quantitative IgG responses to whole RV and the separated RV structural proteins (Fig. 38). Whole RV-specific IgG antibody levels as measured by whole RV ELISA were 202~65 IU/ml (X ~SD) for the control group, whereas the CRS group demonstrated lower levels at 124~55 IU/ml. However, these differences were not statistically significant at the  $p > 0.05$  level. No differences in the responses to the E2 and C protein were determined between the CRS and control group but significant differences were observed in the E1-specific responses. In the control group,

the majority of RV-specific IgG antibody was observed in the anti-E1 response (208~115 AU/ml) which was greater than either the E2- or C-specific IgG response. In the CRS group, the anti-E1 IgG levels were considerably lower (47~25 AU/ml) and significantly lower ( $p=0.004$ ) than those measured in the control group.

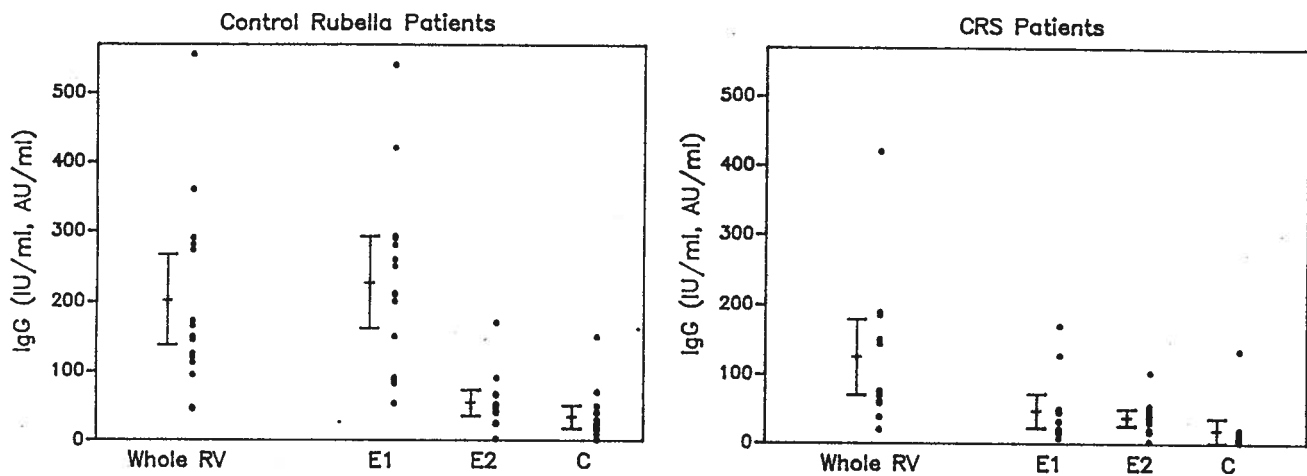


Figure 38: IgG Responses to RV and RV Proteins in Control and CRS Patients. Results are expressed in IU/ml for whole RV-specific IgG and AU/ml for RV protein specific IgG. Error bars indicate ~ 1 Standard deviation around the mean. Values for individual patients are also shown (•). (From Mauracher et al., 1992c)

The RV-specific immune response of CRS patients in this group would normally be considered to be protective, as it was higher than 15 IU/ml of anti-RV IgG, the suggested cutoff value for the definition of protective immunity (WHO, 1971). Yet RV is known to persist in CRS patient populations and RV reinfections have been reported in school aged CRS patients (Hardy et al., 1970), which has been taken to be indicative of an underlying defect in the RV-specific response in these patients. The selective loss of E1 antibodies has been previously described in a small CRS population using immunoprecipitation techniques (Katow and Sugiura, 1985). A subsequent study by others (de Mazancourt et al., 1986) did not report such a decrease in E1 specific

IgG. However, as the CRS patients studied were considerably younger (< 1 year), maternal IgG may have interfered with determination of autologous RV-specific IgG. Furthermore, no steps were taken to correct for the unequal percent distribution of methionine in the RV structural proteins when <sup>35</sup>S methionine labelled preparations were used in their immunoprecipitations. The above study confirms Katow's and Sugiura's findings and suggests that CRS patients have significantly reduced levels of E1 specific antibodies. Although anti-E1 levels were reduced, the majority of CRS patient did have circulating anti-E1 IgG. Therefore the biological activity and affinity of these antibodies was investigated further.

#### **3.4.2. Biological Activity of Sera in CRS Patients**

HAI antibody levels were determined in sera from 11 CRS patients ranging in age from 1 to 27 years of age and were compared to those of control patients described in section III. The median HAI titre of the control group was 1:64 and compared to a median titre of ( 1:8 in the CRS group. HAI values in the CRS patient group did not exhibit a normal distribution and the two populations were therefore compared using non-parametric statistical analysis (Mann-Whitney U test) for the  $1/\log_2$ . The HAI titre showed significantly lower titres in the CRS group as compared to the control group ( $p=0.003$ ). A lack of biological activity of the anti-RV IgG response in CRS patients, as measured by HAI, has been previously described (Cooper et al., 1971; Ueda et al., 1975). Whether this correlated with a concomitant lack of *in vitro* neutralizing antibodies was not determined. In the few CRS patient samples for which both NT and HAI tests were performed in the laboratory, the two tests gave coinciding results, with negative HAI titres correlating to negative NT tests. However it has been demonstrated by the use of monoclonal antibodies, that HA and neutralizing domains do not necessarily represent the same epitopes (Brush, 1988; Waxham and Wolinsky, 1988). A possible mechanism for the lack of HAI activity of the E1 specific IgG in CRS patients is a lack of high affinity antibody. In many CRS patients it was observed that partial HAI was occurring, similar to the soft radial hemolysis pattern described for low affinity anti-RV IgG by the Radial Hemolysis typing test (Hedman et al., 1989). HAI titre determinations have been criticized because of inaccuracy in the two-fold dilution steps, and it has therefore often been suggested that any given HAI titre should be interpreted as a range, plus/minus one dilution from the determined HAI titre. Statistical analysis of HAI titres are nevertheless valid as the variance observed in

any given population reflects both random- and measurement error.

### 3.4.3. Relative Affinity of RV Specific IgG in CRS Patients

Affinity indices were determined for IgG directed to whole RV as well as to the separated structural proteins E1, E2 and C in both CRS- and a control group patients (Fig. 39). Serum from adult control group (n=5) showed high affinity IgG directed to whole virus and E1, but had significantly lower affinity of IgG directed to E2 and C ( $p < 0.001$ ), correlating with the data presented earlier in Figure 35. The CRS patient population was subdivided according to age into a child (n=8, age range 1-4 years) and adult (n=7, age range 17-34) groups.

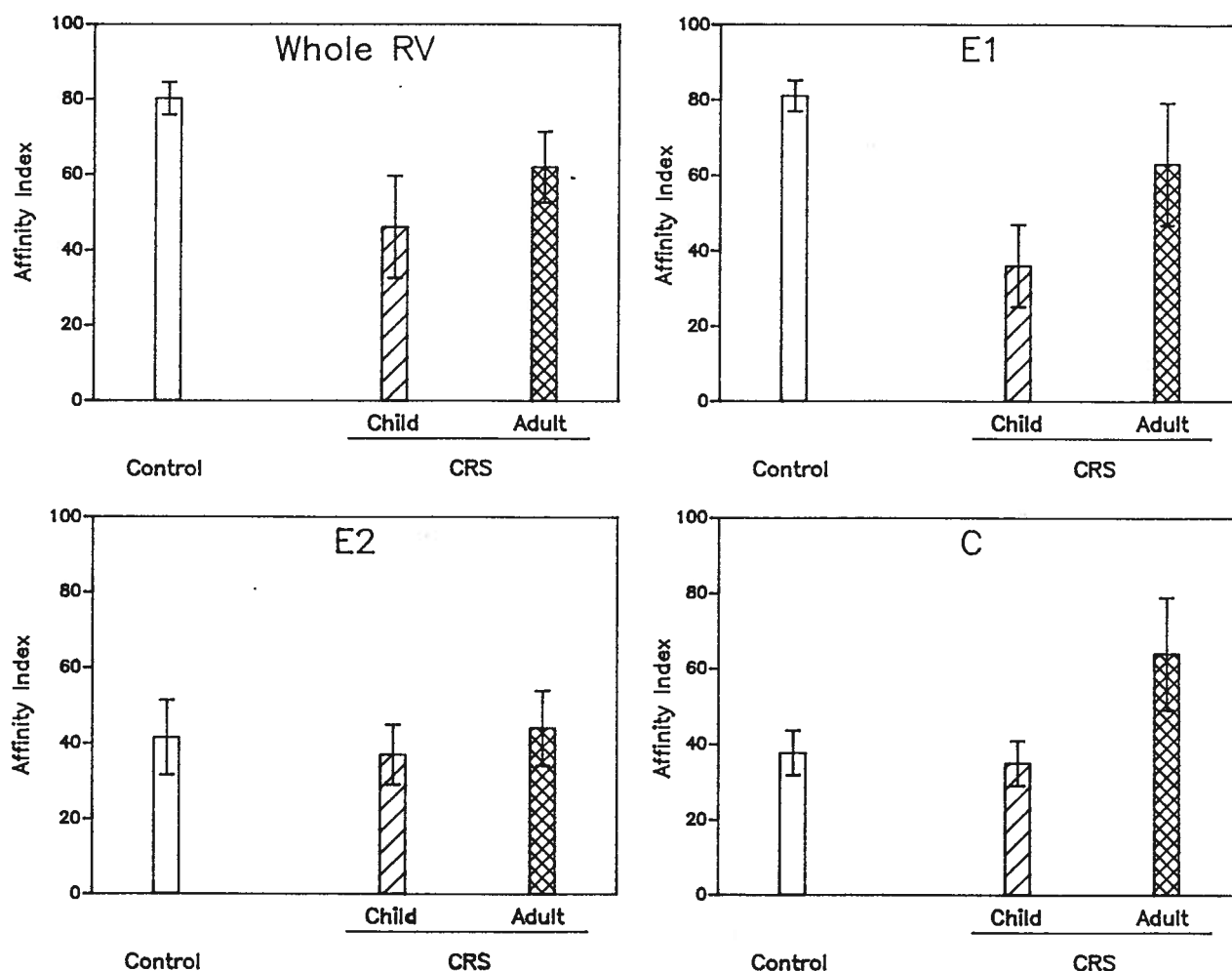


Figure 39. Affinity Indices of IgG directed to RV and RV Structural Proteins in CRS and Control Patients. 15 adult control patients in the convalescent phase of Rubella (white bars) were assayed on affinity ELISA. Results are expressed as the affinity index on the y-axis. A CRS population of 15 patients was divided into childhood group (hatched bars, n=8) and adult groups (cross-hatched, n=7). Error bars are ~SD.

The latter group was age-matched to the control group. The affinity of IgG to whole RV was observed to be significantly lower in both CRS groups ( $p=0.001$ ) as determined by one-way ANOVA. The affinity of anti-E1 antibodies also was significantly reduced ( $p=0.0001$ ) in both CRS groups if compared by ANOVA. The Fisher LSD test also showed significant differences in the means of both the child and adult IgG anti-E1 affinities when compared to the control group. No statistically significant differences were observed in the low affinity response to E2 in any study group ( $p=0.36$ ). ANOVA analysis of the C-specific IgG affinity showed significant differences in the populations ( $p=0.0001$ ). By using post-hoc analysis it was shown that the mean affinity of anti-C IgG in the adult CRS patients was significantly higher than the affinities of either the child CRS- or the control patient's anti-C IgG. No differences were detected in the affinities of anti-C IgG of child CRS- and control patients.

Antibody affinity is an important functional parameter of the humoral response and the inability to switch from low to high affinity antigen-specific responses has been an observed feature of immunological tolerance in animal models (Steward and Steensgaard, 1983). The inability of young CRS patients to produce high affinity IgG to E1 was marked. Age may be a factor in the affinity maturation but it has been recently shown that infants at age one can produce high affinity IgG (O.Meurman and K.Hedman, manuscript in preparation). The adult CRS patients of this study group appear to have partially regained the ability to produce higher affinity IgG to E1 and interestingly were observed to produce high affinity IgG to C protein. It is conceivable that continued antigen stimulation by persistent RV may have facilitated the maturation of the IgG responses in this patient group. The observed decrease in both quantity and affinity of the E1-specific IgG in the CRS population may well be responsible for deficient antibody-mediated viral clearance mechanisms.

#### **3.4.4.Reactivity to Linear and Topographic Epitopes in CRS Patients**

The presence of 2-mercaptoethanol reduces much of the antigenicity of the envelope proteins E1 and E2 but not that of C, by the destruction of epitopes dependent on tertiary protein structure (Mauracher et al., 1992a). In the analysis of CRS patient sera by reducing and non-reducing Western blots it was observed that these subjects were deficient in their ability to produce IgG reactive with reduced E1. In contrast no group differences were observed in the reactivities of E2- and C-specific antibodies in these two populations (Table 8). This observation provided the most consistent feature which differentiated the CRS population from control responders. In respect to the other antibody parameters measured (HAI, affinity, anti-E1 levels) some CRS selective non-responsiveness to linear epitopes in CRS patients in CRS patients has been further investigated

	Control Patients	CRS Patients	p-value
E1	10% ~ 4%	0.5 ~ 1.1	\0.001
E2	46% ~ 41%	31 ~ 40	0.1
C	125% ~ 83%	96 ~ 54	0.5

Table 8. Percentage of Signal Remaining on Western Blot under Reducing Conditions for Control and CRS Patients. Control patients (n=14) were compared to CRS patients (n=24) for the fraction of their RV protein specific IgG reactive to linear epitopes.

patients were always found to lie within the range of control responders; in respect to antibodies to linearized E1 (E1<sub>red</sub>), all CRS patients showed significantly reduced responses to linear epitopes in comparison to normal control patients. Immunoblot results of two representative patients are illustrated in the Figure 40. This

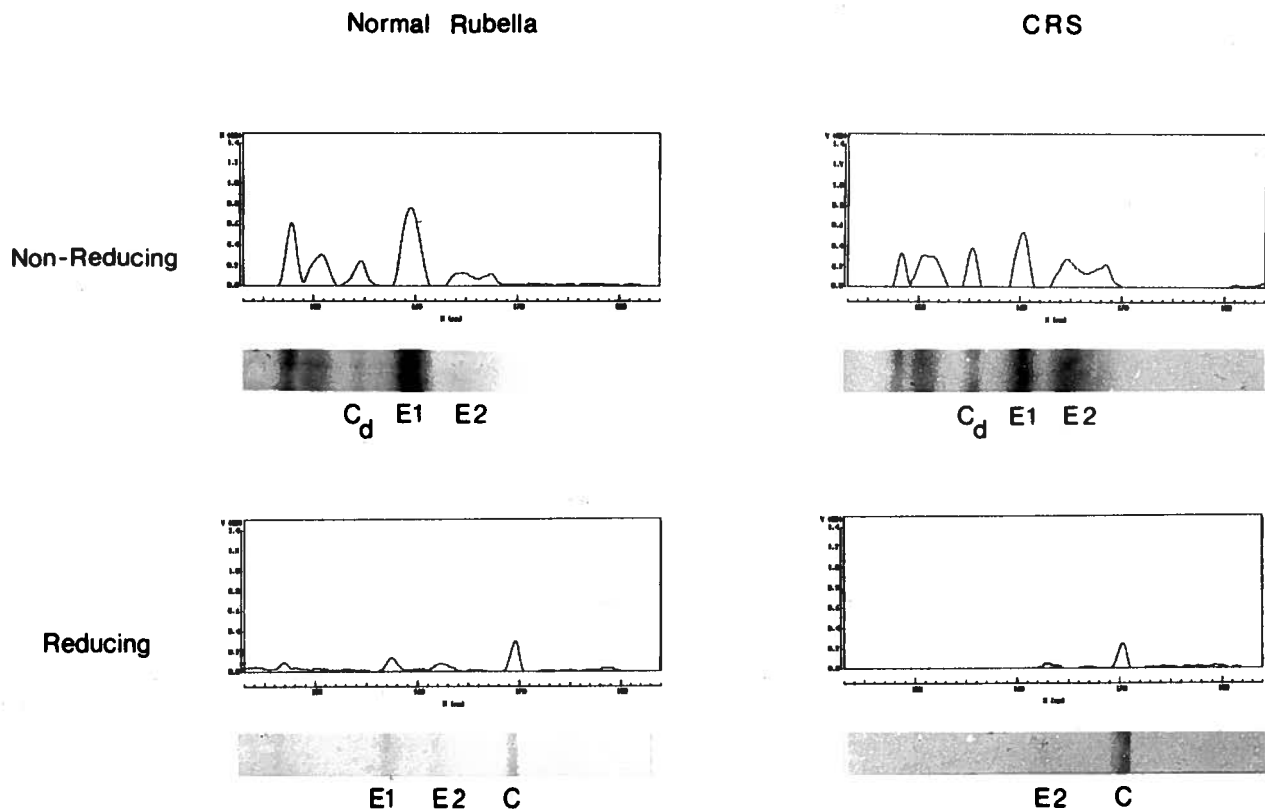


Figure 40. Western Blot under Reducing and Non-Reducing Conditions of Representative Control- and CRS Patients. Two blots, and their corresponding densitometric scan, are shown for a adult control patient and one CRS patient. Serum concentrations were equal for the reducing and non-reducing Western blots.

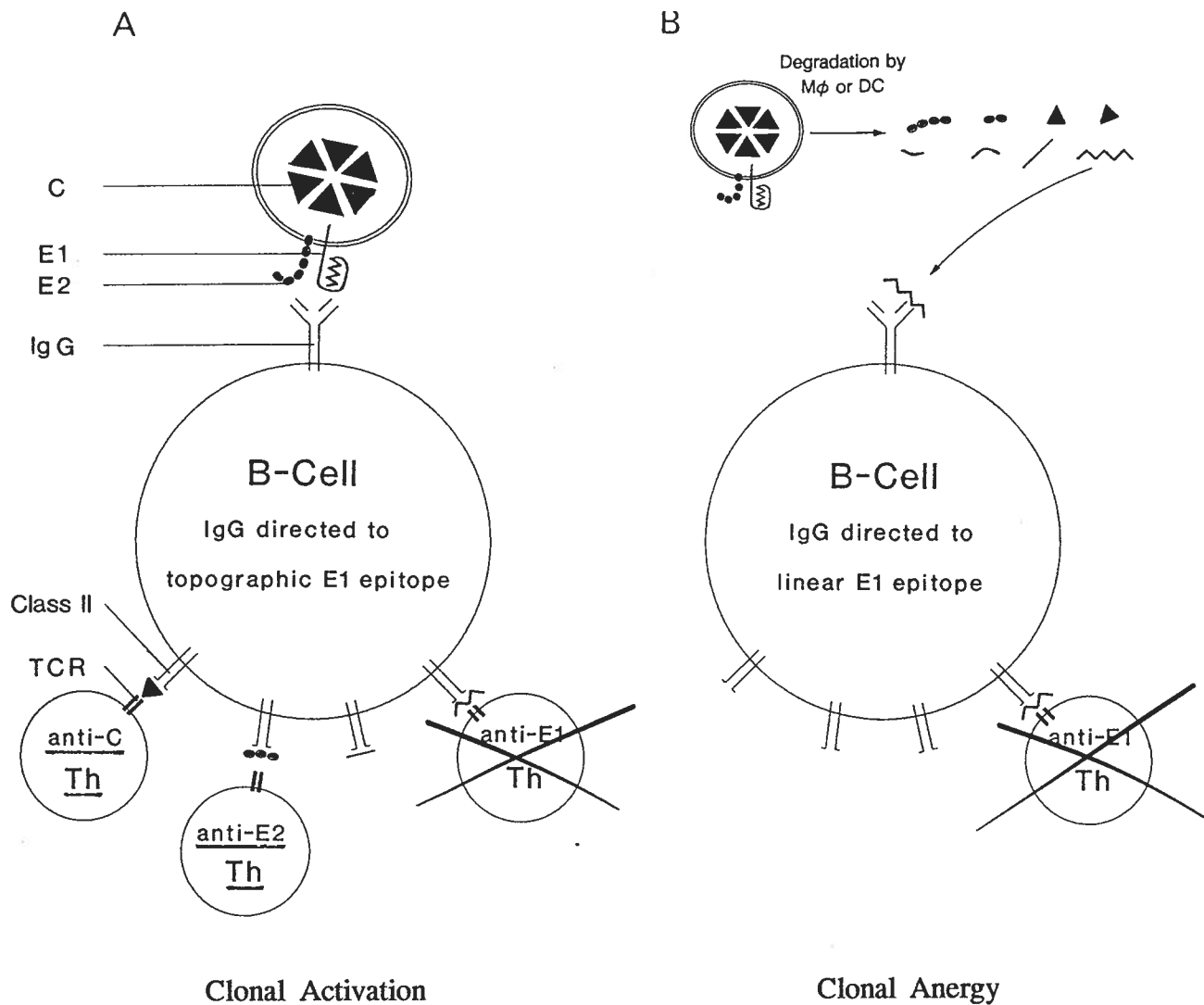


Figure 41. Model for the Production of IgG to Topographic Epitopes in the Absence of E1-Specific Th Cells. A hypothesis is proposed which explains the production the IgG response to topographic epitopes (A) and the lack of IgG responses to linear E1 epitopes (B) in the CRS population by a lack of E1-specific Th cells. Th cells to E1 are proposed to have been tolerized in this population (crossed-out anti-E1 Th) due to exposure of the fetal immune system to RV.

Scenario A depicts a particular clone reactive to a topographic epitope of E1. The IgG product of this clone will be detected on whole RV/E1 protein ELISA or non-reducing Western blot. As the IgG recognizes the intact E1, it will be capable of binding the intact virion encountered in the initial viremic phase. By phagocytosis and presentation of processed antigen in context of class II molecules, this clone will present peptides of all three structural proteins on its surface, and will in turn be able to receive the second activation signal by Th cells (Noelle and Snow, 1990) specific to the three viral proteins. The lack of anti-E1 Th cells does therefore not prevent clonal activation.

On the other hand, the scenario illustrated in situation B, depicts a clone producing IgG to a linear epitope on E1 (VVVY, partially or completely buried within the highly folded E1 protein). The most likely circumstance in which this IgG molecule will recognize its antigen is once the virus and its proteins have been degraded, as an outcome of local inflammatory responses or by exo-peptidases of dendritic cells in the germinal centres of the lymph node (Steinman, 1991, Liu et al., 1992). This clone will therefore bind partially degraded species of the E1 protein, and only present E1 peptides on its class II molecule. However, as Th cells to E1 are lacking, this clone will not receive any further stimulation and will remain in anergy. IgG directed to peptides or linearized E1 will therefore not be produced in patients lacking E1 specific Th cells. This model accommodates the serological findings in CRS patients and lends support to the hypothesis that interuterine exposure has led to immunological tolerance. (From Mauracher et al., 1992c)

by others using solid phase bound peptides representing the a sequence adjacent to the putative neutralizing and hemagglutination regions of RV E1 (Terry et al., 1988), between amino acid residues 213-239 of the RV E1. It was demonstrated that Rubella patients as well as RV vaccinated individuals showed reactivity to this peptide in the weeks following virus exposure (Mitchell et al., 1992b). The CRS patients on the other hand did not show reactivity to this peptide which again suggests the inability of this population to produce IgG to linear E1 epitopes (Mauracher et al., 1992b, Mitchell et al., 1992b). Is the non responsiveness of CRS patients to linearized E1 protein as well as E1 peptide based on an underlying deficiency of E1 specific Th cell responses and does this represent a specific tolerization of the E1 specific cellular response. If so, the ability of CRS patients to make IgG to conformationally dependent epitopes has to be explained. It is unlikely that these epitopes are T cell independent as E1 does not contain either multivalent epitopes nor are the carbohydrate moieties on this protein thought to be antigenic. A model to explain production of IgG directed to topographic epitopes of E1 in the absence of E1-specific Th cells is proposed in figure 41.

#### **3.4.5. Analysis of Sequential Serum Samples from CRS Patients**

Three CRS patients were retrospectively studied pre- and post MMR vaccination over a 2 to 6 year period. Results obtained following IgG immunoblot analysis of sequential sera from these patients are shown in Figure 42 and serological work-up is summarized in Table 9. The cord blood from patients A and B exhibited strong E1 responses in presence of both E2- and C-specific IgG as well as IgG specific to the reduced proteins; no cord blood was available for patient C. Cord blood contains maternal IgG at equal or higher titres than the maternal serum, and it was therefore not surprising that this sample contained reduced E1 reactive IgG. Following the first year of age, these patients lose the reduced E1-specific IgG and HAI antibody responses which become undetectable in all three patients. Patients A and B did not respond to vaccine, with antibody levels remaining similar pre- and post-MMR vaccine. Patient C was unique in that no RV-specific antibodies were detected in the pre-MMR vaccine sample and the post-vaccine samples contained E1-specific IgG only. However these antibodies were not reactive to linearized E1 nor did they show any HAI activity. Cord blood showed maternally derived HAI activity and high affinity E1-specific responses which were then replaced by endogenously produced IgG of lowered affinity with no HAI activity.



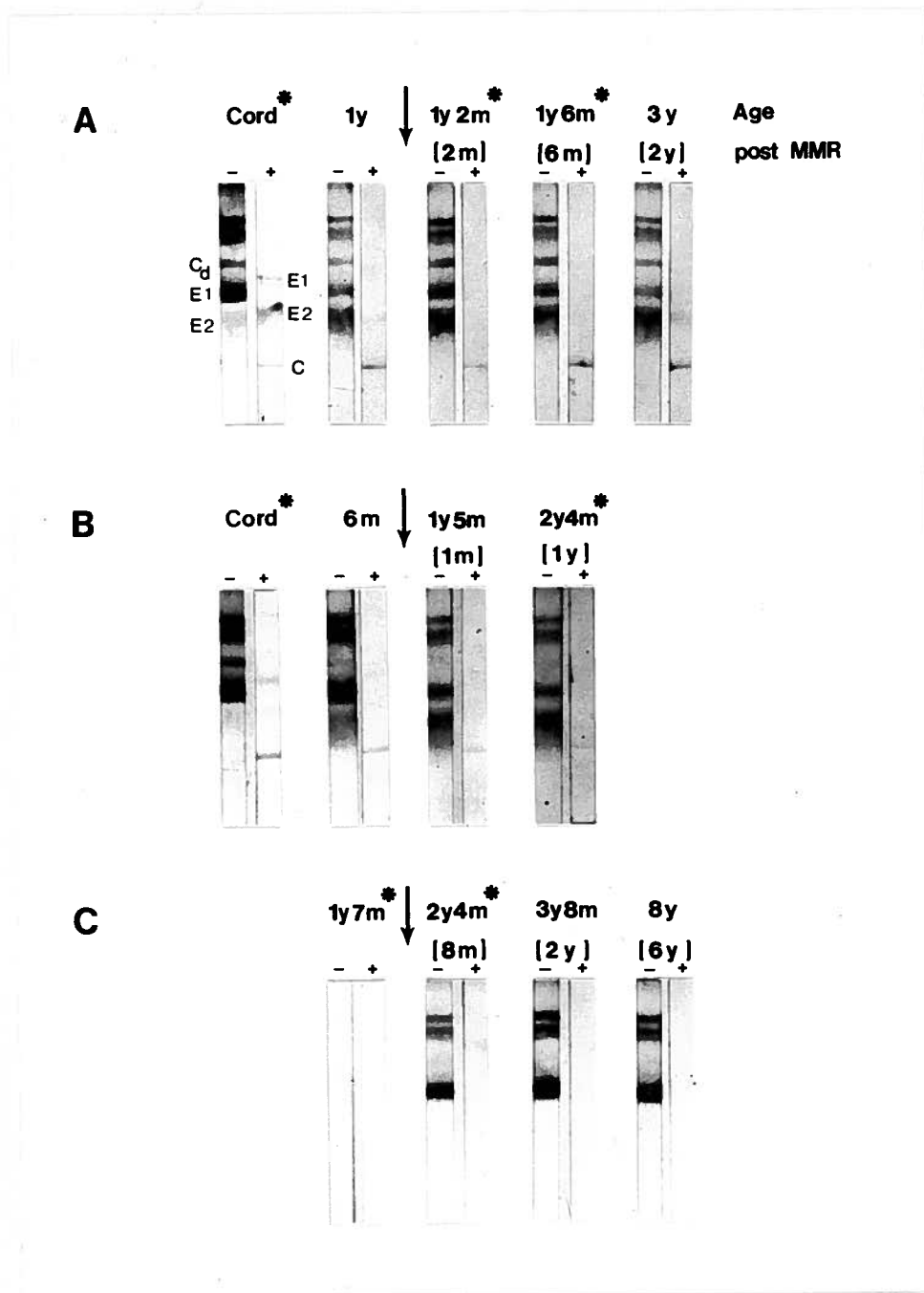


Figure 42. Western Blot Analysis under Reducing and Non-Reducing Conditions for Three MMR Vaccinated CRS Patients. Patients A, B and C were prospectively studied following MMR vaccine (arrow). A cord sample was available for patient A and B. Patient age is indicated on the top of each series, and time following MMR vaccination is indicated within square brackets [].

		Patient A			Patient B		Patient C	
Age :		Birth	1y 2m	1y 6m	Birth	2y 4m	1y 7m	2y 4m
Post MMR:			2m	6 m		1y		8m
Avidity Index	Whole RV	77.9	51	48	77	60	/	16
	E1	81	19	29	78	44	/	32
	E2	35	37	32	36	55	/	/
	C	34	32	37	33	26	/	/
IgG (IU/ml)	Whole RV	145	163	150	112	76	1	69
	E1	82	17	17	53	17	3	50
	E2	24	58	41	25	50	3	3
	C	17	19	20	10	5	1	1
HAI		1:32	1:8	< 1:8	1:32	< 1:8	< 1:8	< 1:8
anti-E1 <sub>red.</sub>		yes	no	no	yes	no	/	no

Table 9. Serological Examination of MMR Vaccinated CRS Patients. Patient samples in this table are indicated by an asterisk on the Western blots in Figure 42.

#### 3.4.6. Evidence for Th Tolerance in CRS Patients

An immunological mechanism for the prolonged persistence of RV in the CRS infant remains speculative. The virus is not cleared from fetal or infant tissues although maternally transferred IgG, as well as in some cases autologous IgM, can neutralize the virus *in vitro*. It is therefore likely that immunoglobulins are of little use in bringing a well established viral infection under control and that the cellular response is of primary importance. The cellular response plays a key role in preventing primary viral infections in the human, as demonstrated by clinical findings in X-linked agammaglobulinemia (XLA) and in congenital thymic aplasia (CTA) patients. XLA patients are deficient in IgG production but produce normal levels of T cells. These patients are usually diagnosed once their maternally derived IgG has been catabolized, late in the first year of life, resulting most often in the patient's susceptibility to chronic bacterial infections. These patient also have difficulties in clearing

enteric viral infections (ie: polio virus) but have little difficulty in terminating systemic viral infections (Saulsbury et al., 1980). On the other hand patients with complete CTA, incapable of producing T cell responses, often succumb to overwhelming viral or mycobacterial infections (Cooper and Butler, 1989).

If cellular responses are of prime importance then the long term persistence of RV in fetal tissues can be readily explained, as maternal T cells do not cross the placenta and fetal T cells responses are largely suppressed *in utero* and for the first months after birth (Papadogiannakis et al., 1990). The gradual decline in the shedding of RV in CRS patients over the first two years of life may be due to the initiation of the endogenous cellular response to RV. Although CRS patients have been shown to have a specific deficiency in their lymphoproliferative responses to whole virus (Fuccillo et al., 1973) others have reported that a non responsiveness to RV is strongly dependent on the time period of intrauterine exposure, with CRS patients who were exposed after 12 weeks of gestation showing near normal responses (Buimovici-Klein and Cooper, 1985). Non responsiveness of lymphocytes measured by whole RV proliferative assays remains unresolved, and the investigation of lymphoproliferative responses in CRS- and control patients using synthetic peptides representing RV proteins may help in a more definitive analysis of the T cell immunity.

In the comparison of the B- and T-cell responses of CRS and control patients to individual RV proteins, it was demonstrated that CRS patients showed strong lymphoproliferative responses to E2 and C, but were selectively deficient in their responses to E1 (Chaye et al., 1992). As these experiments were performed with vaccinia virus expressed proteins, the background response to vaccinia in some of these patient may have caused false positive responses, in spite of steps taken to correct for vaccinia-specific signals. Preliminary results of lymphoproliferative assays using 30 aa peptides representing the complete E1, E2 and C regions have shown that 3 out of 4 CRS patients are completely non-responsive to E1 peptides, whereas all CRS patients react to E2 and C peptides. Of the control responders, 12 out of 12 adult control responders reacted to peptides from all three RV proteins (Dr. Dawei Ou, Dept. of Pathology, UBC, personal communication). These observation support the above hypothesis that the selective antibody non responsiveness to linearized E1 or E1 peptides represents an underlying immunological tolerance of the T cell response to E1.

Antigenic sites recognized by antibody may be regulated by Th cells and has been described in detail for the murine response to staphylococcal nuclease or sperm whale myoglobin (Berzofsky, 1985). Cognate interactions

between B- and T cells are thought to regulate T cell-dependent antibody responses, with the activated Th cell providing the secondary signal to the B cell, via class II-CD4 interactions (Noelle and Snow, 1990). The resting B cell can bind antigen by surface IgG which is thought to provide the first signal for cell activation. Antigen is then phagocytosed, degraded and re-expressed on the cell surface in context of class II molecules (Tony and Parker, 1985). Th cells, recognizing peptides originating from the phagocytosed antigen, can then bind to the B cell and provide the secondary stimulus to induce  $G_0$  to  $G_1$  transition of the B cell with final progression of the B cell to antibody secretion being promoted by IL-4 and IL-5. The existence of this pathway of B cell activation is supported by observations of a hierarchy of help for B cells responding to internal or external antigens of influenza virus in adoptive Th cell transfer experiments using athymic mice (Scherle and Gerhard, 1988). In this model B cells expressing antibody to surface antigens (HA or NA) internalize whole virion and can be activated by Th cells specific to either internal or external proteins (M, NP, HA or NA). In contrast, B cells producing IgG to internal proteins (ie: NP) can only be activated by NP-specific Th cells. It is believed that the B cell will encounter NP only when virions have been degraded, the NA-specific cell will therefore only bind and phagocytose NP antigen. A more recent experiment has described responses of mice which have been made transgenic for the G protein of Vesicular Stomatitis virus (VSV) (Zinkernagel et al., 1990). If these mice are immunized with cloned and expressed G protein no antibody responses are mounted. However if whole VSV virus is used as the inoculum IgG responses to all VSV proteins, including G protein, are made. This experiment was designed to further support the hypothesis that B cell tolerance is regulated at the Th cell level, but also demonstrates that B cells, producing antibody to viral surface structures, can be activated by Th cells with activities to other viral proteins.

A similar scenario is proposed to exist in CRS patients where the individual, *a primo* to the stimulation of the RV-specific IgG response, has lost E1-specific T cells as a result of intrauterine tolerization of the immune system. The model takes the observations made by Scherle and Gerhard one step further by differentiating not only between internal and external proteins of a virus but also between internal and external epitopes of the immune dominant viral protein (See Fig. 41).

### **3.4.7. Summary and Conclusion**

The responses of the humoral immune response in CRS patients was found to differ from the response of adult control patients in the following manner:

- L No significant differences in the IgG responses between CRS and control patients was detected when using whole RV ELISA.
- L When these two populations were analyzed by RV protein-specific ELISA, a significantly lower ( $p=0.004$ ) IgG response to E1 was demonstrated. No significant difference was shown in the E2- and C-specific response.
- L The affinity of IgG directed to whole RV or E1 was significantly lower in the CRS patients as compared to adult control patients.
- L The CRS patient population was characterized by their inability to produce IgG to linear E1 epitopes. IgG was produced to linear E2 and C epitopes. This is also reflected by the significantly reduced responses to E1 peptide (aa 213-239), as demonstrated by others.
- L The CRS patients showed significantly lowered HAI titres. It appears that the E1-specific antibodies present in this group are not biologically active, in the sense that lack HAI activity.
- L A comparative study of B and T cells responses revealed that both serological and lymphoproliferative responses to E1 are significantly reduced in this population, as shown by Chaye et al..

The observations on the RV-specific humoral and cellular responses of CRS patients identify this population to be selectively tolerant to the E1 protein of RV. As this protein appears to be immunodominant and provides the main target for both serological and lymphoproliferative responses it is hypothesised that viral persistence in CRS patients is the outcome of the lack of a major part of the T cell response. Similar dominance of one protein acting as the main T cell antigen has also been reported in other viruses [ie: influenza A (Wraith, 1987) and BHV I (Hutchings et al., 1990)]. The reactivity of cytotoxic T cells in Rubella and CRS patients has not been investigated but it is hypothesised that protein specificities of the Tc cells are similar to that of the Th cells measured by lymphoproliferative assays (Thomson and Marker, 1989; Ou et al., submitted). Why the E2- and C- specific T cells may still be present in the tolerized patient population remains speculative, but the same properties which make these proteins poor T cell antigens may also make them poor toleragens. Cross-reactivity of T cell epitopes of RV capsid or the RV encoded nonstructural (ns) protein with other common viral capsid

or ns proteins may also be possible. The capsid structure has been highly conserved in many RNA viruses and it may be possible that peptide sequences of RV C protein are shared with other capsid proteins or RNA binding proteins. However amino acid homologies between RV C protein and those of the related SFV and SV are between 10 and 20% only (Frey and Marr, 1988). These authors report that significant homologies exist in the nonstructural protein coding region for RV and that of many other RNA viruses. The ns proteins of RV have not been considered as T cell antigens but may potentially play a role in T cell immunity as is the case for DNA viruses (ie: EBNA in Epstein-Barr virus). An amino acid sequence homology search for C protein of RV in GeneBank has not identified any sequence homologies with other viral capsid proteins but has identified short stretches of homology with bacterial and viral RNA/DNA binding proteins. The possibility of anti-RV B-cell activation by cross-reactive Th cells may therefore be valid.

The finding of predominantly low affinity anti-RV IgG in the CRS group may also be taken as indirect evidence for T cell tolerance. In the absence of a strong Th cell response it may be conceivable that B cells do not obtain sufficient stimulation to undergo multiple replication cycles during which affinity maturation can occur. This may explain not only low affinity antibody but also the observed fall in anti-E1 IgG titres in the CRS patients.

Similarities exist in the description of tolerance in CRS patients and the perinatally LCMV infected mouse model. Murine LCMV membrane proteins contain conformationally dependent neutralizing domains (Wright et al., 1989), yet the anti-LCMV IgG in these perinatally infected mice was not capable of clearing the viral infection. In this model, ineffective virus clearance correlated with low Tc cell responses and low delayed type hypersensitivity, but is unrelated to the levels of *in vitro* neutralizing antibody or NK cells (Thomson and Marker, 1989). Although IgG does play an auxiliary role in the rapid clearance of virus in healthy animals (Cerny et al., 1988), the persistence of LCMV in susceptible mice is thought to be solely due to absent LCMV-specific Tc cell (Oldstone, 1989). Nevertheless, virus specific Th cells must be present as IgG production to viral proteins requires Th support - LCMV infected athymic mice only produce IgM specific to LCMV. Further it has been well established that in the neonatally infected mice IgG directed to LCMV proteins are of low affinity and are commonly present as CIC or as deposited as IC in the kidneys or other organs (Buchmeier et al., 1980). This response patterns shows striking similarities to the observations made on low affinity anti-RV IgG responses

(Fig. 39) and high levels of anti-RV IgG CIC (Coyle et al., 1982) in CRS patients. The protein specificity of the cellular response or IgG reactivity to linearized proteins have not been analyzed in LCMV tolerant mice and could reveal a similar scenario as in the CRS patients, where protective immunity may be dependent on the cellular response to a single protein.

The selective non responsiveness of CRS patients to linearized E1 in their serologic responses may have relevance to the interpretation and future development of immunodiagnostic assays for Rubella and other systemic viral infections. CRS patients are characterized by a persisting RV infection, yet are seropositive with commercially available solid phase immunoassays and therefore are interpreted to have "protective immunity". It is well established that neither the endogenously produced IgG nor the maternally derived IgG, having *in vitro* neutralizing activity, clears RV in the CRS infants. Therefore, it has to be questioned whether anti-RV IgG has a function in the clearance or the prevention of Rubella *in vivo*. It can be speculated that the only reason why the presence of IgG correlates with protective immunity in the healthy responders is because it reflects the presence of a functional T cell response. Serological assays for RV should therefore be designed to detect IgG which will correlate to the presence of Th cells specific to the E1 protein. As suggested by the model shown in Figure 41, responses to topographic and structurally dependent E1 epitopes may arise in the absence of E1 Th cells whereas IgG to linearized E1 are dependent on E1 specific Th cells. Therefore it might be more appropriate to use denatured E1 protein as the solid-phase antigen. Such an immunoassay would be capable of differentiating between the responses of control patients and the non-protective responses of CRS patients. Much effort is currently being invested to produce expressed E1 in native form, for example by the use of baculovirus expression systems (Oker-Bloom, 1989), as it is believed that IgG specific to the intact virion or its surface proteins are most relevant to protective immunity. Interactions of antibodies with the structurally dependent neutralizing domains of the virus leading to neutralization in the tissue culture well should not be directly extrapolated to be representative to protective immunity in the organism. This concept was also underlying the design of the experiments described in this thesis, where the biological function of purified proteins was assessed in order to prove that these proteins had maintained their native structure.

On the basis of the presented data, it would be reasonable to speculate that more specific serological RV assay, correlating better with protective immunity, would entail the use of recombinant E1, followed by

deglycosylation and cystine cleavage and blockage. A positive signal in such an assay will likely correlate well with functional T cell immunity and protection. Bacterially expressed RV proteins have been described and produced by independent groups (Londesborough et al., 1992; Dr.S.Gillam and H.Chaye, UBC, personal communication) and E1 has been expressed at high concentration in CHO cells (Dr.T.Hobman, University of California (San Diego), LaJolla, CA) and could potentially be employed in ELISA test using linearized RV E1 protein. The use of insect cell derived E1, used in native conformation for anti-RV IgG ELISA, may be an expensive way of measuring irrelevant IgG and the use of synthetic peptides, cyclized or not, may be a costly and elaborate alternative for arriving at a product providing similar result as the linearized E1 ELISA.



## SUMMARY AND PERSPECTIVES

The hypothesis that intrauterine RV exposure leads to the development of RV-specific tolerance was tested by a comparative analysis of the RV protein-specific antibody responses in CRS and control patients. In order to examine the antibody responses of these patient groups a series of techniques had to be developed or optimized. ELISA techniques using whole RV were improved by including non-ionic detergent, resulting in disrupted virions and leading to increased sensitivity of the assay. The introduction of buffers, containing heat denatured blocking proteins, further improved the performance of RV ELISA as well as ELISAs using other antigen systems (Mauracher et al., 1991b). For the determination of RV protein-specific antibody responses, both Western blot and ELISA using separated proteins were developed. Western blotting techniques were developed using non-reducing conditions (Mauracher et al., 1992a; Zhang et al., 1992). This approach provided a better alternative than immunoprecipitation and allowed the detection of IgG, IgA and IgM to the individual RV structural proteins. Furthermore it showed that SDS-PAGE under low temperature and non-reducing conditions did not selectively destroy the antigenicity of RV antigens. Preparative SDS-PAGE followed by electroelution was then employed to produce separated preparations of the individual RV structural proteins E1, E2 and Cd. That proteins retained their antigenicity and much of their structural integrity was shown as both separated E1 and C were demonstrated to have maintained their biological functions. Optimal conditions for coating these proteins onto ELISA microtitre plates were determined and pooled preparations of each protein were used to examine RV protein-specific IgG quantities and affinities.

IgG affinity assays were initially evaluated using whole virus preparations. Two techniques, the chaotropic elution ELISA and the one-well inhibition ELISA were examined in detail for the determination of relative average affinities of anti-RV IgG in human sera. The chaotropic elution ELISA using 8 M urea as a denaturant gave reproducible results and demonstrated affinity maturation of the anti-RV response following RV infection in adults. It was more applicable than the inhibition affinity assay as it was independent of RV-specific IgM and required less antigen, which was an important consideration once affinity assays were used in separated RV protein ELISAs. Biological activity of antibodies were determined by HAI, an assay which has long been considered the standard to which other RV serology assays are compared.

The separated RV proteins were analyzed for their structural integrity. This was felt to be important as the

majority of E1 and E2 epitopes had been shown to be structurally dependent (Mauracher et al., 1992b). As biological activity of proteins is generally dependent on structure, it was inferred that intact RV protein function would indicate structural integrity. The function of E1 protein is well defined and includes HA activity which has been localized to a defined region on the protein and is known to be sensitive to denaturation (Terry et al., 1988; Qui et al., 1992). The ability of separated E1 to compete for HAI IgG with native virions showed that the structurally dependent HA domain had remained intact in the E1 preparations. During the investigations of alternative methodologies for RV protein purification it was discovered that the C protein became detergent-soluble at pH below pH 5.5. This led to the further investigation of the solubility shift of this protein and resulted in the proposal of the intralysosomal uncoating mechanism of RV nucleocapsid (Mauracher et al., 1991a). This event is believed to be dependent on structural changes in the protein and has recently been shown to be applicable in SFV also, which has lead to the more broadly applicable proposal of intralysosomal capsid uncoating in the Togaviridae family (Schlegel and Kempf, 1992). Purified C protein was shown to have retained its biochemical property to undergo the low pH induced solubility change, indicating that at least one of this protein's biological functions had remained intact throughout the separation protocol. It may be of interest to investigate compounds which may interfere with this property of the C protein, such as the WIN compounds (Badger et al., 1988) which have been shown to inhibit the uncoating of picorna viruses. If low pH-induced uncoating is shown to be a common mechanism for RNA viruses which enter the cell by endocytosis such as Retroviridae or Flaviviridae, then such compounds may prove to become a viable alternative to anti-viral drugs based on nucleotide analogs.

Once the immunological techniques had been established, it became possible to investigate the RV-specific antibody responses of adults with uncomplicated outcomes of Rubella in comparison with those of the CRS patient group. Healthy RV responders predominantly produced antibodies directed to the E1 protein which were of high affinity and appeared rapidly following infection. Of these, on average 90% were directed to conformationally-dependent epitopes with 10% being directed to linear epitopes. The E2-specific response was interesting in that it exhibited slow onset with IgG increasing in quantity as late as one year following infection. It remains unclear if this indicated that RV antigen may persist in the lymph or if crossreactivity between RV E2 and other antigens or self-constituents exists. The affinity of both anti-E2 and C IgG was observed to remain low throughout the first several years following Rubella infection, whereas E1-specific IgG rapidly matured to

high affinity in the months following Rubella. This demonstrated that differential affinity to individual proteins of a pathogen existed (Mauracher et al., 1991) and indicated the importance of investigating the protein-specific immunity in the response to pathogens. The immunodominant role of E1 in the antibody response was also demonstrated in the lymphoproliferative response (Chaye et al., 1992), indicating that the E1 protein may be the most suitable candidate for the development of subunit vaccines.

The comparative analysis of humoral responses to RV between CRS and normal control patients showed that the CRS patient group had significantly lowered IgG responses to the E1 protein, as measured on RV protein ELISA. These results supported findings by others (Katow and Sugiura, 1986). The most prominent feature which differentiated the IgG responses of CRS patients from those in control patients was the selective inability of the CRS group to produce IgG to linearized E1. A model has been proposed which hypothesises that CRS patients have been selectively tolerized to the E1 protein of RV. This is reflected in serological responses in that IgG directed to linear epitopes is absent and the affinity of IgG to whole RV as well as E1 is low. Studies on the serological and cellular responses of CRS- and control patients to RV structural proteins suggest that the Th cell responses to E1 are significantly reduced in CRS patients (Chaye et al., 1992) and ongoing studies using peptides representing sequences of the RV structural proteins have provided further evidence that lymphoproliferative responses to E1 peptides are absent in most CRS patients whereas responses to E2 and C remain intact (Ou et al., 1992).

This thesis has provided evidence to support the hypothesis that intrauterine RV exposure can lead to immunological tolerance. Tolerance is proposed to be specific to the E1 protein and the absence of normal cellular and serological responses may explain viral persistence in the CRS patient. Viral persistence may also be involved in the pathogenesis of RAA and it now is possible to determine if a similar deficit exists in the cellular responses of patients developing arthritis following RV infection.

Models for tolerance induction in the human are rare and it is proposed that further studies on well defined CRS patients will contribute to the understanding of the development of the human immune system. With the availability of peptides from all viral proteins, the fine specificity of both the B and T cell response can be studied. Genetic background of the CRS patients was not taken into account and will always remain a variable in human studies and may be a complicating factor in the detailed T cell studies of CRS and control patients. The selective tolerance to E1 remains intriguing and a good explanation why E2 and C responses are still

present in the CRS patients has not been found. An answer to this question is important as it may teach us what characteristics make a membrane- or an internal protein good T cell antigens. Ultimately such answers may lead to more immunogenic vaccines or conversely they may provide us with clues how proteins can be treated to result in better graft acceptance.

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