ANALYSIS OF THE STRUCTURAL PROTEINS OF RUBELLA VIRUS

By

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B.Sc. (Microbiology), University of Alberta, 1985

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pathology)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June 1988

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ABSTRACT

Complications of rubella virus infection, including congenital rubella syndrome and the association of rubella virus with joint inflammation, emphasize the need for continued research on rubella virus. The finding that the association of rubella virus infection with joint manifestations is more pronounced with wild strains than with vaccine strains suggested the possibility of strain variation.

Several different techniques have been employed in order to compare six rubella virus strains and identify variations in their structural proteins. Differences in biological activities were detected, including the extent of virus production and the ability of various cell types to support replication of rubella virus (tissue tropism). However, the strains were shown to have remarkably similar electrophoretic patterns. Variation appeared to result from alterations in glycosylation. Efforts to isolate the protein components of the two envelope glycoproteins were unsuccessful, and it was therefore not possible to localize variation to either the protein or the carbohydrate components. Future work employing more sensitive methods for examination of fine molecular structure and the correlation of these structures with biological activity will further our understanding of the pathogenesis of rubella virus infection.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr. J.K. Chantler, for the opportunity to carry out this project in her laboratory, and for her generous support and guidance throughout the course of this study. I would also like to thank the members of my supervisory committee, Drs. J.B. Hudson, G. Krystal, W.R. McMaster, P.E. Reid and G. Weeks for their guidance and helpful suggestions. My thanks also go to Ms. Karen Lund, Ms. Georgia Tai and Mr. Malcolm Barth for helpful discussions.

I would also like to express my profound gratitude for the never-ending support of my parents and family.

INTRODUCTION

I. Rubella virus - Molecular Biology

Rubella virus (RV) is a small enveloped virus in the family Togaviridae and is the sole member of the genus Rubivirus. Electron microscopy shows it to be a roughly spherical particle, 50-70 nm in diameter with a 30 nm electron-dense core. This particle is surrounded by a lipid-containing envelope, acquired by budding through the host cell membrane, into which virus-specific glycoproteins have been inserted (59,151,154). The virus has a single-stranded, positive-sense (infectious) RNA genome of about 10 000 nucleotides (47,159) or about 3.5×10^6 daltons (89,154). The genomic RNA sediments at 40S and contains both a 5' methyl cap structure and a 3' poly(A) tract (107). A subgenomic capped and polyadenylated mRNA species, which hybridizes by Northern blot to the 3' portion of the 40S genomic RNA, also exists in infected cells (107). This subgenomic RNA of approximately 3500 nucleotides (47,159) is translated into a 110 000 molecular weight precursor polyprotein (p110) which is co-translationally cleaved into the three structural proteins of rubella, the envelope proteins E1 and E2, and the core protein C. The gene order of these three proteins on the polycistronic subgenomic mRNA species has been shown to be NH_2-C-E2-E1-COOH (105).

A review of early reports on rubella virus, all dating from the

1970s, revealed disagreement in the number and size of rubella virus proteins, with different investigators describing from three to twelve virus-specific proteins produced during infection (15,89,154,156). This lack of consistent data on the number of structural proteins and their molecular weights may in part have been due to difficulty in producing high viral titres and purifying intact rubella virions. Furthermore, while most of the early investigators studied preparations of purified virus, one report (15) was a study of intracellular virus. Since infection by rubella virus does not inhibit cellular metabolism (15,59,154), it is probable that the true number of viral proteins was masked by host proteins.

More recently, consensus has been reached that the virus consists of three structural proteins, the C or core protein, which is an unglycosylated protein of about 33k daltons, and two glycosylated envelope proteins, E1 and E2. E1 has a molecular weight of about 58k daltons, while E2 has been variously reported as a series of two (76,107) or three (163) closely related glycoprotein species with molecular weights of about 42-48k daltons. Peptide maps generated by cyanogen bromide cleavage of E2 species appeared to differ by only a single peptide (161), while other peptide mapping furnished similar maps, but different isoelectric points (65). The consequent suggestion that the same polypeptide exists in more than one charged form is supported by tryptic peptide analyses (105) and by partial amino acid sequencing (76), which showed the E2 species to have identical carboxyl-terminal structures. Together with the fact that the coding capacity of

the subgenomic 24S RNA is sufficient for only one E2 polypeptide, these reports confirm that the E2 species have an identical apoprotein moiety and that the various forms are the result of heterogeneous processing.

II. Structural Organization of Virion

Waxham and Wolinsky (162) have modelled the structural organization of the proteins in the rubella virion from results of gel electrophoresis under non-reducing conditions. Results generated by two-dimensional SDS-PAGE showed that the E1 glycoprotein exists in three distinct species, in a monomeric form (E1), as a disulphide-bonded E1-E1 dimer with a relative molecular weight of 105k daltons and as a 95k dalton disulphide-bonded E1-E2 heterodimer. Non-reduced E2 was shown to occur almost exclusively in the E1-E2 heterodimeric form, and C was reported to exist only in a dimeric form of 78k daltons, associated with the viral genome.

The structure of the rubella virion appears to be unique in the Togaviridae family, as the relative proportions of E1, E2 and C are not equal (162). The model therefore describes the central core of the virion as an association of multiple dimeric units of the C protein with the genomic RNA to form an icosahedral structure (35,162). This capsid structure then acquires an envelope into which repeating hexamers, each composed of five E1 molecules and one E2 molecule, are inserted. It was proposed that one E1-E2 heterodimer is inserted in this hexamer, while the remaining E1 molecules remain as monomers or interact to form

homodimers. That the antigenic domain on E2 is generally inaccessible to recognition by antibodies may thus be explained by its position in the E1-E2 heterodimer of the hexamer (162). Furthermore, the variable accessibility of the multiple antigenic domains of E1 may be the result of E1 configurational changes which depend upon its three potential interactions (E1, E1-E1, and E1-E2).

III. Sequences of Rubella Virus Structural Proteins

The amino acid sequences of the E1, E2 and C proteins of the wild type M33 strain of rubella virus (23) and E1 (47,159), E2 (159) and C (141) of the Therien wild type strain together provide details of a unique togavirus.

The E1 and E2 glycoprotein genes are both preceded by a series of approximately twenty uncharged, mainly hydrophobic, amino acids (23,47,159), typical of signal sequences which direct the translocation of proteins through the endoplasmic reticular membrane. At the carboxy termini of both E1 and E2 lie similar sequences of hydrophobic amino acids which probably function as trans-membrane domains and anchor the glycoproteins to the lipid bilayer. Additionally, there are two long stretches of uncharged amino acids in the coding region for E1 (47) and three long hydrophobic regions in the E2 coding region (23). Both the E1 and E2 sequences are unusually G/C-rich, with a high number of proline and cysteine residues (23,159). The sizes of E1 and E2, as calculated from the amino acid sequences, are in good agreement with

those determined by gel electrophoresis for the unglycosylated forms (present in tunicamycin-treated cells) (47,159). The deduced amino acid sequence for E1 indicates the presence of three potential sites for N-linked glycosylation (23,47,159), while E2 has been reported as having either three (23) or four (159) potential glycosylation sites. The large shift in the mobility of E2 synthesized in the presence of tunicamycin suggests that all (three or four) potential glycosylation sites are filled (159), but whether all three potential sites in E1 are filled is still uncertain. The difference between the molecular weights of the glycosylated and unglycosylated forms of E1 is only 5000 daltons (159), although gel filtration studies by Frey et al. (47) showed that at least two of the sites are full.

The nucleotide sequences of the C protein gene show it to be unusually rich in C (41.6%) and G (31.2%) residues, but poor in A (15.4%) and U (11.8%) (141). Codon usage is non-random, with C and G residues preferentially found in the third position of all codons except those for glutamine (141). Regions with long stretches of up to 35% G or 45% C residues are found throughout the gene, with highest G + C content (80%) in the amino-terminal third. The C protein is strongly hydrophilic as well as rich in proline (14.1%) and arginine (14.4%) residues (141). Clusters of both amino acids are concentrated in the amino-terminal third of the protein (141), and this positively-charged region may be involved in binding the protein to the negatively-charged 40S genomic RNA in the viral nucleocapsid (23).

The amino acid sequences for E2 as determined by Clarke et al. (23) and Vidgren et al. (159) show good agreement for about half the sequence. The disagreement in the remaining half is likely the result of single nucleotide changes which could lead to a switch in frameshift. These changes may reflect strain differences, but could also result from the difficulty in sequencing this region because of its high G + C content.

The amino acid sequence of E1 as determined by Vidgren et al. (159) differs from that determined by Frey et al. (47) in only three positions, all due to single base differences which may have accrued from errors in sequencing or from mutations in the Therien strain of rubella virus acquired during cultivation. Comparison of the sequences of the M33 strain C gene (23) and the Therien strain C gene (141) reveals five single amino acid changes that probably reflect strain differences. Three nucleotides at various positions in the Therien sequence appear to be absent in the M33 sequence, resulting in a different amino acid sequence because of frameshift alterations. However, this may be an artefact that illustrates the difficulty of sequencing the C gene because of its high G + C content.

A comparison of the nucleotide and amino acid sequences of the rubella virus E1, E2, and C proteins reveals no homology with other alphaviruses, including Semliki Forest virus and Sindbis virus (141). The overall base composition of the coding regions, with their high G + C content, is very different from the alphaviruses, and is unique among

RNA viruses. The unusual codon usage, which differs markedly from that generally observed in human cells, may affect the rate of translation by contributing to a shortage of specific isoacceptor tRNA species (141). This may indeed also explain the relatively slow production of rubella virus particles in cell culture.

IV. Monoclonal Antibody Studies

Monoclonal antibodies (mAbs) have been used to isolate regions of the structural polypeptides of rubella virus that bear on the infectivity of the virus. Once identified, these specific regions may be amino acid sequenced, an essential step toward producing a synthetic, non-replicating subunit vaccine for rubella virus.

Numerous recent studies have attempted to define epitopes important for infectivity. Most of the mAbs produced to date by various investigators recognize E1, which implies an immunodominant role for this protein as an antigen, with at least three distinct epitopes having been defined on E1 (52,68,69,153,163). All of the studies have located the haemagglutination site in E1, and neutralizing epitopes have been located in E1 as well as E2 (35,52,68,69,152,161,163).

As well as defining functional epitopes, monoclonal antibodies against E2 have also been used to immunoprecipitate a precursor to the E2 glycoprotein from infected cells (163). This precursor is smaller and lacks the molecular weight heterogeneity of E2 found in the virus,

further supporting the suggestion that post-translational modifications, including association with E1, account for the multiple forms of E2 present in mature rubella virus and reported by so many investigators.

Although several reference strains of rubella virus exist, it appears that there is only one serotype (35,151). However, early morphologic studies demonstrated that rubella virus strains could be differentiated by their ability to form large or small plaques in rabbit kidney (RK) cell culture (43) and by differences in the kinetics of plaque formation. Strain differences have also been detected by neutralization tests, which revealed differences in rate constants for neutralization of six rubella virus isolates (51). Furthermore, although Ho-Terry and co-workers (67) found no difference between the electrophoretic patterns of a wild-type rubella virus strain and the RA27/3 vaccine strain by polyacrylamide gel electrophoresis, the strains could be distinguished by competitive radioimmunoassays. These results suggested the existence of strain-specific antigens, a view supported by the detection of strain-specific epitopes (35) on the E2 glycoprotein of all tested strains.

V. Rubella-Associated Arthritis

Naturally-acquired rubella is a common childhood illness that manifests itself as a generally mild and self-limiting infection characterized by fever, coryza and malaise followed by an atypical rash consisting of round, slightly raised, discrete macules (151). Its

clinical importance, however, lies, firstly, in its ability to cause severe birth defects (congenital rubella syndrome) if a pregnant woman is infected particularly in her first trimester of pregnancy and, secondly, in its association with joint inflammation.

Arthritis associated with rubella virus infection was recognized as early as 1906 (109) and acute joint inflammation has since then been recognized as a common complication of both natural infection and rubella vaccination. Although normally transient and without sequelae, and generally with complete resolution of symptoms within two to three years, recurrent inflammation has, in a small number of cases, been reported for as long as five to fifteen years (17,18,137,147,148). In all these cases, continuing involvement of the virus is suggested by the presence of rubella virus in peripheral blood lymphocytes and synovial fluid. Rubella-associated arthritis (RAA) may also progress to a condition resembling rheumatoid arthritis, characterized by RF (rheumatoid factor)-positivity (91,94). However, in most instances, the inflammation is resolved before permanent joint damage results.

Although RAA has been described in patients of all ages, development of arthritis seems most often to be associated with adolescent and adult females. The incidence of joint inflammation also appears to be more frequent after natural infection than after immunization with the currently licensed vaccine strains HPV77/DE5 and RA27/3: a recent study (147) thus found that 52% of women who contracted natural rubella subsequently developed acute arthritis, as compared with

only 14% of immunized women. It has also been observed that the different vaccine strains (Cendehill, HPV77/DE5 and RA27/3, etc.) vary in their ability to produce joint manifestations.

Isolation of rubella virus from joints affected by inflammation has been reported after both natural infection (60) and immunization (104,166). In a large number of cases, rubella virus has also been isolated from peripheral blood lymphocytes of patients with rubella-associated arthritis (18, Chantler, unpublished). This suggests the possibility that the virus persists at sites other than joints, and that the inflammatory response is the result of viral reactivation which produces immune complexes that accumulate in the joints (45). This hypothesis is supported by an ability to detect rubella-specific immune complexes for up to eight months following rubella vaccination, with an associated higher incidence of joint symptoms (158). Immune complexes have also been implicated in acute rubella-associated arthritis, as suggested, firstly, by a temporal association of the appearance of anti-rubella virus antibodies with the onset of joint symptoms (25,104) and, secondly, by the higher levels of circulating immune complexes in symptomatic patients (158). However, association of elevated levels of immune complexes with development of acute arthritis after rubella immunization was not confirmed by one study (133).

Rubella virus has also been advanced as a cause of chronic inflammatory arthritis of unknown etiology (29,54,57). The evidence for this is twofold: firstly, that rubella virus can persist in humans for

long periods in congenital rubella syndrome, and secondly, the recognized frequency of arthritis associated with acute rubella virus infection and the prolonged occurrence of rubella arthritis in some patients. Several reports have described rubella arthritis progressing to classical rheumatoid arthritis (91,94). Other investigators have isolated rubella virus from several cases of rheumatoid arthritis with no known association with recent rubella infection (16,44), and one study (20) has offered evidence for implicating rubella virus as an etiological agent in approximately 30% of juvenile chronic arthropathies. It has also been suggested that rubella virus may be the cause of a small proportion (<10%) of adult rheumatoid arthropathies.

VI. Correlation of Viral Protein Structure with Pathogenicity

Mutations accumulate spontaneously and very rapidly in the genomes of RNA viruses, and consequently make it difficult to correlate identified gene alterations with specific changes in biological properties (79). Studies of numerous viral systems have shown that changes in pathogenicity are commonly the result of alterations in viral protein structure. Both tissue tropism and virulence have been found to be altered by minor changes in the amino acid sequence of certain viral polypeptides, particularly envelope glycoproteins (82). Although pathogenicity is generally under multigene control (41), even a single amino acid substitution can, in some systems, render a virus avirulent. A correlation between specific nucleotide substitutions and altered viral virulence has been suggested for numerous viruses, including

rabies virus, polio virus and reovirus type 3.

In rabies the pathogenicity of different rabies virus strains for adult mice depends upon the presence of a specific antigenic determinant on the viral glycoprotein, and the ability to use neutralizing monoclonal antibodies for selecting nonpathogenic mutants of rabies virus makes it reasonable to assume that the pathogenic properties of the virus are indeed determined by the glycoprotein. Dietzschold et al. (33) analyzed tryptic peptides of the glycoproteins of the pathogenic parent virus and nonpathogenic variants, and then amino acid sequenced a specific tryptic peptide variant. This work established that the change in pathogenicity coincided with an amino acid substitution at position 333 of the glycoprotein molecule, resulting in the replacement of an arginine residue with either isoleucine or glutamine (33) or glutamine or glycine (130). While amino acid substitutions were also detected at other positions in the glycoprotein, these changes affected only neutralization, not pathogenicity. Thus, an arginine at position 333 appears to be essential for retention of pathogenicity. It has been suggested that the replacement of this arginine residue by another amino acid may cause a significant change in glycoprotein conformation.

Sequence analysis of poliovirus vaccine strains has yielded a similar correlation between specific nucleotide substitutions and altered viral virulence. Five base substitutions in the genome of poliovirus type 3 (P3/Leon/37) may be responsible for the attenuated phenotype of the Sabin vaccine strain (138), while reversion of the

Sabin type 3 poliovirus vaccine strain to a neurovirulent phenotype is consistently associated with a point mutation in the 5' noncoding region of the viral genome (39).

Work with reassortment mutants of influenza virus (which has a segmented genome) has identified the viral haemagglutinin as one of the major determinants of viral virulence. Although it appears that an optimal gene constellation, rather than a single virus gene, is responsible for the pathogenicity of influenza virus (8), infectivity of the influenza virus is directly related to the presence of the haemagglutinin in its cleaved form. The haemagglutinin (HA) is post-translationally modified by cleavage at a connecting peptide region into two subunits, HA1 and HA2, and this cleavage is a prerequisite for viral infectivity. Furthermore, HA cleavage can be correlated with strain virulence and virus production in tissue culture (31). Another study (78), a comparison of a virulent and an avirulent strain, showed that while the HA of the avirulent strain was cleaved only in the presence of trypsin, the virulent strain HA precursor was also cleaved into HA1 and HA2 when trypsin was absent. However, the amino acid sequences of both strains were indistinguishable through the connecting peptide region, and difference in the susceptibility of the HA to cleavage is therefore not directly attributable to the sequence of this peptide. While the apparent molecular weight of the HA1 subunit from the avirulent strain was higher than that of the virulent strain, the molecular weights of the haemagglutinins were indistinguishable when the viruses were grown in the presence of tunicamycin. Since monoclonal antibodies indicated

that at least one epitope on the HA differed between virulent and avirulent strains, and there were no deletions or insertions in the amino acid sequences, it was thought possible that the difference in molecular weight was due to the loss of a carbohydrate side chain in the virulent strain. But further examination of the amino acid sequences detected only one amino acid change in the virulent strain that could affect a glycosylation site in the area of the connecting peptide. This loss of a carbohydrate moiety may permit access of an enzyme that recognizes the basic amino acid sequences and results in cleavage activation of the haemagglutinin in the virulent virus strain.

The revelation that protease activation results in exposure of new terminal amino acid sequences explains in part the role of cleavage in determining viral virulence as well as tissue tropisms. Since the rubella virus structural proteins are specified by a polycistronic message, relative sensitivity to cleavage of the p110 precursor of different strains could determine virulence in this system also.

Differences exist not only in the susceptibility of the haemagglutinin to cleavage and pathogenicity between different subtypes of influenza virus, but even within one subtype (8). These differences exist in the structures of the glycoproteins, as indicated by variation in the molecular weights (electrophoretic mobilities) of the haemagglutinin and the neuraminidase, and in the presence of carbohydrate moieties. For example, the acquisition of virulence by one strain may, as already noted earlier, be associated with a point

mutation which results in the loss of a glycosylation site (31). In avian influenza and Newcastle disease viruses, the susceptibility of the viral glycoproteins to proteolytic cleavage (which determines pathogenicity) is determined primarily by the structure of the glycoproteins (8,100). In Sendai virus as well, the fusion protein F must be cleaved into its F_1 and F_2 subunits to become active. Although conversion of the F protein from its inactive to its active form may be accomplished in vitro by trypsin, trypsin sensitivity can be changed by a single amino acid substitution at the cleavage site of F. The acquisition of trypsin resistance has been traced to a mutation resulting in the replacement of arginine by an isoleucine residue at the cleavage site (trypsin cleaves on the carboxy terminal side of arginine and lysine residues) (73).

Viral particle variation has also been implicated in the establishment of persistent infections. For example, the periodic nature of equine infectious anemia has been attributed to sequential production and release of novel antigenic strains which temporarily escape host immune surveillance (97). The results from this study suggest that structural variation of the EIAV glycoproteins occurs during persistent infection and remains stable during replication in cell culture. The variation can be identified by altered migration rates in SDS-PAGE as well as by generation of distinctive peptide maps, and may be due either to carbohydrate heterogeneity or to alterations occurring in the amino acid sequence. A more recent study of EIAV was directed toward antigenic and biochemical characterization during

persistent infection (119). The EIAV isolates could be distinguished antigenically by neutralization assays and Western blot analysis. Again, virion glycoproteins displayed different electrophoretic mobilities upon SDS-PAGE. Further work, involving tryptic peptide and glycopeptide maps of each virus isolate, revealed biochemical alterations in both the amino acid sequence and glycosylation patterns of the virion surface glycoproteins gp90 and gp45, but no structural variation was observed in the internal viral proteins. Oligonucleotide mapping showed structural variation at the level of the viral genome and indicated that point mutations were probably responsible for this variation.

Since virulence is likely to be determined by a variety of factors that contribute independently to the capability of a virus to cause illness in an infected host, characterization of the molecular determinants of viral virulence is important in understanding the biology of virus infections.

VII. Glycoproteins - Introduction

Many biologically important proteins contain constituents attached to the polypeptide chain. These range from small molecules, such as sulphates and phosphates, to complex structures, including oligosaccharides and nucleic acids. Among these constituents - and perhaps the most important - are the oligosaccharide side chains which, when covalently attached to peptide backbones, characterize the

glycoproteins. Glycoproteins are found in bacteria, viruses, fungi, plants and higher animals and are involved in many cellular functions. These include cell-cell interaction, adhesion of cells to substratum, enzymic activity, histocompatibility and, in the case of enveloped viruses, the attachment of the virus to its host via specific receptor recognition and the elicitation of a specific antiviral response.

Viral glycoproteins are synthesized by an orderly process that involves the insertion of nascent polypeptide chains into the endoplasmic reticulum membrane, processing of amino-terminal peptides and glycosylation of the nascent chains. The maturation and incorporation of the glycoproteins into mature progeny virus involves the migration of the glycoprotein from the endoplasmic reticulum to the smooth membrane, the Golgi apparatus and finally to the plasma membrane. Viral glycoproteins may be found in several distinct states. An unglycosylated polypeptide exists in the cell only when inhibitors of glycosylation are present. A partially glycosylated polypeptide may be found intracellularly as an intermediate product; but by the time the glycoprotein reaches the cell surface, it is fully glycosylated. The fully glycosylated form is associated with or anchored to the cell membrane through a hydrophobic region, and is incorporated into virus particles as part of the envelope. In general, the viral genomes are too small to specify glycosyl transferases, and glycosylation of the viral glycoprotein therefore involves the glycosylating mechanisms of the host cell.

VIII. GLYCOPROTEIN STRUCTURE

The linkage between the oligosaccharide and the protein is a glycosidic linkage that results from a condensation reaction between an amino acid side chain on the protein and the anomeric carbon on the first residue of the oligosaccharide (80). In glycoproteins of higher organisms, carbohydrate linkages to protein may be either N-glycosidic, in which the carbohydrate is linked to the amido nitrogen of asparagine, or O-glycosidic, with the carbohydrate linked to the hydroxyl oxygen of serine, threonine or, rarely, hydroxylysine. O- and N-glycosyl classes of carbohydrates differ not only in the type of sugar-protein linkage, but also in monosaccharide composition, mode of biosynthesis and resistance to inhibitors of glycosylation. N-glycosides are more common than O-glycosides in glycoproteins of higher organisms, but a single glycoprotein may have multiple chains, some of which are O-glycosides and some N-glycosides. The carbohydrates in N-glycosides are generally somewhat longer than in O-glycosides (7-25 residues versus 2-10 residues).

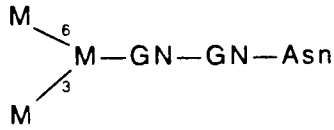
Asparagine-linked oligosaccharides (N-glycosides) on glycoproteins share a common precursor and thus share a common "core" sequence: $M\alpha 1 \rightarrow 3(M\alpha 1 \rightarrow 6)M\beta 1 \rightarrow 4GN\beta 1 \rightarrow 4GN$-Asn (M:D-mannoses, GN:N-acetyl-D-glucosamine) (Figure 1). Sequence differences occur in the sugars attached to the α1,3- and $\alpha\alpha$1,6-linked mannose residues of the core. There are three major types of asparagine-linked oligosaccharides: high mannose, complex and hybrid oligosaccharides (Figure 1). In high

FIGURE 1: STRUCTURES OF THE OLIGOSACCHARIDE CHAINS OF ASPARAGINE-LINKED GLYCOPROTEINS

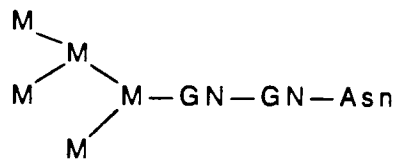
The core structure and modifications of the oligosaccharide chain of asparagine-linked glycoproteins are diagrammed.

STRUCTURES OF THE OLIGOSACCHARIDE CHAINS OF ASPARAGINE-LINKED GLYCOPROTEINS

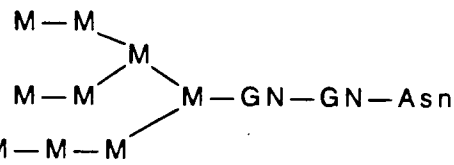
Mα1−3(Mα1−6)Mβ1−4GNβ1−4GN-Asn **or**



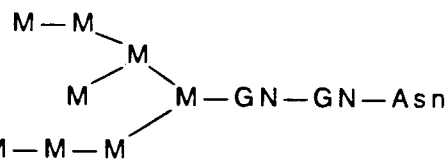
Core Structure



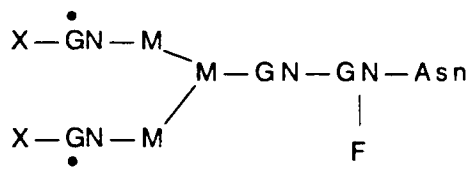
(high-mannose, M5)



(high-mannose, M9)



(high-mannose, M8)



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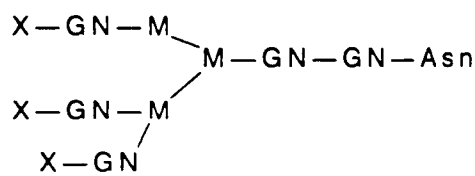

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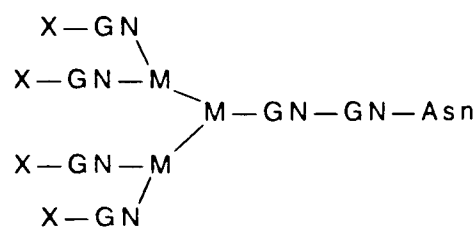

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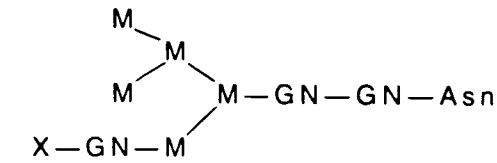

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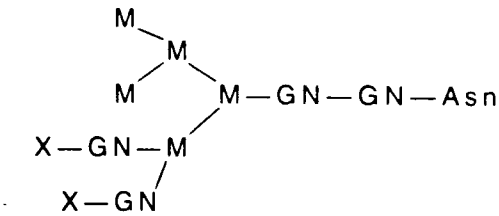

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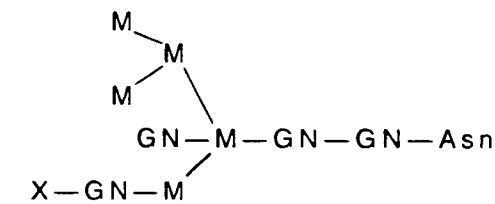
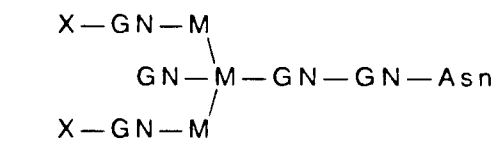


(bisected hybrid)



(bisected complex)

ABBREVIATIONS:

F: fucose
M: mannose
X: variable oligosaccharide
GN: N-acetylglucosamine
Asn: asparagine
●: attachment point for optional branching residues

(From Boehringer Mannheim Biochemica Info)

mannose oligosaccharides, all the residues attached to the core are mannose residues and number five to nine (including the three in the core) in most mammalian glycoproteins. Complex oligosaccharides are characterized by having N-acetylglucosamine (GN) attached to both the α1,3- and α1,6-linked mannose residues in the core. In the hybrid type of asparagine-linked oligosaccharide, the α1,6-linked core mannose has only mannose residues attached to it while the α1,3-linked core mannose has one or more GN-initiated branches attached to it.

IX. VIRAL GLYCOPROTEINS

Two types of oligosaccharide moieties may be found in viral glycoproteins. These are the high mannose type, which contains N-acetylglucosamine and mannose, and the complex type which consists of galactose, fucose and sialic acid in addition to mannose and N-acetylglucosamine. Sialic acid is usually the terminal sugar of the oligosaccharide side chain in the complex moiety (48). Both the high mannose and complex types have been found to be present in the glycoproteins of toga-, myxo- and retroviruses. Hybrid structures have yet to be identified in viral glycoproteins.

Although N-glycosides are more common than O-glycosides in glycoproteins, there is evidence from studies of virus and host cell systems that both types of linkages may exist in the same glycoproteins. While O-linked oligosaccharides are predominant in many cell surface glycoproteins such as glycophorin, and in secreted glycoproteins such as

submaxillary mucins, numerous other cellular glycoproteins, including fetuin, glycophorin and immunoglobulins contain both asparagine- (N-) and serine- or threonine- (O-) linked oligosaccharides (62). Glycoproteins with O-linked or O- and N-linked oligosaccharides have been found in vaccinia virus, coronaviruses, herpes simplex virus and respiratory syncytial virus (RSV) (53). Vaccinia virus haemagglutinin, a structural glycoprotein, has both O- and N-linked oligosaccharide chains (62), with N-linked oligosaccharides predominating, but O-linked chains participating in the expression of biological activity (132). The E1 glycoprotein of mouse hepatitis virus, a coronavirus, seems to be the first identified viral structural glycoprotein that contains only O-linked oligosaccharides (62). Other studies have also suggested the occurrence of O-linked, as well as previously identified N-linked, oligosaccharides in the glycoproteins of herpes simplex virus type I (108).

X. RUBELLA VIRUS GLYCOPROTEIN STRUCTURE

Preliminary analysis of the composition and structure of rubella virus glycoproteins has yielded several features. The first studies reported that, since the glycoproteins could be labelled with tritiated sodium borohydride after oxidation with galactose oxidase, galactose must be the terminal carbohydrate moiety (149). Other carbohydrate components were identified as N-acetyl-D-glucosamine and mannose. Neuraminidase treatment indicated the apparent absence of sialic acid residues in the terminal oligosaccharides of both E1 and E2

(neuraminidase cleaves terminal sialic acid residues) (10,149). Another study showed that while E1 and the E2 species E2a and E2b were all glycosylated, only E1 and E2b were efficiently labelled with ^{3}H-mannose, thus underscoring the possibility that the difference in migration between E2a and E2b is due to differences in glycosylation (106).

Treatment with endoglycosidase H, which cleaves N-linked high mannose or hybrid structures, had no effect on the mobilities of E2a and E2b (106), and thereby suggests that their glycan moieties are not of the high mannose type. The presence of complex glycans, however, is not excluded. The difference in mobility between E2a and E2b can therefore be attributed to both the number and structure of the glycan moieties. The same study reported that the major peptide migrating at the position of E2b apparently contained N-linked glycans since it could not be detected in tunicamycin-treated infected cells. In its place, a smaller protein, representing the unglycosylated form of E2a or E2b or both, was found.

More recently, it was shown (9) that, in infected cells, the counterparts of E1 and E2 migrated as sharp bands, indicating that the heterogeneity in virion E2 is a function of virus maturation. Further analysis indicated that while E1 comprises predominantly simple or high mannose oligosaccharide side chains, the composition of E2 is more heterogeneous, with the higher molecular weight species being relatively low in mannose and high in galactose (the complex glycan pattern), and

the lower molecular weight components high in mannose and low in galactose (the simple pattern).

Another report by the same investigators (10) included the analysis of GP59 and GP43, the intracellular counterparts to E1 and E2 respectively, after digestion with endoglycosidase H. It appeared that while the intracellular species GP59 (E1) and GP43 (E2) were sensitive to endo H, the E1 and E2 present in extracellular virus contained both sensitive and resistant species. Although this finding is not in agreement with previous work by Oker-Blom et al. (106), who found that endo H had no effect, the methods of analysis differed, with the former focussing on digestion of individual glycopeptides and the latter, likely a less sensitive method, on the digestion of whole virions.

PURPOSE OF THIS STUDY

Rubella virus infection remains a serious problem twenty years after the development of a vaccine. Congenital rubella syndrome is still seen too often, even in highly developed countries, and the involvement of rubella virus in transient and chronic joint pain and rheumatoid arthritis following natural infection and immunization has only begun to be studied. The development of a vaccine of high efficacy and few complications thus remains a priority, but must be preceded by in depth study of the molecular characteristics of the virus.

Numerous reports have noted that the incidence and severity of joint manifestations varied in dependence on the different rubella virus strains. Several other biological differences have also been reported, including variation in neutralization kinetics, haemagglutinating activity and, from this study, tissue tropism, which may be defined by the ability of the virus to enter specific cell types and establish a productive infection. Tissue tropism may be characterized by the extent of virus production and the time, post-infection, at which maximum cytopathic effect is observed, and plays an important role in the course of infection.

The molecular basis for these biological variations is still poorly understood. The variations may be the result of mutations in the nucleotide sequence which lead to alterations in the amino acid sequence. The biological variation may also be the result of variable

processing, with differences in the carbohydrate moieties of the glycoproteins possibly resulting in conformational variation and differential presentation of antigenic epitopes. Precedence for both nucleotide sequence mutations and processing differences resulting in biological variation exists in other viral systems. It is perhaps most likely that rubella virus strain differences result from a combination of types of variation.

With these facts in mind, preliminary studies were made in efforts to correlate the incidence of joint symptoms caused by wild type and vaccine strains of rubella virus with tissue tropisms in vitro. Further to these experiments, specific rubella virus variants, chosen on the basis of differences in biological activity were analyzed by polyacrylamide gel electrophoresis for alterations in structural protein make-up and by peptide analysis for alterations in the molecular structure of the viral structural proteins. Although non-structural proteins can also play a role in determining virulence by directly or indirectly influencing viral replication, these proteins have yet to be fully characterized and therefore studies were concentrated on the structural proteins. It was expected that these studies would enable correlation of biological differences among rubella virus strains with specific molecular alterations, a correlation that has been accomplished in other viral systems.

MATERIALS

I. ABBREVIATIONS

1. APS: ammonium persulphate
2. BSA: bovine serum albumin
3. DOC: deoxycholic acid
4. EDTA: ethylenediamine tetraacetic acid
5. FBS: foetal bovine serum
6. HIFBS: heat-inactivated foetal bovine serum
7. NP40: Nonidet P40
8. PAGE: polyacrylamide gel electrophoresis
9. PBS: phosphate-buffered saline
10. PBS+: phosphate-buffered saline with calcium and magnesium
11. PEG: polyethylene glycol
12. PMSF: phenylmethyl sulphonylfluoride
13. SDS: sodium dodecyl sulphate
14. TEMED: N,N,N',N'-tetramethylethylenediamine
15. TPS: Tween, phosphate, saline buffer
16. Tris: tris (hydroxymethyl) aminomethane

II. MATERIALS

1. Cells

Vero (African green monkey kidney), Raji, Cess, and U937 cells were obtained from the American Type Culture Collection (ATCC). All cells were maintained as continuous cultures.

2. Virus Stocks (Original)

M33	ATCC-wild type
HPV77/DE5	Meruvax I - Merck, Sharp and Dohme 1979
RA27/3	Meruvax II - Merck, Sharp and Dohme 1982
Thomas	Congenital rubella syndrome isolate 1978
Therien	adapted in Finland 1984
1B2	plaque-purified patient isolate 1981

3. Electrophoresis chemicals

All chemicals for one- and two-dimensional gel electrophoresis were purchased from Bio-Rad, except SDS (BDH).

4. Enzymes

Staphylococcus aureus V8 protease: purchased from Miles Laboratories Ltd.
Endoglycosidase F (Endo F): purchased from Genzyme

5. Miscellaneous materials

Hydrogen fluoride was generously donated by Dr. Dutton, Department of Chemistry, UBC.
Nitrocellulose was purchased from Bio-Rad and Schleicher & Schuell.
Film for autoradiography: Kodak X-Omat RP x-ray film
Staphylococcus aureus protein A: Calbiochem
Protein A - Sepharose: Pharmacia

III. SOLUTIONS (not described in METHODS section)

1. TNE (pH 7.4) - 0.01 M Tris
   0.1 M NaCl
   1 mM EDTA

2. TPS (pH 7.6) - 10 mM sodium phosphate
   0.9% NaCl
   0.05% Tween-20

3. PBS (pH 7.0) - 0.15 M NaCl
   7.5 mM Na_2HPO_4
   2.5 mM $NaH_2PO_4.H_2O$

4. PBS+ (pH 7.3) - 0.14 M NaCl
   2.7 mM KCl
   8 mM Na_2HPO_4
   1.5 mM KH_2PO_4
   0.9 mM $CaCl_2$

5. SDS-PAGE sample buffer - 0.0625 M Tris-HCl (pH 6.8)
   2% SDS
   10% glycerol
   5% β-mercaptoethanol
   0.001% bromphenol blue

6. Staphylococcus aureus V8 protease buffer - 0.0625 M Tris-HCl (pH 6.8)
   10% glycerol
   0.1% SDS
   0.001% bromphenol blue

7. Endoglycosidase F reaction buffer - 0.1 M sodium phosphate (pH 6.1)
   50 mM EDTA
   1% NP40
   0.1% SDS
   1% β-mercaptoethanol

8. Neuraminidase reaction buffer - 0.1 M Na acetate (pH 5.0)
   1 mM Tris-HCl (pH 7.6)
   2 mM PMSF

9. SDS-PAGE running buffer (pH 8.3) - 0.025 M Tris
   0.1% SDS
   0.192 M glycine

10. Western Blot Transfer Buffer I (pH 8.3) - 0.013 M Tris
    0.096 M glycine
    20% methanol

11. Western Blot Transfer Buffer II (pH 9.9) - 10 mM $NaHCO_3$
    3 mM Na_2CO_3
    20% methanol

12. Gel fixative - 25% methanol
    7% acetic acid
    2% glycerol

13. Culture medium (Vero cells) - 199 + 10% FBS + 1% antibiotic/antimycotic (Penicillin 10 000 U/ml Streptomycin 10 000 µg/ml Amphotericin-B 25 µg/ml)

14. Culture medium (Raji cells) - RPMI + 15% FBS + 1% antibiotic/antimycotic

15. Culture medium (Cess and U937 cells) - RPMI + 10% FBS + 1% antibiotic/antimycotic

16. Culture medium (post-infection) -
    Vero cells - 199 + 5% HIFBS + 1% antibiotic/antimycotic
    Raji, Cess, U937 cells - RPMI + 5% HIFBS + 1% antibiotic/antimycotic

IV. GEL SOLUTIONS

A. One-Dimensional SDS-PAGE gels

1. non-gradient separating gel (10% acrylamide) -
   10% acrylamide (0.8 bis:30 acryl.)
   0.375 M Tris-HCl (pH 8.6)
   0.1% SDS
   0.1% TEMED
   0.033% APS

2. gradient separating gel (7.5% to 20% acrylamide) -
   stock I - 7.5% acrylamide (0.3 bis:60 acryl.)
   0.558 M Tris-HCl (pH 8.6)
   0.1% SDS
   0.1% TEMED
   0.01% APS

   stock II - 20% acrylamide
   0.558 M Tris-HCl (pH 8.6)
   0.1% SDS
   0.1% TEMED
   0.006% APS

   ratio stock I:stock II 1.4:1

3. stacking gel - 4.6% acrylamide (0.3 bis:60 acryl.)
   0.128 M Tris-Hcl (pH 6.8)
   0.1% SDS
   0.1% TEMED
   0.07% APS

B. Two-Dimensional PAGE gels (with IEF gels)

1. Sample buffer - 9.5 M urea
   2% NP40
   2% ampholines
   5% β-mercaptoethanol

2. Sample overlay - 8 M urea
   1% ampholines

3. Anode electrode solution - 0.01 M H_3PO_4

4. Cathode electrode solution - 0.02 M NaOH

5. SDS equilibration buffer (pH 6.8) - 10% glycerol
   5% β-mercaptoethanol
   2.3% SDS
   0.0625 M Tris

6. Isoelectric Focussing Tube gels - 4% acrylamide (1.62 bis: 28.38 acryl.)
2% NP40
2% ampholine (4:1 pH 5-7:pH 3.5-10)
0.01% APS
0.07% TEMED
9.2 M urea

METHODS

I. Virus Stock Preparation

Virus stocks were prepared by infecting Vero cell monolayers or Raji, Cess, or U937 cell suspensions at an MOI of between 1 and 10 for an adsorption period of 4 hours, after which the inoculum was removed and replaced with fresh medium. Supernatants were collected when cytopathic effect (CPE) was observed, at approximately 3 to 5 days post-infection. These were centrifuged at 3000 rpm for 15 minutes (in an IEC Centra-7R centrifuge) to remove cellular debris, and titrated by plaque assay.

II. Plaque Assay

Serial ten-fold dilutions (10^{-1} to 10^{-6} or 10^{-8}) of virus stocks were prepared in 199 medium + 2% HIFBS and were used to infect Vero cell monolayers for a 4 hour adsorption period. The inoculum was aspirated and the monolayers were overlaid with 0.5% agarose in 2 x 199 medium and incubated at 35°C. Plaques were counted after 10 to 14 days.

Modification for RA/27 strain:
The monolayers were overlaid a second time 3 days after the first overlay with 0.5% agarose in 2 x 199 medium containing neutral red.

III. Precipitation with Polyethylene glycol (PEG)

Supernatants from virus-infected cell cultures were clarified by centrifugation at 3000 rpm for 15 minutes in an IEC Centra-7R centrifuge. An equal volume of 20% (w/v) PEG (M.W. 3350) in PBS was added to each sample. Samples were incubated on ice for 2 hours and the precipitates were collected by centrifugation at 10 000 rpm for 10 minutes in a Sorvall centrifuge.

IV. Immunoprecipitation

Virus particles, precipitated with PEG from supernatant medium, or cell pellets, were taken up in 1 ml 1% NP40 (v/v) in TNE, vortexed and incubated on ice for 30 minutes. Cell debris was pelleted in an Eppendorf centrifuge for 3 to 5 minutes. Rabbit antiserum (50 ul) was added to each supernatant and the samples were vortexed and incubated on ice for 2 hours (or overnight at 4°C). S. aureus protein A (100 ul) was added to all samples, which were vortexed again and incubated on ice for 2 hours. Immune complexes were pelleted by centrifugation and washed twice in 1% NP40 (v/v), 0.5% DOC (w/v), 0.1% (w/v) SDS in TNE (pellets were dispersed by sonication). The pellets were taken up in sample buffer prior to gel electrophoresis.

V. One-Dimensional SDS-PAGE

SDS-PAGE was carried out by the method of Laemmli (86). Gradient

separating gels or single-concentration 10% acrylamide gels were poured, allowed to polymerize and overlayed with a stacking gel in which a template was embedded. Samples (supernatants precipitated with PEG or immunoprecipitates) were taken up in SDS sample buffer, boiled for 3 minutes, centrifuged briefly and applied to the sample wells. Gels were run at 32 mA per gel until the bromphenol blue marker dye reached the bottom of the gel. Gels were then either fixed and dried under vacuum for autoradiography or electroblotted onto nitrocellulose paper for immunodetection.

VI. Two-Dimensional PAGE

Two-dimensional PAGE gels were carried out by the method of O'Farrell (103). In the first dimension, isoelectric focussing (IEF) gels were poured in glass tubes to a height of 12.5 cm. The tubes had been prepared by pretreating them in a dichromate cleaning solution overnight, rinsing them in dH_2O and thereafter washing them again overnight in ethanol saturated with potassium hydroxide (KOH). After one more rinsing, they were left to dry and capped at one end with 3 or 4 layers of Parafilm.

The tube gels were overlaid with 20 ul of 8 M urea, followed by dH_2O. Polymerization was allowed to take place for 30 to 60 minutes, after which the Parafilm plugs were removed and the tube gels loaded into the gel tank containing anode buffer. The initial overlay was sequentially replaced with 10 µl of sample buffer, 10 µl of sample

overlay and, finally, the cathode electrode solution. The tube gels were pre-electrophoresed for 15 minutes at 200 v, 30 minutes at 300 v and 30 minutes at 400 v. Overlay buffers were removed and the samples, dissolved in sample buffer, layered on top of the gels. Samples were sequentially overlaid with 10 µl sample buffer, 10 µl sample overlay and, finally, cathode electrode solution. Gels were run for 18 hours at 400 v, removed from the tubes, equilibrated in SDS equilibration buffer for 2 hours at room temperature and stored at -20°C until required.

A single-concentration 10% SDS-PAGE gel was used for electrophoresis in the second dimension. The tube gels were embedded in 1% agarose (in SDS equilibration buffer) applied to the top of the stacking gels, and the slab gels were run at 32 mA per gel until the bromphenol blue marker dye reached the bottom of the gel. The slab gels were then fixed, dried and autoradiographed.

VII. Western Blotting

A. Transfer

Following electrophoresis, the gels were sandwiched between sheets of nitrocellulose paper that had previously been wetted in blotting buffer, and placed in a Hoeffer TE42 transfer apparatus. Transfer was carried out, with cooling, at 50 mA overnight, followed by 2 to 4 hours at 300 mA, or, after a brief equilibration period (30 minutes at 50 mA), for 4 hours at 300 mA. Following transfer, the blots were washed for 2 x 10 minutes in TPS and air-dried.

B. Detection

The nitrocellulose sheets were blocked in warm (40°C) 3% gelatin in TPS for 20 minutes (to reduce background) and washed in TPS for 10 minutes. The sheets were then incubated for 1 hour at room temperature (r.t.) while being agitated with a primary rabbit polyclonal antibody of anti-rubella antiserum diluted 1:200 in TPS, and were then washed twice in TPS for 10 minutes. The antiserum had been previously prepared by column-purification on DEAE-Sephacel columns and adsorbing the serum against Vero cells to reduce non-specific binding. Secondary incubation with biotinylated goat anti-rabbit antiserum (Vectastain), diluted 1:200 in TPS, was carried out for 1 hour at r.t. with agitation. Again, the sheets were washed twice for 10 minutes in TPS prior to colour development using biotinylated horseradish peroxidase complexed to avidin (Vectastain) and prepared in TPS. The sheets were incubated with this complex for 30 minutes at r.t. with agitation. This was followed by a 5 minute wash in PBS. Colour development was accomplished by immersing the sheets in a solution of 0.015% H_2O_2 and 3 mM 4-chloro-1-naphthol (dissolved in methanol) in PBS at r.t. with agitation for 5 to 15 minutes. The sheets were then rinsed in dH_2O and air-dried. In some cases, the samples were radioactively labelled, and the sheets also autoradiographed either before or after the immunological detection of antigens.

VIII. Digestion by Hydrogen Fluoride

Rubella virus samples were prepared from infected Vero cell

supernatants precipitated with PEG. The precipitates were taken up in 0.5 ml PBS and lyophilized. Duplicate samples were treated with hydrogen fluoride (HF) under both mild (1 hour, 0°C) and harsh (3 hours, 23°C) conditions. Samples were then either taken up in dH_2O and lyophilized again before solubilization in SDS sample buffer, or taken up directly in sample buffer. Thereafter they were loaded on to 10% polyacrylamide mini gels, which were electrophoresed at 32 mA per gel. The gels were then either stained with Coomassie blue stain and dried under vacuum or were blotted onto nitrocellulose paper. The nitrocellulose sheets were developed as previously described and then stained briefly in amido black stain.

IX. Staphylococcus aureus V8 Protease Digestion

One-dimensional gradient gels were poured and run as usual. Individual sample lanes were cut out and equilibrated for 30 minutes, without stirring, in 0.125 M Tris-HCl, pH 6.8 containing 0.1% SDS. Previously prepared gradient gels were overlaid with the same buffer, and the gel strips placed horizontally through the buffer 10 to 15 mm above the separating gel. The buffer was then aspirated and replaced with stacking gel, overlaid with 0.1% SDS. After polymerization of the gels, the electrode compartments were filled with SDS running buffer, and the gels overlaid with V8 protease buffer with or without V8 protease. The protease was employed at a concentration of 8 ng per square millimeter of slot surface, or 2.5 µg/ml. Electrophoresis was carried out at 32 mA per gel until the bromphenol blue marker dye

approached the bottom of the stacking gel, at which point the current was turned off for 30 minutes to allow for digestion to take place. Following electrophoresis, the gels were either fixed and dried for autoradiography or electroblotted onto nitrocellulose paper for immunodetection.

RESULTS

I. ONE-DIMENSIONAL SDS-PAGE

1. Introduction

The simplest and most common method for characterizing viral proteins is one-dimensional SDS-PAGE, which separates proteins by molecular weight (size). This method can be used in preliminary studies to determine the molecular weights of proteins of interest and can also indicate major differences in specific proteins of several strains of a single virus. In conjunction with Western blotting and immunological detection procedures, SDS-PAGE can thus offer much information and answer many questions about basic protein structure.

2. Choice of Primary Antibody for Immunodectection

Rabbit polyclonal antisera had been prepared against many of the rubella virus strains examined in this study, but despite previous reports of the cross-reactivity of anti-rubella virus antisera, there was still a possibility that one antiserum might cross-react more strongly than any of the other antisera.

Identical rubella virus protein samples were electrophoresed in triplicate, transferred to nitrocellulose sheets and developed with antisera prepared against three different strains. Results are shown in

Figure 2, and reflect the high degree of cross-reactivity among antisera. In each panel, the indicated antiserum detected the three structural protein groups of the three major virus strains (Therien, HPV77/DE5 and M33). Even so, it appears that antiserum prepared against the HPV77/DE5 strain most strongly detects the viral proteins of the strains examined (panel 2). This antiserum also detects the numerous degradation products in each sample lane, which disappeared in other experiments when greater care was taken to prevent digestion by proteases. High molecular weight species (HMWS) probably representing E1 or E2 dimers or polymers were also clearly detected, but decreased in intensity or disappeared altogether in other experiments when higher concentrations of β-mercaptoethanol were used. As a result of this experiment, rabbit anti-HPV77/DE5 antiserum was used as the primary antibody for immunodetection in the majority of further experiments.

3. One-Dimensional SDS-PAGE

With the optimal antiserum for detection determined, a basic comparison of the structural proteins of six rubella virus strains was made. Earlier studies had determined that rubella virus infection of Vero cells produced satisfactory viral titres in the supernatant and preliminary strain comparisons were therefore made using this cell type. This cell line was also found to be satisfactory for studies of intracellular proteins and thus could be used for both kinds of studies while the use of lymphoblastoid cell lines was limited to studies of extracellular proteins.

FIGURE 2: DETECTION OF RUBELLA VIRUS STRUCTURAL PROTEINS BY THREE POLYCLONAL RABBIT ANTISERA

Vero cell cultures (10 x 10^6 cells) were infected with rubella virus stock preparations. Supernatants from 72 to 96 hours post-infection were collected and precipitated with 10% PEG. The resulting pellets were resuspended in SDS sample buffer (60 µl) and the samples (15 µl) electrophoresed and transferred to nitrocellulose paper for immunodetection.

The E1, E2 and C polypeptides are indicated. Also of note are the high molecular weight species (HMWS) and the species appearing between the E1 and E2 regions, both of which are greatly decreased by the addition of higher concentrations of β-mercaptoethanol, suggesting that these species are disulphide-linked polymers. Other minor bands (a "laddering" effect) are believed to result from sample degradation.

DETECTION OF RUBELLA VIRUS STRUCTURAL PROTEINS BY THREE POLYCLONAL RABBIT ANTISERA

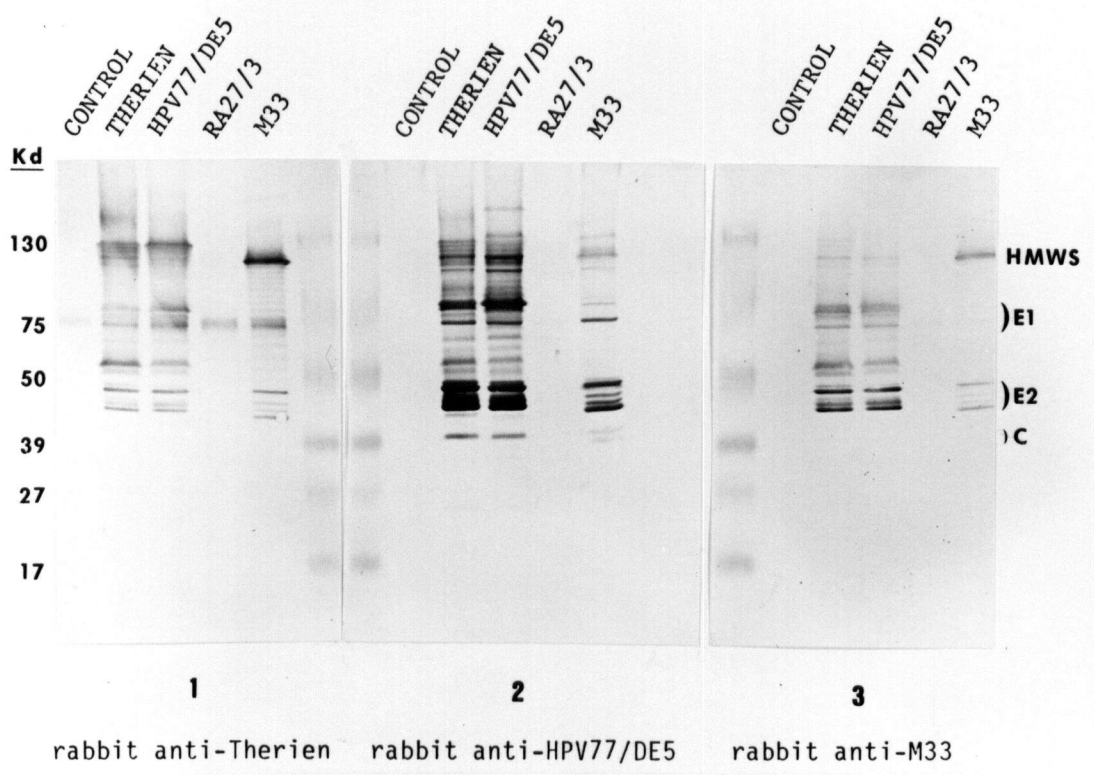


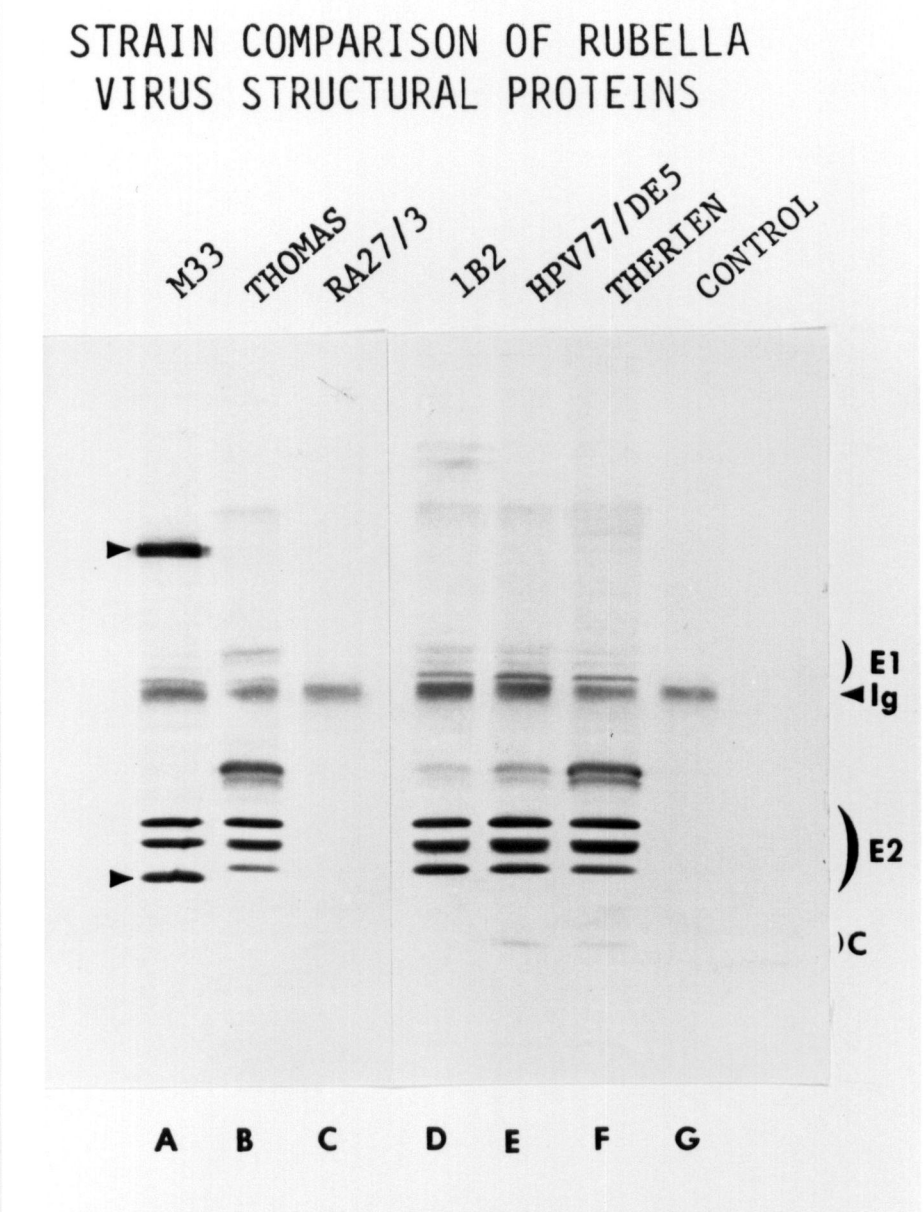
Figure 3 illustrates the great similarity of the protein patterns produced by the Therien, HPV77/DE5 and 1B2 strains. The E1 glycoprotein is detected as one major and several minor bands just above the band representing IgG. The latter is commonly seen in Western blots of immunoprecipitated protein samples and results from recognition of the rabbit antiserum used in the immunoprecipitation protocol by the goat anti-rabbit secondary antibody in the immunodetection system. E2 appears as a series of three or four bands (labelled), and the C protein appears very faintly (lanes E and F). The inconsistent presence of the C, or core, protein on Western blots may possibly be attributed to a loss of antigenicity upon transfer to nitrocellulose sheets since it appears consistently by autoradiography. Also, since a change in transfer buffer, from a Tris-glycine (150) to a carbonate (36) buffer system which returns proteins to a more native form after electrophoresis, resulted in stronger and more consistent detection of C, this explanation is deemed the most satisfactory. The species migrating below the Ig band, but above the E2 region may represent altered conformational forms of E2 and are not consistently observed.

Figure 3 also shows that the patterns of proteins of the Thomas, M33 and RA27/3 strains differ from the protein patterns of the other three strains. The Thomas strain, although producing a pattern for the E2 glycoprotein species identical to those of the first three strains, gives a different pattern for E1, with two major E1 species migrating at the same rate and another E1 species migrating more quickly than in the other strains. This band is not clearly observed in Figure 3, but has

FIGURE 3: STRAIN COMPARISON OF RUBELLA VIRUS STRUCTURAL PROTEINS

Vero cell cultures (10 x 10^6 cells) were infected with rubella virus stock preparations. Cells were collected at 72 hours post-infection, washed in PBS and resuspended in SDS sample buffer (100 µl) containing 46 ul β-mercaptoethanol (2x normal volume). Samples (10 µl) were electrophoresed and the resulting gel transferred to nitro-cellulose paper. The blot was developed with rabbit anti-Therien antiserum as the primary antibody.

The E1, E2 and C species are indicated. Of interest are the high molecular weight species and displaced band in the E2 region of the M33 strain (arrows). Also of note are the species appearing between the E1 and E2 regions which are present inconsistently on Western blots and may represent altered conformational forms of E2.


STRAIN COMPARISON OF RUBELLA VIRUS STRUCTURAL PROTEINS
M33
THOMAS
RA27/3
1B2
HPV77/DE5
THERIEN
CONTROL
E1
Ig
E2
C
A
B
C
D
E
F
G

been noticed as a characteristic of this strain (see Figure 6).

The M33 strain shows the most marked differences, with the presence of large quantities of a high molecular weight species (arrow), most likely a dimer of E1 and E2. While this species was also detected in the protein patterns of the other strains (Figure 2), and could be greatly reduced by the addition of higher concentrations of a reducing agent, the M33 high molecular weight species appeared more resistant to reducing agents. The E2 region is also different from that of the other strains. Examination of the E2 group reveals that the fastest migrating, and therefore the smallest, species is displaced relative to the smallest species in the other strains (arrow).

The RA27/3 strain did not produce any pattern, a failure that reflects the much lower virus titres (10-100 fold) obtained by infection with this strain than with the other strains. PAGE analysis using highly concentrated RA27/3 supernatant virus resulted in detection of RA27/3 proteins, and thereby suggests that detection problems in this study are solely a reflection of low protein concentration.

4. Time Course of the Production of Rubella Virus Structural Proteins

An experiment designed to explore the course of infection and production of rubella virus proteins was carried out in Vero cells. Cell supernatants were collected and the culture medium replaced every 24 hours until maximum cytopathic effect (CPE) was observed, about 4 to

5 days post-infection. Parallel infected cell monolayers were also photographed at 24 hour intervals. Figure 4 is a comparison of the CPE produced by five rubella virus strains at 72 hours post-infection. The CPEs produced by the M33, Therien and 1B2 strains are the most marked when compared with the control, while that produced by HPV77/DE5 is less intense, but still obvious. Infection by the RA27/3 strain, however, appears to leave the cell monolayer intact, even up to five days post-infection. This result agrees with the protein analysis in Figure 3 and is perhaps not unexpected, since plaque titration data have shown infection with RA27/3 to produce lower virus titres than any of the other strains. Infection with this strain is thus less productive, and host cells are able to survive intact for longer periods.

A time course of the appearance of structural proteins is shown in Figure 5. Patterns for the E2 region of all the strains except RA27/3 are distinct 48 hours after infection, while the E1 patterns are not clearly detectable until much later, about 96 to 120 hours (4 to 5 days). In the RA27/3 sample, a single band, which may represent the C protein, appears faintly at 96 hours and, by 120 hours, has become quite strong. This band is often the only protein species detected in unconcentrated samples of RA27/3. The detection of protein patterns correlates well with the appearance of cytopathic effects.

5. Detection with Monoclonal Versus Polyclonal Antisera

Since a monoclonal antibody directed against an E1 epitope was

FIGURE 4: COMPARATIVE CYTOPATHIC EFFECT OF RUBELLA VIRUS INFECTION IN VERO CELLS (72 HOURS POST-INFECTION)

Vero cell cultures (5×10^6 cells) were infected with rubella virus stock preparations at an MOI of 1 to 10. Photographs of cytopathic effect (CPE) were taken at 24 hour intervals.

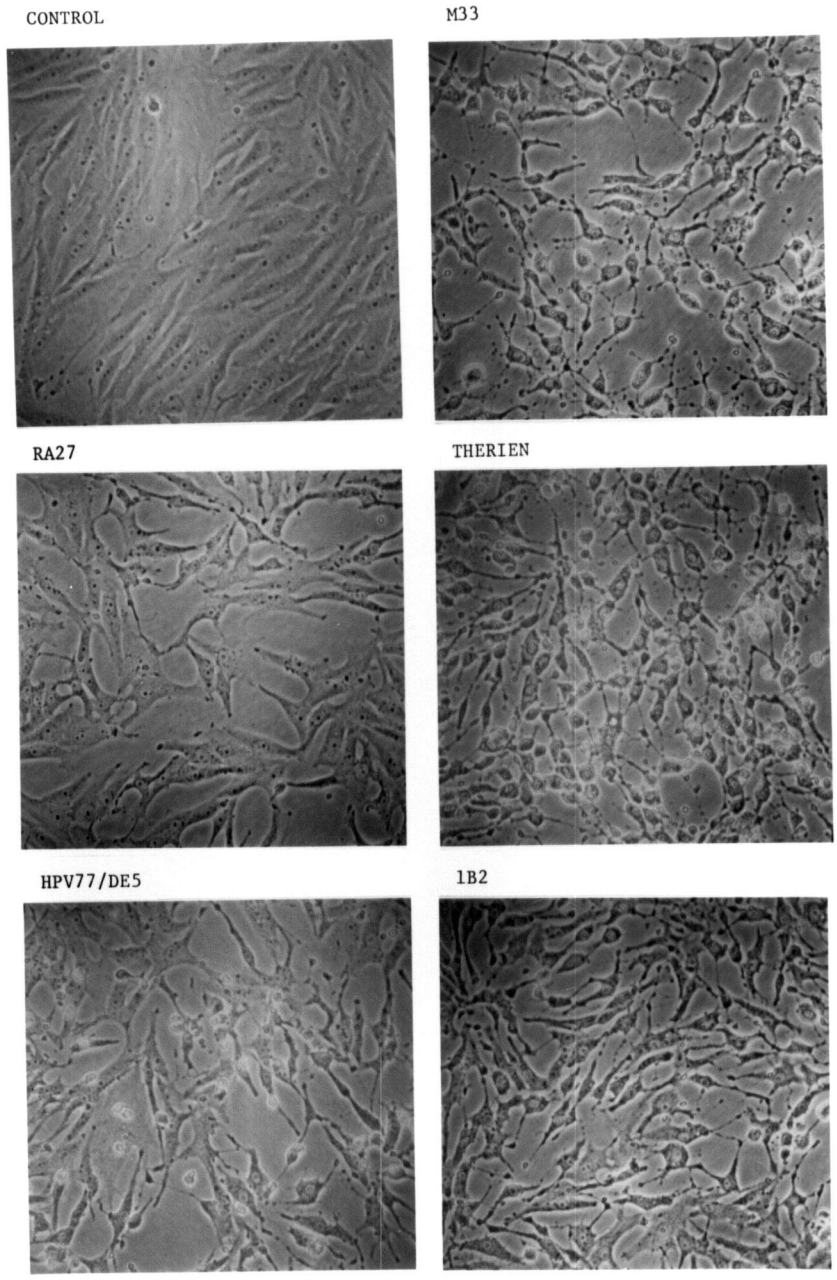
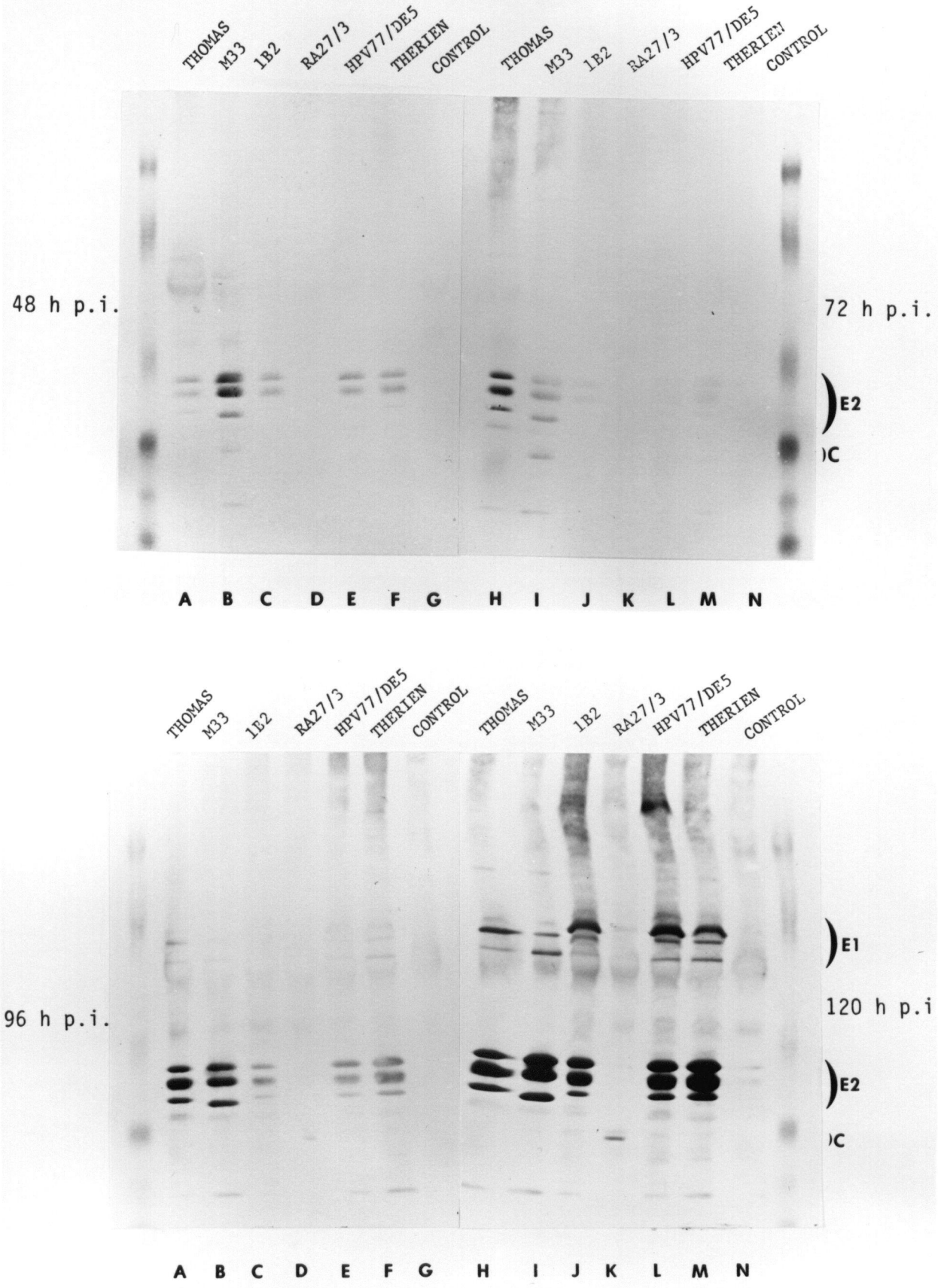

CONTROL
M33
RA27
THERIEN
HPV77/DE5
1B2

FIGURE 5: TIME COURSE OF PRODUCTION OF RV STRUCTURAL PROTEINS

Vero cell cultures (10 x 10^6 cells) were infected with rubella virus stock preparations and supernatants collected and replaced every 24 hours. Supernatants were precipitated with 10% PEG and resulting pellets resuspended in SDS sample buffer (100 µl). Samples (5 µl) were electrophoresed, transferred to nitrocellulose paper and developed with rabbit anti-HPV77/DE5 antiserum as the primary antibody.

TIME COURSE OF PRODUCTION OF RV STRUCTURAL PROTEINS



available, the proteins detected by the monoclonal and polyclonal antisera were compared. Figure 6 (lanes H-N) shows the pattern produced by development with rabbit anti-HPV77/DE5 polyclonal antiserum, and lanes A-G the pattern detected by a monoclonal antibody against M33 E1. While the polyclonal pattern is that consistently seen, the detection of a low molecular weight band in the M33 sample by the monoclonal antibody is an interesting finding. More specifically, the E1 patterns of the Therien, HPV77/DE5 and 1B2 strains show E1 to migrate with an approximate molecular weight of 68k daltons, while the Thomas E1 migrates much faster (this band is barely detectable in Figure 6), and the M33 E1 migrates to the E2 region. This detection of a low molecular weight species by the monoclonal antibody represents the most striking strain difference yet observed among rubella virus strains and is most likely explained by the epitope to which the monoclonal antibody reacts only being accessible in the unglycosylated form of E1 in this particular strain. The detection of the Thomas E1 species at a molecular weight of approximately 58k daltons may be explained by the same mechanism, although in this case, an underglycosylated form, rather than an unglycosylated one, is recognized.

II. TWO-DIMENSIONAL GEL ELECTROPHORESIS

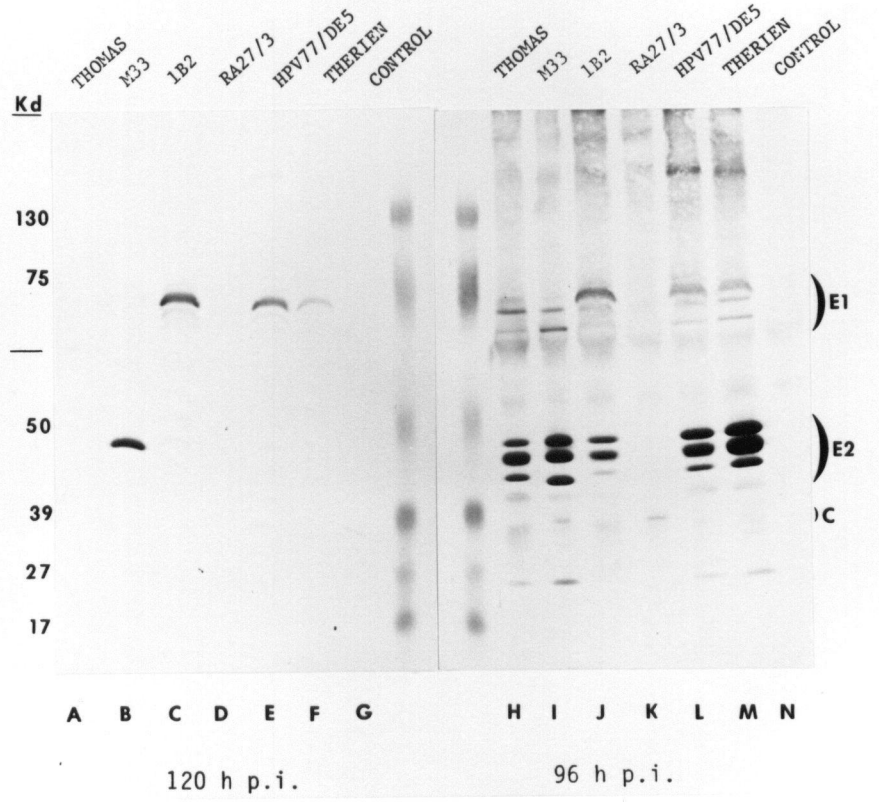
1. Introduction

Although a number of biological differences, including plaque formation, haemagglutination activity and antigenicity, have been

FIGURE 6: COMPARATIVE DETECTION OF RV E1 GLYCOPROTEIN WITH POLYCLONAL AND MONOCLONAL ANTISERA

Vero cell cultures (10 x 10^6 cells) were infected with rubella virus stock preparations and supernatants collected and replaced every 24 hours. Supernatants were precipitated with 10% PEG and the resulting pellets resuspended in SDS sample buffer (100 µl). Samples (5 µl) were electrophoresed, transferred to nitrocellulose paper. Sample lanes A through G were developed with the monoclonal antibody, 2B5 (directed against the M33 E1) and lanes H through N, with rabbit anti-HPV77/DE5 antiserum.

COMPARATIVE DETECTION OF RV E1 GLYCOPROTEIN
WITH POLYCLONAL AND MONOCLONAL ANTISERA



detected among rubella virus strains, analysis of the structural proteins of rubella virus by one-dimensional (1-D) SDS-PAGE yielded differences in only three (M33, Thomas, RA27/3) of the six strains examined. Since the extent of the biological differences indicated the likelihood of further, perhaps more subtle, molecular variation among the virus strains, the method of two-dimensional (2-D) gel analysis was used to explore this possibility.

In the O'Farrell (103) two-dimensional gel system, proteins are separated in the first dimension on the basis of charge by isoelectric focussing. Thereafter, the proteins are separated in the second dimension on the basis of molecular weight (size), as in one-dimensional SDS-PAGE. This system allows for the resolution of two or more distinct proteins which may have the same mobility (molecular weight) under reducing conditions.

2. Results and Discussion

As seen in Figure 7, the protein patterns generated by two-dimensional gel electrophoresis of six rubella virus strains are remarkably similar. The most obvious difference is the lack of a clear pattern for the RA27/3 vaccine strain. As previously mentioned, this strain grows to much lower titres (10 to 100-fold less) in cell culture than the other strains, and supernatants from RA27/3-infected cells therefore contain substantially less viral protein for analysis than is found for any other rubella virus strain. Until more concentrated

FIGURE 7: STRAIN COMPARISON OF RUBELLA VIRUS STRUCTURAL PROTEINS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

Vero cell cultures (10 x 10^6 cells) were infected with stock virus preparations and labelled overnight three to four days post-infection with 50 µCi/ml ^{35}S-methionine following a two hour starvation period in 1/10 methionine-containing medium. Supernatants (5 ml/sample) were collected, precipitated with 10% PEG and immunoprecipitated with rabbit anti-HPV77/DE5 antiserum (50 µl). Immunoprecipitates were resuspended in 2D-PAGE sample buffer (45 µl) and the samples (20 µl) loaded onto tube gels for electrophoresis in the first dimension.

The pH gradient formed in the first dimension was pH 5.2 to 7.0.

Gels were autoradiographed for four weeks at 4°C.

Legend: One-Dimensional Gel

A Control

B Therien

C HPV77/DE5

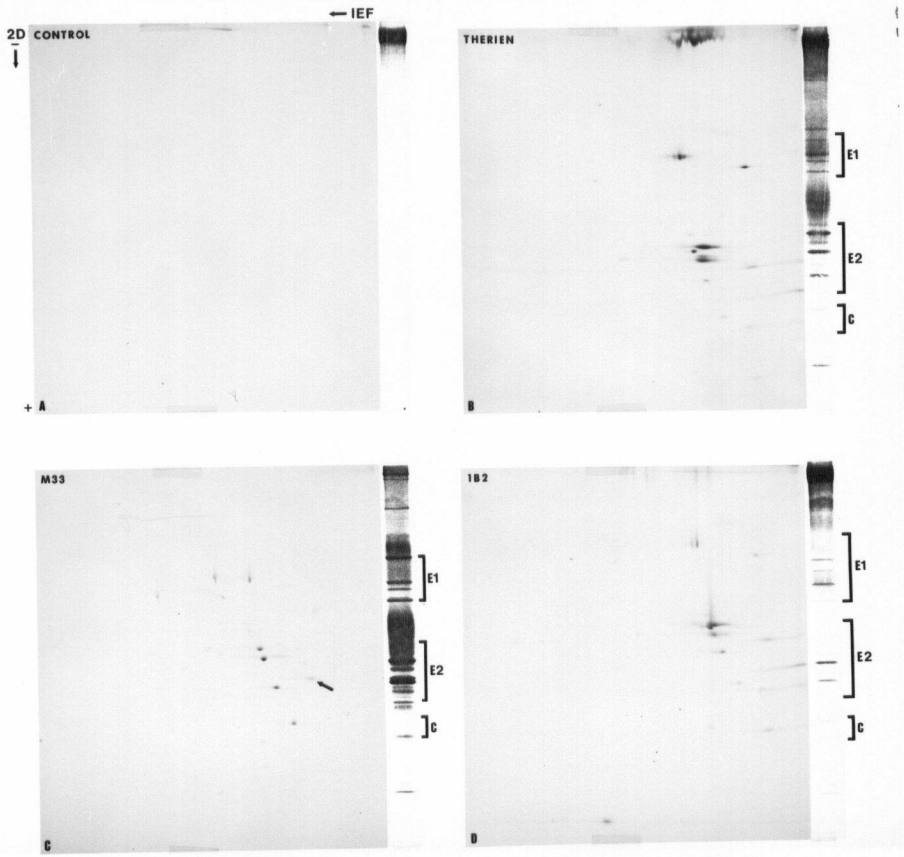
D M33

E RA27/3

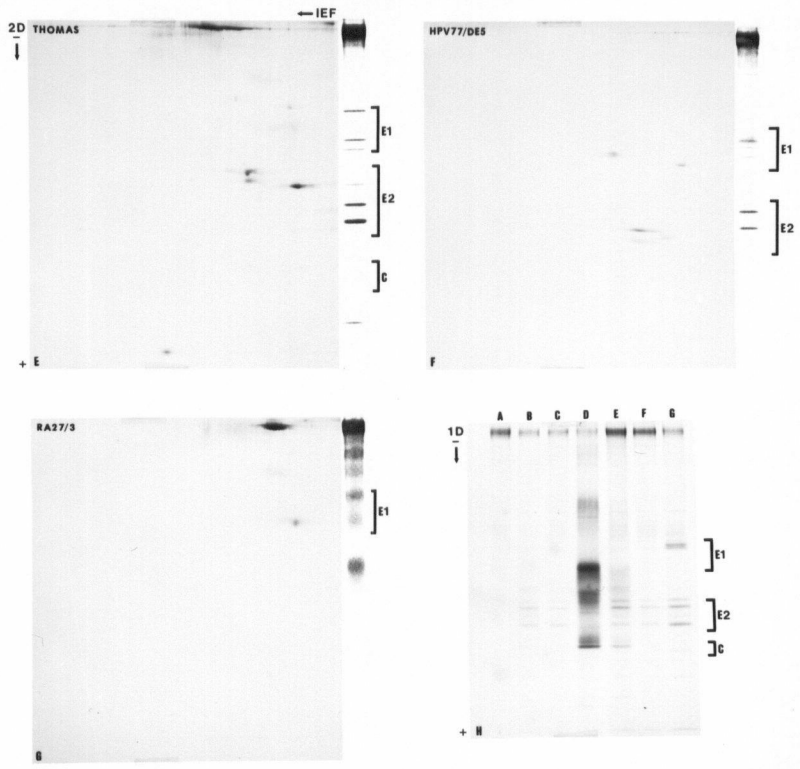
F 1B2

G Thomas

STRAIN COMPARISON OF RUBELLA VIRUS STRUCTURAL PROTEINS
BY TWO-DIMENSIONAL GEL ELECTROPHORESIS



STRAIN COMPARISON OF RUBELLA VIRUS STRUCTURAL PROTEINS
BY TWO-DIMENSIONAL GEL ELECTROPHORESIS



protein samples of the RA27/3 strain can be obtained, comparison of this pattern with those of the other strains is impossible.

The most significant difference among the strains examined is seen by comparison of the M33 wild type strain with any of the other strains, most notably Therien and 1B2. As indicated by the arrow in Figure 7c, the M33 pattern shows a single displaced spot in the E2 region relative to those in the Therien and 1B2 patterns. This may be the same displaced protein species detected by 1-D SDS-PAGE. The identity of the protein remains undetermined, but it may be the M33 E1 protein which was shown to co-migrate with the E2 species in 1-D SDS-PAGE. This could be substantiated by using the techniques of Western blotting and immuno-detection with a monoclonal antibody directed against E1. Electro-phoretic conditions for transfer of the proteins from a 2-D gel onto nitrocellulose paper and conditions for development may, however, need to be altered, since Western blots developed with even polyclonal antisera failed to reveal all protein spots detected by autoradiography (data not shown). Because two-dimensional SDS-PAGE affords greater resolution of proteins than one-dimensional electrophoresis, each resolved spot probably contains less protein. In part, this was suggested by the fact that samples had to be radiolabelled to a higher specific activity than for one-dimensional SDS-PAGE.

Two-dimensional SDS-PAGE thus failed to detect any differences in the structural proteins of rubella virus that could not be detected by one-dimensional SDS-PAGE. For routine examination of the structural

proteins of new rubella virus strains, it was therefore decided to reject this technique in favour of the faster, less complicated and less labour-intensive technique of one-dimensional SDS-PAGE. This decision was also prompted by the difficulty of interpreting two-dimensional SDS-PAGE patterns.

III. TISSUE TROPISM

1. Introduction

Biological differences among strains of a virus can manifest themselves in many ways, including variation in the kinetics of neutralization, haemagglutinating activity and tissue tropism. Earlier experiments in this laboratory had revealed several differences in biological activity among the strains, including differences in plaque formation, haemagglutination and tissue tropism (particularly in chondrocytes). As well, previous studies on the relative permissiveness of various reticular cell types to infection by rubella virus showed variation. The Therien, HPV77/DE5, 1B2, Thomas and M33 strains all gave comparable titres in mixed peripheral blood mononuclear cells and Raji cells (Epstein-Barr virus-transformed human B-cells), while the RA27/3 strain replicated very poorly or at undetectable levels (Table I) (Chantler, unpublished). These results have great bearing for the potential development of persistent infections, as in congenital rubella syndrome and rubella-associated arthritis.

Table II shows the results of plaque assays carried out with virus-infected Vero, Cess (EBV-transformed human B-cell line) and U937 (human monocyte-like cell line) cells. Again, although the first five rubella virus strains replicated satisfactorily in all the cell lines tested, the RA27/3 strain replicated poorly in both Vero and U937 cells and appears not to replicate at all in Cess cells.

2. Permissiveness of Cess and U937 Cells to Different Rubella Virus Strains

Further information on the degree of expression of rubella virus proteins by lymphoblastoid cell lines was obtained by one-dimensional SDS-PAGE of both intracellular and supernatant virus. Very little protein was found intracellularly (data not shown) and thus while both intra- and extracellular proteins from infection of Vero cells could be analyzed, only supernatant viral proteins produced by infection of these cell lines were examined. Protein samples obtained from Cess cell infection gave patterns similar to those obtained from infected Vero cells (Figure 8). As before in other work, no RA27/3 pattern could be detected, but in this case, rather than low production of virus particles relative to the other strains, the evidence from plaque assays (previously described) suggests that RA27/3 cannot replicate in this cell line. The M33 strain again gave the most strikingly different protein pattern, with its high molecular weight dimer and displaced band in the E2 region. In this cell type, however, the displaced band is not the smallest species, but rather, the second smallest, with a faster

TABLE I: REPLICATION OF RUBELLA VIRUS IN DIFFERENT CELL LINES

Virus Strain	Cell Line		
	Mixed PBMC	B-Cell Line Raji	T-Cell Line CCRF-CEM
1B2	+++	+++	++
Therien	+++	+++	++
Thomas	+++	++	NT
M33	++	++	NT
HPV77/DE5	+++	+++	++
RA27/3	+/-	-	NT

NT - Not Tested

(From J.K. Chantler, unpublished)

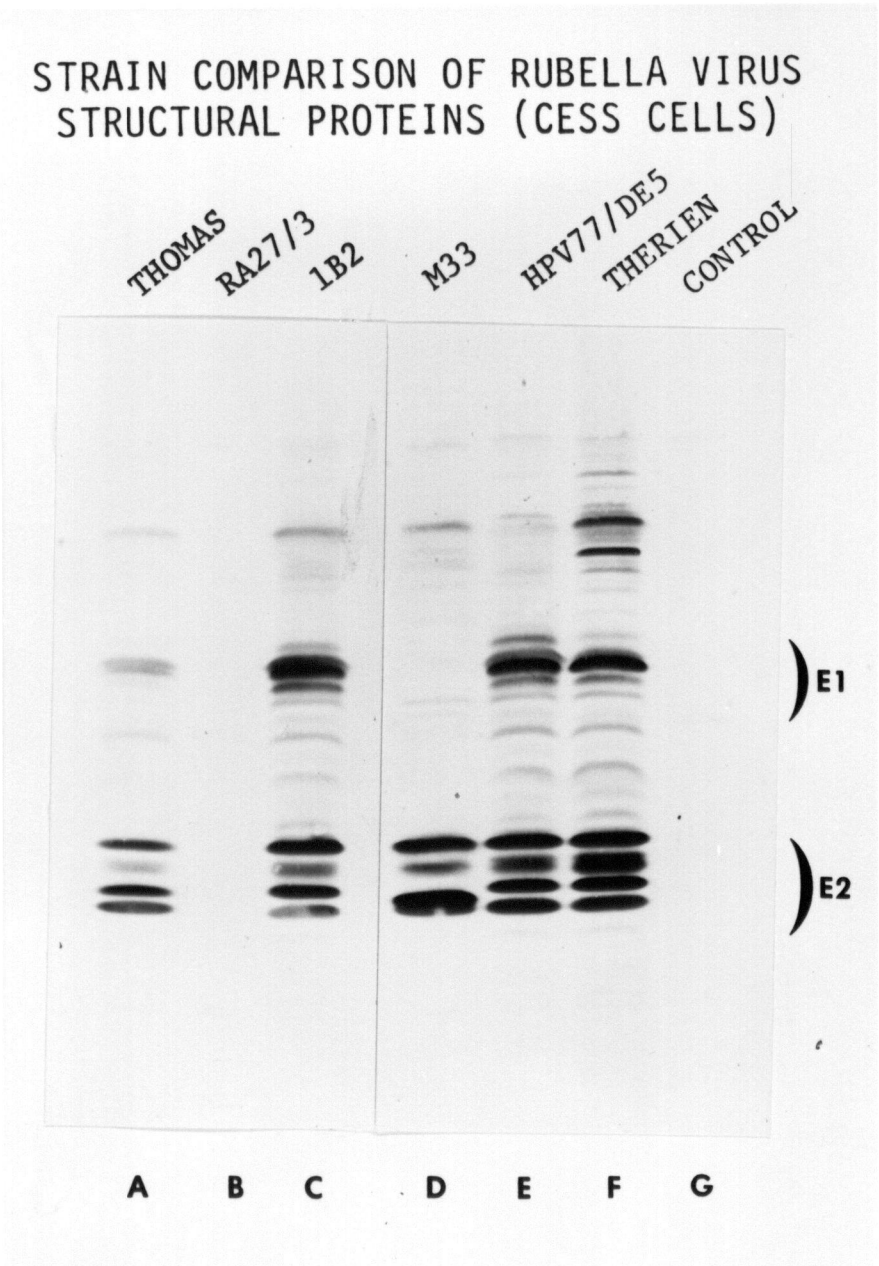
TABLE II: PLAQUE TITRATION OF RUBELLA VIRUS IN VERO, CESS AND U937 CELLS

Virus Strain	Cell Line		
	Vero (pfu/ml)	Cess (pfu/ml)	U937 (pfu/ml)
1B2	3.8×10^7	4.4×10^8	8.5×10^7
Therien	2.5×10^7	4.2×10^8	2.7×10^8
Thomas	1.4×10^7	3.5×10^8	5.3×10^7
M33	1.2×10^7	3.1×10^8	4.9×10^7
HPV77/DE5	1.7×10^8	5.1×10^8	1.6×10^8
RA27/3	1.3×10^6	$<10^2$	1.1×10^6

FIGURE 8: STRAIN COMPARISON OF RUBELLA VIRUS STRUCTURAL PROTEINS (CESS CELLS)

Cess cell cultures (5 x 10^6 cells) were infected with rubella virus stock preparations. Cell supernatants were collected at 72 hours post-infection and the viral proteins precipitated with 10% PEG. Precipitates were resuspended in SDS sample buffer (100 µl) and 5 µl samples used for electrophoresis.

Of particular note are the high molecular weight species and the displaced E2 band in the M33 strain. Also of interest is the low molecular weight band in the E2 region of all the strains which appears below the displaced M33 band. The other minor bands ("laddering" effect) are believed to result from sample degradation.


STRAIN COMPARISON OF RUBELLA VIRUS
STRUCTURAL PROTEINS (CESS CELLS)
THOMAS
RA27/3
1B2
M33
HPV77/DE5
THERIEN
CONTROL
E1
E2
A
B
C
D
E
F
G

migrating species, running just below the M33 displaced band, being detected in all the viral strains. E1 species appear only very faintly in the M33 and Thomas strains and, since the E2 bands appear in approximately equimolar concentrations with the E2 bands of the other strains, suggest that recognition of this protein by the antiserum used for detection is somehow impeded, possibly as a result of glycosylation differences which have altered or obscured the epitopes recognized by the antiserum.

Rubella virus infection of U937 cells generated the same patterns as those seen in Cess cells (Figure 9). But like RA27/3 infection of Vero cells, and unlike that of Cess cells, the lack of pattern may be attributed to lower production of virus particles than by other rubella virus strains. Again, the pattern produced by the M33 strain is the most different, with its faint E1 species and its displaced band in the E2 region, the latter a characteristic of this strain in all cell types examined to date.

IV. RUBELLA VIRUS GLYCOPROTEIN ANALYSIS

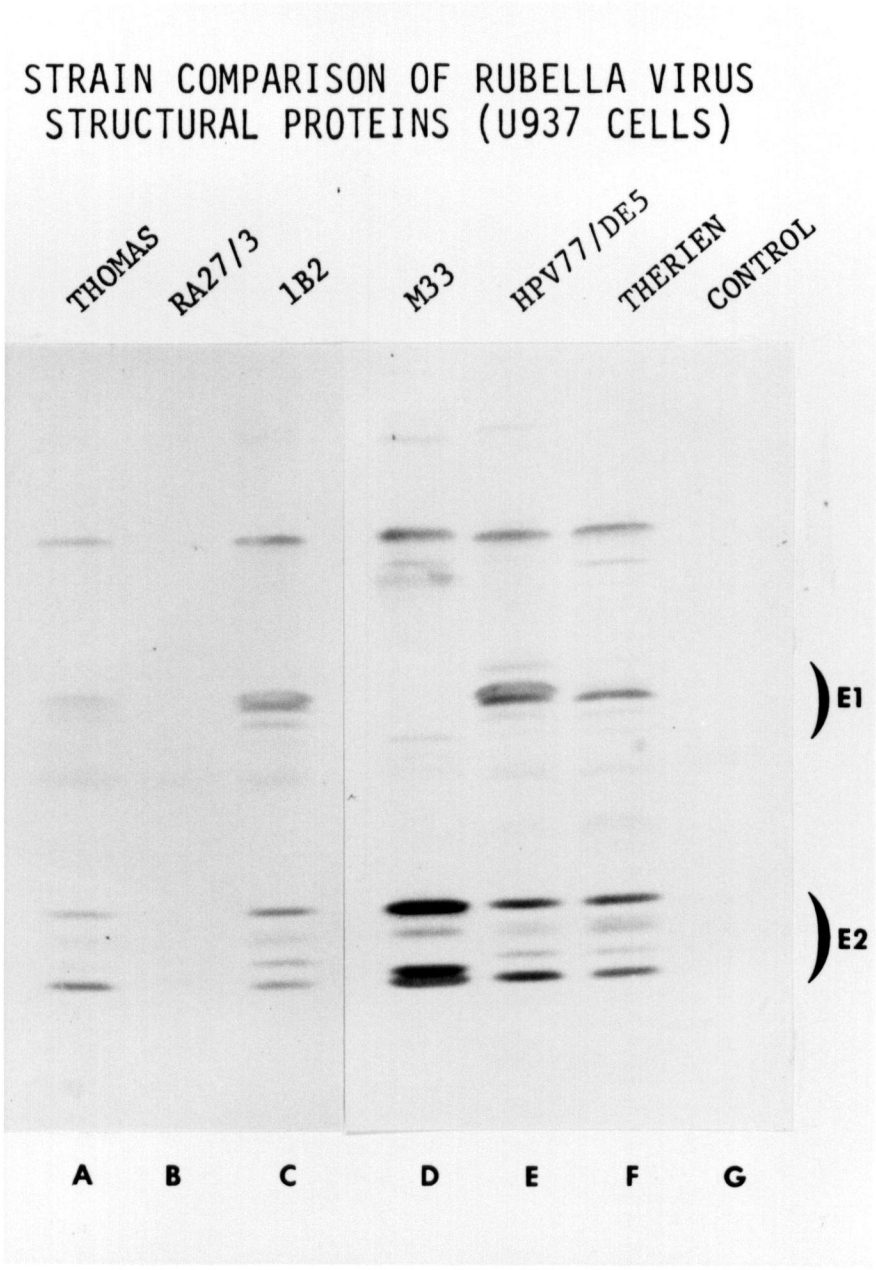
1. Deglycosylation - Introduction

In the study of glycoproteins, it is often advantageous to be able to isolate the polypeptide backbone free of attached glycan moieties. This facilitates the analysis of either component or, indeed, the identification of the type of linkage involved. Deglycosylation may be

FIGURE 9: STRAIN COMPARISON OF RUBELLA VIRUS STRUCTURAL PROTEINS (U937 CELLS)

U937 cell cultures (5 x 10^6 cells) were infected with rubella virus stock preparations. Cell supernatants were collected at 72 hours post-infection and the viral proteins precipitated with 10% PEG. Precipitates were resuspended in SDS sample buffer (100 µl) and 5 µl samples used for electrophoresis.

Of note are the high molecular weight species and the displaced E2 band in the M33 strain. Also of interest is the low molecular weight species appearing below the displaced M33 band, also seen in Figure 8.


STRAIN COMPARISON OF RUBELLA VIRUS
STRUCTURAL PROTEINS (U937 CELLS)
THOMAS
RA27/3
1B2
M33
HPV77/DE5
THERIEN
CONTROL
E1
E2
A
B
C
D
E
F
G

accomplished by either chemical or enzymatic methods.

2. Chemical Deglycosylation - Hydrogen Fluoride

Highly glycosylated proteins are well known to be difficult to purify. Microheterogeneity due to varying degrees of glycosylation contributes to the difficulty, making it especially hard to establish the presence of one unique peptide species. However, it is possible to achieve satisfactory deglycosylation by means of hydrogen fluoride (HF), which will cleave glycosidic linkages, and thereby remove microheterogeneity as well as reduce the molecular weight of the (glyco)protein.

Exposure of glycoproteins to anhydrous hydrogen fluoride at 0°C will cleave all the linkages of neutral and acidic sugar moieties within one hour while leaving the peptide bonds and glycopeptide linkages of amino sugar moieties intact (99). Harsher conditions, of three hours at 23°C will cleave the O-glycosidic linkages of amino sugars, but leave peptide bonds and N-glycosidic linkages intact. And since treatment of glycoproteins with HF results in little or no degradation of the sugars themselves, quantitative recovery is possible.

Rubella virus glycoproteins were treated with hydrogen fluoride in an attempt to further demonstrate the type of glycosidic linkages in these glycoproteins. Unfortunately, the results obtained by this technique were uninterpretable, because the positive and negative

control proteins, as well as the rubella virus test proteins, were completely hydrolyzed by the acid (data not shown). This may have been due to a large excess of HF, since the addition of the HF to the reaction vial was inexact at best, and the system employed had been developed for large samples of glycoprotein. Since generation of accurate data depends on digestion with hydrogen fluoride under anhydrous conditions, it is also possible that despite all precautions, some water entered the system. Overall, then, this system failed to furnish any further information on the structure of rubella virus glycoproteins.

3. Enzymatic Deglycosylation - Endoglycosidase F

The use of enzymes as means for deglycosylating glycoproteins eliminates the hazards associated with highly toxic and corrosive chemicals. Another obvious advantage of enzymatic deglycosylation is the specificity of cleavage which may be achieved. For example, exoglycosidases will only act on terminal residues, while endoglycosidases may be active at sites within the glycoprotein structure. Furthermore, endoglycosidases have been identified that operate only on specific types of structures or at specific cleavage sites.

For this study of rubella virus glycoprotein deglycosylation, endoglycosidase F (Endo F) was chosen. This enzyme is active on N-linked glycosides, the type believed to be present in rubella virus from

studies with monensin and tunicamycin. It cleaves high mannose, biantennary hybrid or biantennary complex structures, but will not cleave bisected hybrid structures, triantennary complex or tetraantennary complex structures (Figure 1, p19).

Preliminary experiments on the digestion by Endo F of a crude preparation of rubella virus proteins precipitated with PEG were unsuccessful (with no decrease in the molecular weights of the glycoproteins being detected). Since this preparation contains large amounts of protein (including protein in the foetal bovine serum of the culture medium), this failure was attributed to the possibility that the small amount of enzyme added to the reaction mixture was being overwhelmed by substrate. It was therefore thought essential to develop a methodology by which individual rubella virus glycoproteins could be isolated.

A first attempt at isolation of a rubella virus glycoprotein involved careful disruption of the virus particles and selective precipitation of the E1 glycoprotein with a monoclonal antibody. This was carried out by resuspending PEG-precipitated HPV77/DE5-infected cell supernatants in an SDS/Tris buffer, boiling the samples for 90 seconds and then centrifuging to recover the pellet. NP40 (1 ml) was added to each of two sample pellets to dilute out residual SDS, while the remaining two pellets were resuspended in a 1:1 mixture of NP40 and SDS/Tris buffer to retain a high SDS concentration, thus preventing reassociation of E1 and E2 subunits into dimers. All four pellets were

then immunoprecipitated with a monoclonal antibody, 2B5 (directed against M33 E1), followed by goat anti-mouse IgG and, finally, by S. aureus protein A containing 5% bovine serum albumin (BSA). Figure 10 shows the results of this experiment. Despite the differences in initial sample treatment, each lane on the nitrocellulose sheet shows successful isolation of the rubella virus E1 glycoprotein.

However, a disadvantage of this method lies in the immuno-precipitation of a complex of glycoprotein, monoclonal antibody and S.aureus protein A which forms a dense pellet that can only be dispersed by sonication. Because of the large quantity of protein in the pellet, not all of it viral, conditions were deemed unfavourable for digestion by enzymes. It was therefore felt that this method of isolating glycoproteins was not ideal, and that treatment with Endo F of immune complexes precipitated with S. aureus protein A was not feasible.

An attempt to precipitate the glycoprotein/monoclonal antibody immune complex by a centrifugation step, thereby avoiding the addition of S. aureus protein A altogether, was made. However, this also failed, likely a result of the formation of an immune complex of insufficient size to be precipitated in this manner.

A modification of the first method, with protein A-sepharose replacing S. aureus protein A, was attempted. Protein A-sepharose is a highly purified form of S. aureus protein A and has the advantage of producing less solid pellets upon centrifugation. As before, HPV77/DE5-

FIGURE 10: IMMUNOPRECIPITATION OF THE E1 GLYCOPROTEIN

Vero cell cultures were infected with HPV77/DE5 and the supernatants collected at 48 hours post-infection followed by precipitation with 10% PEG. Resulting pellets were differentially treated prior to immunoprecipitation with the monoclonal antibody 2B5 (75 µl), goat anti-mouse Ig (10 µl) and S. aureus protein A + 5% BSA (100 µl).

Sample Pretreatments:

A HPV77/DE5: SDS concentration kept high

B HPV77/DE5: SDS concentration kept high

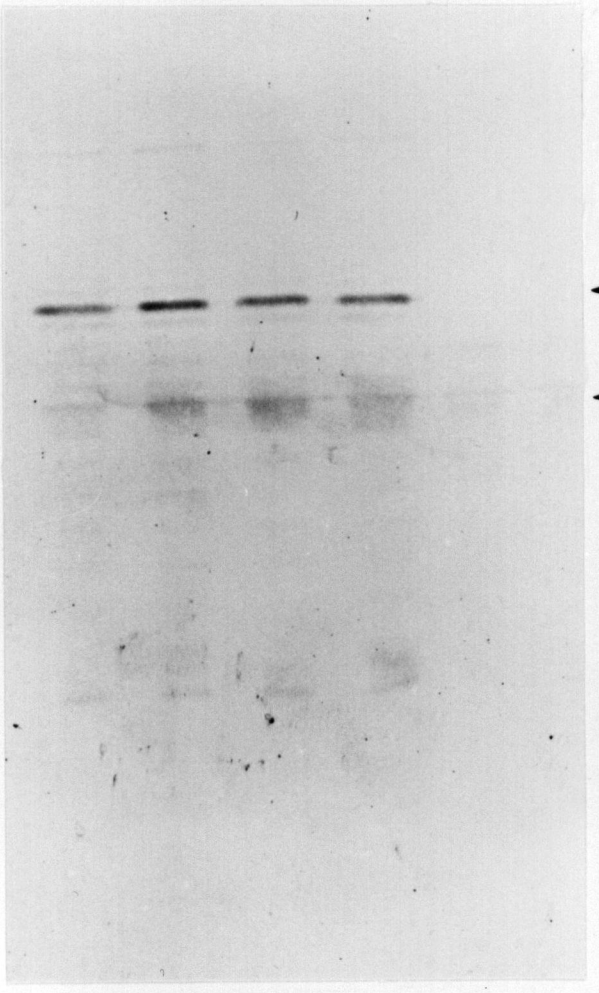
C HPV77/DE5: SDS diluted out

D HPV77/DE5: SDS diluted out

E Control: SDS concentration kept high

F Control: SDS diluted out

IMMUNOPRECIPITATION OF THE E1 GLYCOPROTEIN



infected cell supernatants were precipitated with PEG, the resultant pellets resuspended in SDS sample buffer, boiled and immunoprecipitated as previously described, except that protein A-sepharose was used to precipitate the immune complexes. The results of this experiment are shown in Figure 11, and although not as striking as those shown in Figure 10, demonstrate once again, that successful isolation of E1 was achieved.

The method for isolating the rubella virus E1 glycoprotein using protein A-sepharose was used in an experiment designed to test digestion of E1 by Endo F as well as by neuraminidase. Purified E1 was digested by Endo F at concentrations of 0.5 and 2 units (enzyme from NEN) for 1 hour at 37°C. Identical samples were digested by neuraminidase at a concentration of 0.01 units for 4 hours at 37°C. Neuraminidase was considered a negative control in these experiments as this enzyme functions to cleave terminal sialic acid residues, which do not exist in the rubella virus glycoproteins. Neither enzyme treatment produced any effect on the mobility, and thus the molecular weight, of the E1 glycoprotein (data not shown).

Since the ideal method of isolating rubella virus glycoproteins would eliminate the need for introduction of additional protein species into the system, one other procedure was investigated. Rubella virus proteins were electrophoresed on 10% acrylamide "mini" gels on which stained molecular weight markers were also run. Gel slices corresponding to the regions on the gel to which the rubella virus

FIGURE 11: COMPARISON OF METHODS FOR ISOLATION OF THE E1 GLYCOPROTEIN

Immunoprecipitation protocol:

A HPV77/DE5: monoclonal antibody 2B5 (75 µl) goat anti-mouse Ig (10 µl) protein A-sepharose (150 µl)

B Control: monoclonal antibody 2B5 (75 µl) goat anti-mouse Ig (10 µl) protein A-sepharose (150 µl)

C HPV77/DE5: monoclonal antibody 2B5 (75 µl) goat anti-mouse Ig (10 µl) S. aureus protein A + 5% BSA (100 µl)

D Control: monoclonal antibody 2B5 (75 µl) goat anti-mouse Ig (10 µl) S. aureus protein A + 5% BSA (100 µl)

E HPV77/DE5: rabbit anti-HPV77/DE5 antiserum (40 µl) protein A-sepharose (150 µl)

F Control: rabbit anti-HPV77/DE5 antiserum (40 µl) protein A-sepharose (150 µl)

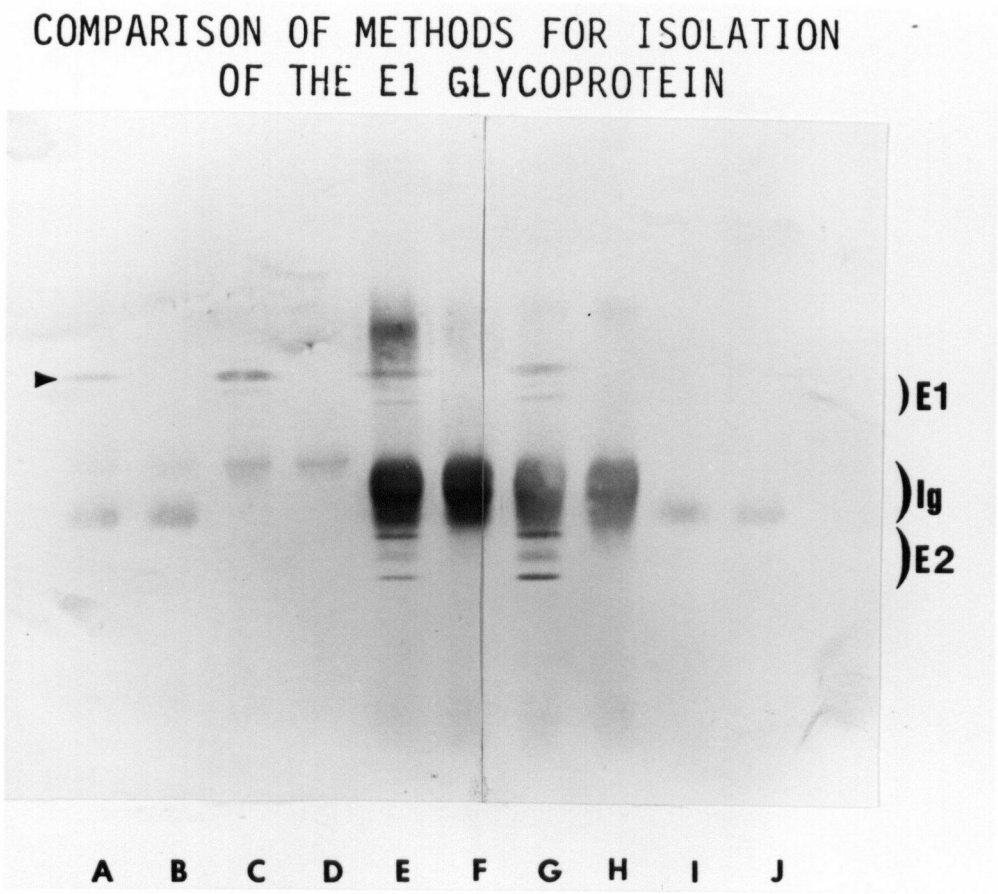
G HPV77/DE5: anti-HPV77/DE5 (40 µl) S. aureus protein A + 5% BSA (100 µl)

H Control: anti-HPV77/DE5 (40 µl) S. aureus protein A + 5% BSA (100 µl)

I HPV77/DE5: goat anti-mouse Ig (10 µl) protein A-sepharose (150 µl)

J Control: goat anti-mouse Ig (10 µl) protein A-sepharose (150 µl)

Lanes A and C show successful isolation of the E1 glycoprotein (arrow). Secondary bands in lanes A through D represent Ig species; the smaller Ig band in lanes A and B is precipitated with protein A-sepharose and the larger band in lanes C and D is precipitated with S. aureus protein A + 5% BSA.


COMPARISON OF METHODS FOR ISOLATION
OF THE E1 GLYCOPROTEIN
E1
Ig
E2
A B C D E F G H I J

glycoproteins were expected to migrate were excised and extruded through 18 gauge needles. Samples were boiled in Endo F reaction buffer for 90 seconds prior to the addition of enzyme at a final concentration of 0.2 milliunits/ml (enzyme from Genzyme). Samples were incubated overnight at 37°C, and both the gel slurry and its eluate were electrophoresed on gradient acrylamide gels as previously described. Neither preparation proved to be satisfactory as the gels appeared to electrophorese inconsistently and the resulting Western blots could not be interpreted (results not shown).

4. Discussion of Digestion of Rubella virus Glycoproteins by Endoglycosidase F

More work must evidently be done with endoglycosidases if meaningful insight into the composition and structure of rubella virus glycoproteins is to be gained. The first step in such work must be the development of a means for isolation of individual glycoproteins. One possible method which has very recently shown potential involves immunoprecipitation of specific glycoproteins by monoclonal antibodies, PAGE separation and ultimate recovery of each glycoprotein group by preparative gel electrophoresis followed by electroelution.

Success with digestion of rubella virus glycoproteins by endoglycosidases appears to be difficult to achieve. This may be due to the structure of the glycan moieties, as yet unstudied. It is possible that the N-glycosides present in rubella virus glycoproteins consist of

structures of such high complexity that access to the cleavage site by the enzyme used in this study (Endo F) is prevented. If so, an enzyme with a different specificity might be employed, or, failing that, alterations in the complexity of the structures involved might be made. These could include reduction of glycoproteins to glycopeptides, or reduction of the complexity of the oligosaccharide chain by preliminary digestion with specific exoglycosidases. The latter method may result in conversion of a substrate sterically unfavourable for digestion by endoglycosidases into a more favourable form. Employment of a mixture of enzymes of varying specificities may also serve to deglycosylate the protein of interest by sequentially removing branch structures.

During the course of this study, another enzyme, often a contaminant in preparations of Endo F, became available in purified form from several biotechnology companies (Boehringer Mannheim, Genzyme). This enzyme, glycopeptidase F (GPase F), has a broader range of activity than Endo F. It is active on N-glycosides and cleaves most high mannose, hybrid and complex oligosaccharide structures, but will not cleave oligosaccharides attached to an N- or C-terminal asparagine residue, nor will it cleave O-linked oligosaccharides. It may thus be a more useful enzyme for isolation of polypeptide backbones free of attached glycan groups. Like Endo F, it is sensitive to SDS, and it is therefore necessary to dilute the SDS as well as to include a non-ionic detergent (NP40) in the incubation buffer in order to retain enzymic activity.

Both Endo F and GPase F may be found to be more active on glycopeptides than glycoproteins. If so, the use of proteases with narrow specificities should be considered for the production of glycopeptides, especially prior to digestion with GPase F, because a non-specific protease will produce many small fragments. This would increase the likelihood of an asparagine residue, to which oligosaccharide groups are attached, being the N- or C-terminus of a fragment, a structure on which GPase F is not active.

Although technical complications still exist, deglycosylation of the rubella virus glycoproteins remains as a promising research tool for studying the effects of carbohydrate moieties on biological function.

V. PEPTIDE MAPPING

1. Introduction

Peptide mapping involves the reduction of proteins to peptides by either chemical or enzymatic means and characterization of the resulting peptides by a technique such as electrophoresis. Since it is not possible to identify relationships between proteins on the basis of electrophoresis alone, the technique may be used as means for assessing whether proteins of similar size are the product of a single gene, but have undergone differential processing. For example, proteins may vary in mobility as a result of slight chemical modifications or post-translational cleavage events.

Both chemical and enzymatic cleavage methods produce specific cleavages at given amino acids. Chemical methods result in complete cleavage at a small number of specific amino acids, and thus tend to produce larger peptides (50-100 residues) than those furnished by enzymatic cleavages (5-20 residues). Both types of cleavages may be controlled by a variety of factors which include pH, temperature, protein to enzyme ratio (enzymatic cleavage), reaction period and protein conformation.

The choice of a proteolytic enzyme depends greatly on the amino acid composition of the protein in question. One proteolytic enzyme of high specificity which has been found to be particularly useful for peptide analysis is Staphylococcus aureus V8 protease, which cleaves at the carboxy-terminal side of glutamic acid residues in a bicarbonate buffer (pH 7.8) or acetate buffer (pH 4.0), and at aspartic and glutamic acid residues in a phosphate buffer (pH 7.8) (70). This protease was employed in a modification (146) of the Cleveland technique (24) for the digestion of proteins in two-dimensional SDS-PAGE, and that modification has become popular for the comparative analysis of proteins because of its relative simplicity and the fact that it generates a pattern of peptide bands that is highly reproducible and characteristic of both the protein and the enzyme used. The resolving power of the method depends largely on the molecular weight distributions, relative amounts and protease susceptibilities of the individual proteins in the sample. Limitations of the method lie primarily in its inability to resolve the numerous small peptides generated by extensive digestion. Resolution of

very complex samples may be further limited by difficulties in identifying all peptides generated by digestion of a given protein and by the possibility that two peptides, although generated from different proteins, have the same mobility in the second electrophoretic dimension.

2. Results and Discussion

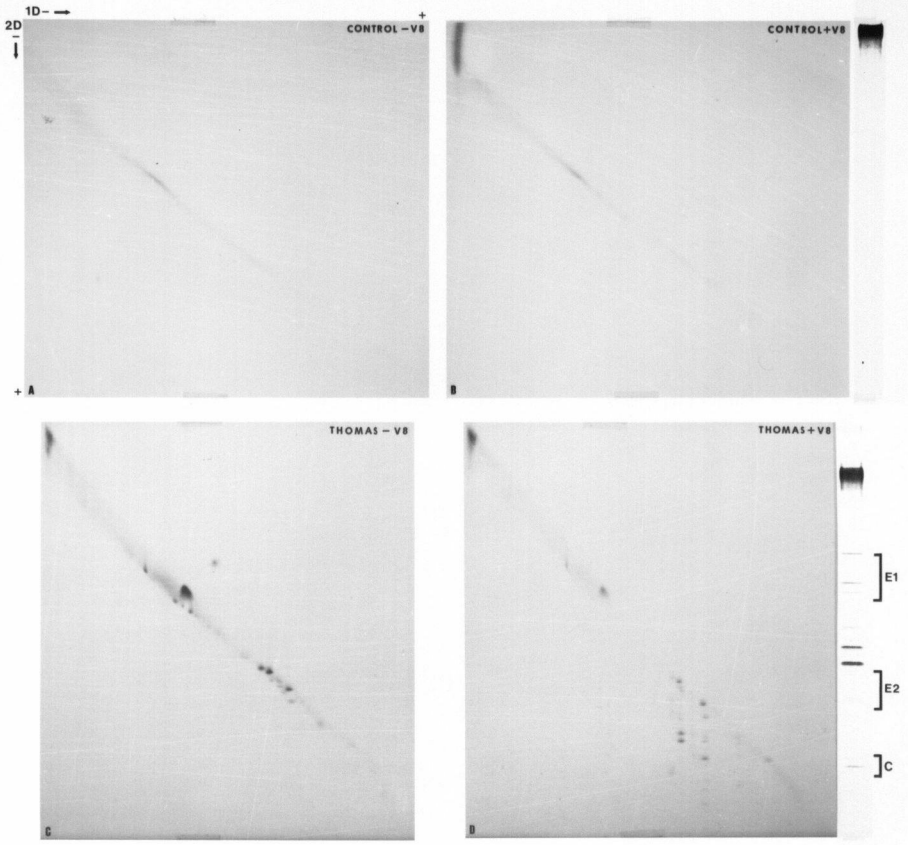
As already noted earlier, Western blots developed with a monoclonal antibody directed against an epitope of E1 indicated that the rubella virus strains examined in this study have E1 glycoproteins of varying mobilities. More specifically, in the M33 strain, the protein band detected by the monoclonal antibody (E1) was found to have an approximate molecular weight of 48k daltons, and thus co-migrated with the E2 species of all the rubella virus strains. This suggests that proteins in this molecular weight range varied not only in the extent of processing, specifically glycosylation, but actually represented two different proteins. To examine this possibility, digestion of the rubella virus proteins by V8 protease was used.

As seen in Figure 12, digestion patterns were obtained for the E2 species of all the rubella virus strains, except RA27/3 (pattern not shown), but no patterns for digestion of E1 could be detected. The best result was that obtained by digestion of the Thomas strain (D), which produced several fragments from the two spots corresponding to the two most clearly labelled E2 species in the control figure (C). Although

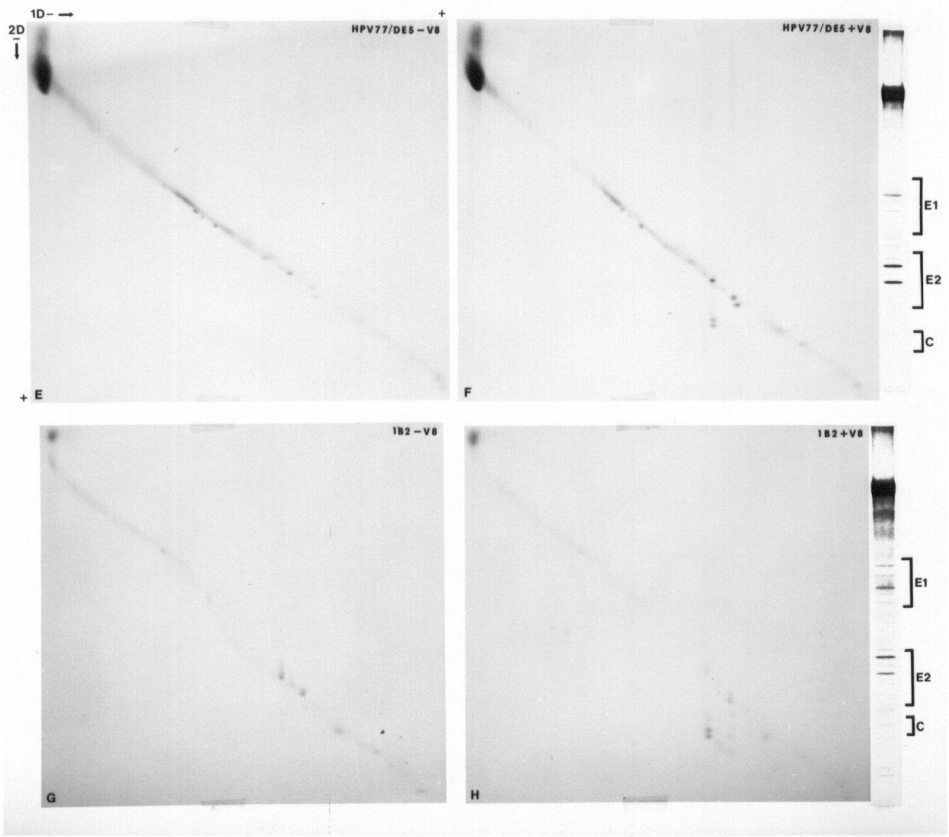
FIGURE 12: RUBELLA VIRUS STRUCTURAL PROTEINS:
S. AUREUS V8 PROTEASE DIGESTION

Vero cell cultures (10 x 10^6 cells) were infected with rubella virus stock preparations. At the first indication of CPE (M33 and RA27/3: 48 hours post-infection, all other strains: six days post-infection), the cells were starved for two hours in 1/10 methionine-containing medium and labelled overnight with 50 µCi/ml ^{35}S-translabel. The labelled supernatants (5 ml/sample) were precipitated with 10% PEG, the resulting pellets resuspended in 1% NP40 in TNE containing 2mM PMSF (a protease inhibitor) and immunoprecipitated with rabbit anti-HPV77/DE5 antiserum (50 µl). Immunoprecipitates were resuspended in SDS sample buffer (60 µl) and 10 µl samples were electrophoresed through the first dimension gel. Dried gels were autoradiographed for four weeks at 4°C.

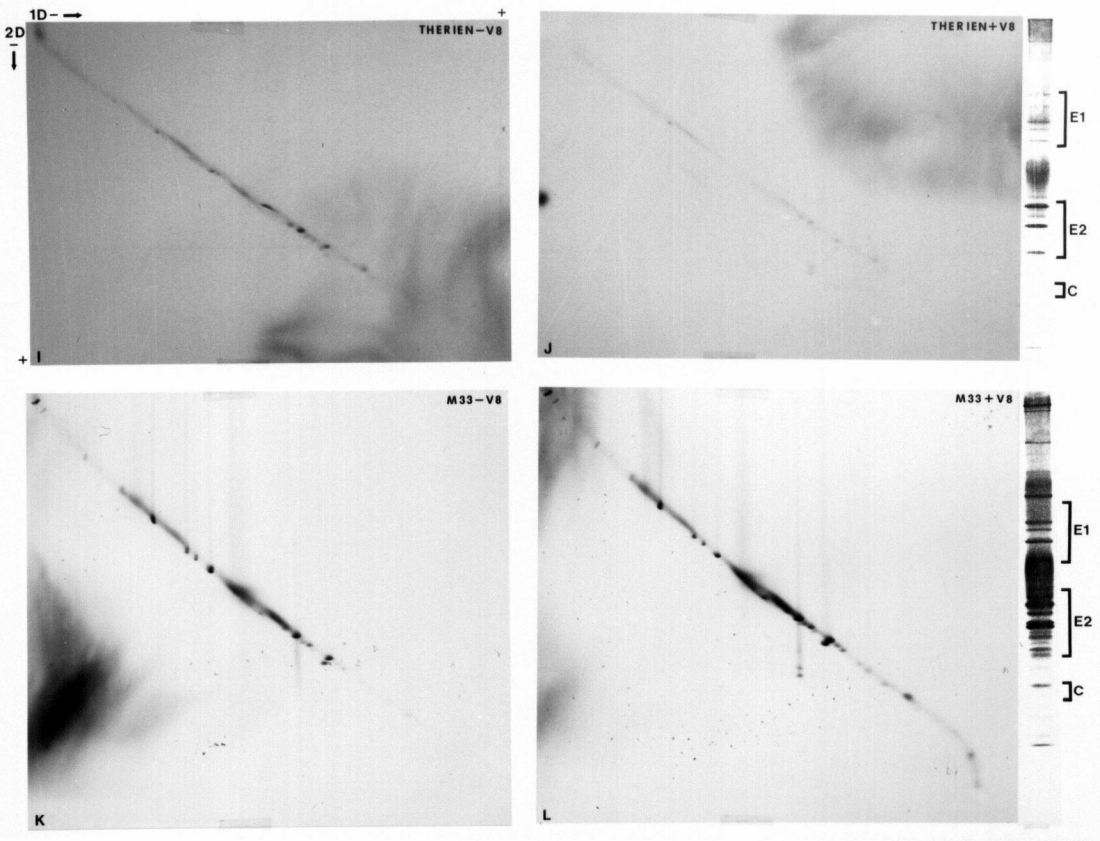
RUBELLA VIRUS STRUCTURAL PROTEINS:
STAPHYLOCOCCUS AUREUS V8 PROTEASE DIGESTION



RUBELLA VIRUS STRUCTURAL PROTEINS:
STAPHYLOCOCCUS AUREUS V8 PROTEASE DIGESTION



RUBELLA VIRUS STRUCTURAL PROTEINS:
STAPHYLOCOCCUS AUREUS V8 PROTEASE DIGESTION



many of the fragments are indistinct, it is clear that at least one of the bands appears in both lanes, indicating that they are probably identical. If, however, the two parental E2 spots are different forms of the same species, it would be expected that more of the digestion pattern would correlate. Digestion patterns for the other rubella virus strains examined show distinct bands for only one E2 species (F,H,J,L). In each case, two fragments are detected from one of the two major E2 species corresponding to those in the controls (E,G,I,K), but cannot be compared with fragments resulting from digestion of the other E2 species.

Published sequences of the E2 glycoprotein show that there are thirteen potential sites for cleavage by V8 protease, and if all the sites are recognized, digestion by the enzyme would therefore generate fourteen peptide fragments. However, one of the potential cleavage sites lies within a potential glycosylation site and two other cleavage sites occur immediately carboxy-terminal to two other potential glycosylation sites. If these glycosylation sites are indeed filled, the V8 protease may be incapable of cleaving the protein because of conformational inhibition or steric hindrance by the glycosidic groups. Thus, theoretically indicated generation of fourteen peptides by V8 may not be achievable in practice.

The digestion patterns presented here must also be considered in light of the detection system used. Initial attempts to Western blot the final gels and immunodetect the peptides generated by digestion with

V8 protease failed. Although there was some evidence of digestion of the proteins, only one or, occasionally, two peptides were visualized (data not shown), and useful comparison of digestion patterns was therefore impossible. The most likely explanation for this inability to detect the peptide fragments immunologically lies in the need to retain intact antigenic epitopes. It is probable that generation of fragments was accompanied by destruction of epitopes recognized by the antisera, and the majority of the fragments consequently remained undetected.

Autoradiography was next tested as a means for detecting peptides. Protein samples labelled with either ^{35}S-methionine or ^{35}S-translabel (labelled methionine and cysteine residues) were digested by V8 protease and electrophoresed. Results with ^{35}S-Met-labelled samples were unsatisfactory, with few peptides being detected. However, digestion of ^{35}S-translabelled proteins was much more successful. This may be explained by once again examining the amino acid sequence for E2. Comparison of mole percentages of amino acid residues present in the E2 protein as reported by Clarke et al. (23) and Kalkkinen et al. (76) shows consistency in methionine (2.3-2.5%) and cysteine (5.0-5.3%) residues; and when these residues are identified within the sequence, it can be seen that labelling with ^{35}S-Met alone will show up a maximum of five out of a possible fourteen generated fragments, while ^{35}S-translabel will label ten fragments. In either case, several potential fragments may go undetected if this method of detection is used.

The situation is even more difficult for the E1 glycoprotein.

There are thirty-four potential V8 cleavage sites in this protein and none exist within or adjacent to any of the three potential glycosylation sites. Thirty-five E1 peptide fragments may thus be generated by V8 protease digestion. Again, if mole percentages of methionine (0.6-0.7) and cysteine (4.9-5.0) residues are considered, and these residues located within the sequence, ^{35}S-Met alone will only label a maximum of three of the possible thirty-five fragments, but ^{35}S-translabel could label a possible eighteen fragments. Even if some of the labelled peptide fragments of E1 were sufficiently hot to be visualized by autoradiography, it must also be borne in mind that if thirty-five fragments are indeed generated by V8 protease digestion, some of the individual fragments would be small enough to migrate completely through the acrylamide gel. In this work a 7.5 to 20% gradient gel was used. This acrylamide concentration may not be optimum for recovery of possible small E1 fragments, and future work with this technique should therefore include some experimentation with different gel formulations and acrylamide concentrations.

If interpretable results are to be obtained from protease digestion, some means of visualization that could detect all the fragments generated by digestion will have to be developed; for only when all fragments are visualized, would it be possible to gain information about the identity of similarly-sized proteins. Possible solutions to this problem include radioactively labelling tyrosine residues with ^{125}I, but since tyrosine residues do not occur in each possible fragment, this will also leave some fragments undetected. The

best solution appears to be one which completely avoids the need for protein-labelling. Silver staining of the second dimensional gel might be a satisfactory technique for visualization. This is a sensitive technique and may be completed in a day, eliminating the long wait for results when autoradiography is employed. It does, however, have drawbacks, including the high cost of the reagents and difficulty in reducing background staining, although this latter complication may be minimized by experimentation to find the right conditions for staining.

SUMMARY AND CONCLUSION

Major variation in the biological activities of several rubella virus reference strains has been detected within the last decade. Differences in haemagglutinating activity, neutralization kinetics, association with joint inflammation and, from this study, tissue tropism all play important roles in determining the course of rubella virus infection, including which cell types will be infected, how much progeny virus is produced and how quickly infection will spread, as well as the occurrence of complications and the establishment of persistent infections. The variety and extent of this biological variation among strains suggests the existence of underlying differences in molecular structure. Mutations in the nucleotide sequences could result in amino acid substitutions, resulting, in turn, in conformational changes or in additions or deletions of side chain structures. These changes can alter the ability of the virus to enter a particular cell type (tissue tropism) and set up a productive infection. Changes in side chain structures, namely carbohydrate moieties, can also result in alterations of virus activity, especially if receptors on host cells no longer recognize the structures. Thus, biological activity can be vastly altered by changes in molecular structures.

An attempt was made in this study to examine six rubella virus strains for gross differences in structural protein composition. One-dimensional SDS-PAGE analysis revealed limited strain variation, with three strains (Therien, HPV77/DE5, 1B2) producing apparently identical

protein patterns and obvious differences in proteins appearing in only the M33, Thomas and RA27/3 strains. Since the many differences in biological functions detected among these strains cannot be accounted for solely by this variation in protein pattern, other means designed to detect more subtle strain differences were employed. Two-dimensional electrophoresis was one such method, since it is capable of resolving proteins of the same molecular weight (size) by isoelectric focussing. However, this technique failed to provide any additional evidence of strain variation due to gross protein differences.

Since an important question yet to be answered about rubella virus structure involves the localization of strain differences to either the protein core structure or the carbohydrate side chain moieties, attempts were also made to strip the rubella virus glycoproteins of their side chains. If this had been successful, proof of whether the strain variation resulted from genetic mutation or post-translational modification (as suggested by the presence of several forms of each glycoprotein) may have been provided. Unfortunately, despite several attempts at deglycosylation, the location of strain differences remains unknown.

Tissue tropism is another characteristic that has been found to vary among rubella virus strains. While basic patterns of the structural proteins were observed in studies of fibroblastic (Vero) and lymphoblastoid (Cess and U937) cell lines infected with five of the six rubella virus strains examined, the sixth strain (RA27/3) appeared to

selectively replicate in only two of the three cell lines, and even in these cell lines, did not replicate to the same extent as the other virus strains. Furthermore, an additional E2 species running just below the displaced M33 E2 band was found in U937 and Cess cells, but not in Vero cells, suggesting that processing (glycosylation) may be different between fibroblastic and lymphoid cells.

An experiment to compare the patterns of structural proteins detected by a monoclonal antibody directed against the E1 protein in comparison with the polyclonal anti-rubella virus antiserum resulted in the observation that, in the M33 strain, the E1 band detected by the monoclonal antibody migrated to the E2 region. It was therefore likely that the different proteins migrating in this region were not solely the result of differential processing of a single species, but also included a second protein species. The S. aureus V8 protease digestion experiments were designed to examine this possibility, and the preliminary evidence obtained suggests that this is indeed the case, with only one peptide generated being detected in both major constituents of the E2 region.

It is apparent from this study that the variation in biological activity of the six rubella virus strains examined results from subtle variations in the molecular structure of the virus and not gross alterations in polypeptide structure of the structural proteins. While it remains a possibility that the variation may result from alterations in the non-structural proteins as well, this remains speculation only

until more characterization of these proteins has been done. Methods of fine structural analysis, including such techniques as enzymatic deglycosylation, peptide mapping and high performance liquid chromatography (HPLC) remain to be adapted to rubella virus as means for detecting and localizing the potentially subtle variations or mutations. Only when these strain variations are detected and correlated with biological function will it be possible to attempt development of a vaccine which will provide a better means for preventing the complications of rubella virus infection.

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