

CHARACTERIZATION AND ISOLATION OF A B-CELL
SURFACE RECEPTOR FOR DIABETOGENIC EMC-D VIRUS
AND STUDIES ON THE RELATIONSHIP BETWEEN
RECEPTOR NUMBER AND PATHOGENESIS IN
VIRUS-INDUCED IDDM IN MICE

CENTRE FOR NEWFOUNDLAND STUDIES

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MANSJANG P. BALDEH



**CHARACTERIZATION AND ISOLATION OF A β -CELL SURFACE
RECEPTOR FOR DIABETOGENIC EMC-D VIRUS AND STUDIES
ON THE RELATIONSHIP BETWEEN RECEPTOR NUMBER AND
PATHOGENESIS IN VIRUS-INDUCED IDDM IN MICE**

BY

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Abstract

The diabetogenic variant of EMC virus, EMC-D, can infect mouse pancreatic β -cells and may induce a type 1 diabetes-like syndrome in SJL/J mice, but not in C57BL/6J mice. A number of reports suggest an association between pathogenicity and the number of β -cell receptors available for EMC-D virus binding. Yet a great deal more is known about EMC-D virus, than about the receptor structures that allow its entry into β -cells. The aim of this project was to characterize the virus receptor on BMP cells and to see if the receptor plays a role in EMC-D virus-induced diabetes in SJL/J mice.

The nature of the EMC-D virus receptor was investigated by *in vitro* attachment of ^3H -labelled EMC-D virus to BMP cells. Virus binding was saturable and reached equilibrium after 2 h at 4°C . Although Scatchard analysis revealed only a single high affinity ($K_d = 1.2 \text{ nM}$; 4×10^5 sites/cell) binding site, Hill analysis of the saturation binding data strongly suggested that EMC-D virus has more than one binding site on BMP cells. Pretreatment of the cells with protease, neuraminidases, or with lectins, showed that the receptor is a protein and that sialic acids are required for virus binding to BMP cells. Pretreating cells with phospholipases C and D or with enzymes specific for N-linked carbohydrates had no significant effect on virus binding.

BMP cell surface receptor regeneration after protease treatment took about 6 h, and was inhibited by cycloheximide, but not by tunicamycin. HPLC analyses of total and neuraminidase-releasable sialic acids suggested that about 30% of the total sialic acid on BMP cells is Neu5Ac; Neu5Gc on BMP cells seemed resistant to *V. cholerae* neuraminidase. It is concluded that the receptor for EMC-D virus on BMP cells is a sialylated glycoprotein, and virus binding involves Neu5Ac, and possibly Neu5Gc, but not N-linked sugars.

The Hill analysis result is supported by the detection of two affinity purified putative receptor proteins (M.W. 97,000 & 70,000 daltons) from BMP cells. Chromatofocusing studies on these proteins confirmed that the putative virus receptor contains Neu5Ac. Monoclonal antibodies raised against the putative receptor blocked EMC-D virus infection of BMP cells and virus binding to β -cells from SJL/J mice. FACS analyses of virus binding and anti-EMC virus anti-idiotypic antibody binding to β -cells, showed that cells from SJL/J and C57BL/6J mice contain about the same number of EMC-D virus receptors. It is concluded that the inability of EMC-D virus to cause IDDM in C57BL/6J may not be due to lack of, or a reduction in the number of virus receptors on β -cells from C57BL/6J mice. It is speculated that anti-idiotypic antibodies may play a role in the initiation of β -cell destruction in virus-infected SJL/J mice, possibly by molecular mimicry.

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List of Abbreviations

AEMC-----	Anti-EMC virus antibody
BB-----	Biobreeding rat
BMP-----	Baby mouse pancreatic cells
C57BL/6J-----	A mouse strain, resistant to EMC-D virus-IDDM
CVA-----	Coxsackievirus A
CVB-3-----	Coxsackievirus B type 3
DOC-----	Sodium deoxycholate
EMC-----	Encephalomyocarditis virus
EMC-B-----	A non diabetogenic variant of EMC virus
EMC-D-----	A diabetogenic variant of EMC virus
GalNAc-----	N-acetylgalactosamine
GlcNAc-----	N-acetylglucosamine
FACS-----	Fluorescent activated cell sorter
FMD-----	Foot-and-mouth disease virus
³ H-----	Tritium
HPLC-----	High performance liquid chromatography
HRV-14-----	Human rhinovirus type 14
ICAM-1 -----	Intercellular adhesion molecule-1
IDDM-----	Insulin-dependent diabetes mellitus (type 1 diabetes)

MDBK-----Mardin-Darby bovine kidney cells

Neu5Ac-----N-acetyl neuraminic acid (a sialic acid)

Neu5Gc-----N-glycolyl neuraminic acid (a sialic acid)

NOD-----Non-obese diabetic mouse

Phosphate buffer --- 0.02 M sodium phosphate buffer, pH 8.0

SJL/J-----A mouse strain, susceptible to EMC-D virus-induced IDDM

VAP-----Virus attachment protein

Chapter One

INTRODUCTION

1.1. General Properties of Viruses

1.1.1. Brief historical background

In 1892 the Russian scientist Dmitrii Iwanowskii reported that when sap removed from tobacco leaves was passed through filters which were designed to trap bacteria, the filtrate contained infectious agents which, when inoculated into fresh tobacco plants, produced tobacco mosaic disease. Iwanowskii concluded, erroneously, that the disease causing agent was a toxin secreted by bacteria present in sap of tobacco leaves. Six years later, in 1898, Beijerinck working in The Netherlands repeated Iwanowskii's experiments and demonstrated transmission of tobacco mosaic disease in bacteria-free filtrates in which no microscopic organism could be detected. Beijerinck called this pathogen of tobacco mosaic disease, which is smaller than common bacteria, "contagious living fluid" or "virus". However, Beijerinck did not invent the word "virus". Before 1898, the term "virus" was used by medical and biological scientists to denote poison or any infectious microorganism, irrespective of its nature (Knight, 1974).

Today, we know a lot more about viruses than their ability to cause disease in plants. We can define a virus as an infectious, replicating microorganism containing either a single stranded or a double stranded DNA or RNA genome, which is surrounded by a protein coat, called the capsid. In some viruses the capsid structure is enclosed within a lipoprotein bilayer which forms an envelope. Viruses use the cellular synthetic machinery to direct synthesis of components required for their replication and, since they depend heavily upon host cell metabolic components for multiplication, they reproduce only inside living cells. Viruses vary in morphology from spherical to rod-like and they measure anywhere between 22 nm to 300 nm in diameter. In contrast, bacteriocoeci and erythrocytes measure approximately 1000 nm and 7500 nm in diameter, respectively.

1.1.2. The picornavirus family

Viruses can be classified on the basis of whether or not they contain a lipoprotein envelope, or on the basis of their polarity (otherwise called sense) in which case the viral mRNA is designated as the plus sense (positive strand) while its complementary sequence, which does not function as mRNA, is known as the minus sense (negative strand). The picornavirus family contains small (30 nm in diameter), non-enveloped, single stranded RNA viruses, shaped in the form of an icosahedron. On the other hand, orthomyxovirus,

paramyxovirus, togavirus and retrovirus families are classified as enveloped, single stranded RNA viruses. Other enveloped RNA virus families with single stranded RNA genomes include coronaviruses, arenaviruses and rhabdoviruses. DNA virus families include adenoviruses and papovaviruses which are non-enveloped and contain double stranded genomes. Poxviruses and herpesviruses also contain double stranded DNA but they are enveloped. Based on host range and physico-chemical properties such as acid stability and buoyant density, the picornavirus family has been divided into four genera: enteroviruses, rhinoviruses, aphthoviruses and cardioviruses.

The enterovirus genus includes polioviruses, coxsackieviruses, echoviruses, enterovirus types 68 to 72, hepatitis A virus and a number of non-human enteroviruses. These viruses inhabit the alimentary tract in humans, and are able to survive in the acidic conditions found in this environment. Polioviruses can also replicate in motor neurons within the central nervous system (Rueckert, 1991).

In contrast to enteroviruses, rhinoviruses have the characteristic feature of being acid labile and are adapted mainly to the nasopharyngeal region. They are the major cause of common cold in adults and children. The genus contains about one hundred and ten human serotypes and two bovine

serotypes (Rueckert, 1991).

The aphthovirus genus consists of foot-and-mouth disease (FMD) virus types 1 - 7 which rarely affect humans but infect cloven-footed animals especially cattle, goats, pigs and sheep. FMD virus is acid labile and it has been suggested that the acid lability of the virus may be due to the high concentration of the basic amino acid, histidine, which lines the interfaces of the pentamer (Rueckert, 1991). The pentamer is the basic building block of the virus capsid and is made up of five copies of four virus proteins (VP), termed VP1 to VP4. These virus proteins are described in section 1.1.3.

The cardiovirus genus includes encephalomyocarditis (EMC) virus, which is the virus I worked with and therefore forms the basis of this thesis. EMC virus was first isolated from non-human primates (Helwig & Schmidt, 1945) and later from pigs (Muriene *et al.*, 1960). Some of the viruses within this genus constitute what has been referred to as the EMC virus group, which includes Columbia SK virus, Maus Elberfeld (ME) virus, MM virus and Mengovirus (Mahy, 1988). These viruses have been shown to be immunologically indistinguishable from EMC virus by neutralization assay (Dick, 1949), complement-fixation test and by cross protection assay (Warren *et al.*, 1949). Theiler's murine encephalomyelitis virus (TMEV) is a member

of the cardiovirus genus but it is not part of the EMC virus group (Mahy, 1988). Like enteroviruses, cardioviruses are acid stable but at pH 6.0, in the presence of 0.1 M chloride or bromide ions at 37°C, EMC virus disintegrates and releases infectious RNA (Dunker & Rueckert, 1969; Dunker & Rueckert, 1971).

The name encephalomyocarditis, resulted from the ability of the virus to produce encephalomyelitis and myocarditis in laboratory mice. Although cardioviruses are generally regarded as murine viruses, EMC virus has been recovered from human patients as well as from a variety of animals which include birds, blood-sucking arthropods, pigs, elephants and squirrels (Dick, 1949; Tesh, 1977). Serological surveys have revealed EMC virus infection of swine in England and Hawaii (Tesh, 1977) and the virus has been isolated from zoo animals such as apes and monkeys which were found moribund, partially paralysed or dead (Helwig & Schmidt, 1945; Roca-Garcia & Sanmartin-Barberi, 1957). In humans, the virus has been found in patients suffering from encephalitis and meningitis (Jungeblut & Dalldorf, 1943; Dick, 1949; Gajdusek, 1955) and serological surveys have revealed that an epidemic of EMC virus infection in humans occurred in the Phillipines (Smadel & Warren, 1947). Other studies indicate that, like poliomyelitis, EMC virus infection in humans tends to occur primarily during childhood and

adolescence. Tesh (1977) believes that in humans, EMC virus infection is fairly common but it is either asymptomatic or unrecognized by the attending physician.

Encephalomyocarditis virus has several variants. Based on its organ tropism in mice, the strain described by Muriane and associates (1960) was classified into two main variants: the E-variant and the M-variant (Craighead, 1966). The E-variant of EMC virus is neurotropic and produces a rapidly fatal infection, while the M-variant is myocardiotropic and usually causes a non-fatal illness with few signs of central nervous system involvement (Craighead, 1966). The M-variant was also shown to induce diabetes in particular strains of mice (Craighead and McLean, 1968). Yoon and collaborators later isolated two more variants by repeated plaque-purification of the M-variant; their isolates were designated as D-variant and B-variant (Yoon *et al.*, 1980). The D-variant was established as highly diabetogenic in certain strains of mice while the B-variant was found to be non-diabetogenic.

1.1.3. The picornavirus capsid

The capsid of picornaviruses measures 22 nm to 30 nm in diameter and is made up of sixty copies of each of four virus proteins (VP) named VP1, VP2, VP3 and VP4, in order of decreasing molecular weight. Among members

of the picornavirus family, VP1, VP2 and VP3 are similar in size to one another. The number of amino acid residues in picornaviral capsid proteins ranges from 209 - 302 for VP1, 218 - 372 for VP2, and 221 - 246 for VP3. VP4, which is much smaller, comprises only 68 to 85 amino acid residues (Rueckert, 1991). VP0 is a precursor picornaviral capsid protein; it appears during viral maturation which occurs after the viral RNA has been packaged and the capsid is being assembled to form an infectious particle. VP0 is cleaved by a virus-coded protease to form VP4 and VP2 (see Fig. 1-1); this process, the cleavage of VP0, is one of the final steps in capsid assembly and is believed to be related to stabilization of the mature virus particle (Rueckert, 1991).

One important function of the capsid is to protect the viral genome from nucleases in the environment. Another function is as a determinant of host range and tissue tropism in non-enveloped viruses, because it serves as an attachment protein, which recognizes specific receptors on the surface of susceptible cells. In addition, the picornaviral capsid determines antigenicity of the virion through variations in the size of the loops which connect eight strands in a β -barrel structure. This structure has been described in HRV-14 (Rossmann *et al.*, 1985), poliovirus type 1 (Hogle *et al.*, 1985), FMD virus (Acharya *et al.*, 1989), and Mengo virus (Luo *et al.*, 1987).

1.1.4. Brief description of the picornavirus genome

Although this thesis is concerned primarily with cell surface receptors for EMC virus, a preamble on the structure of the picornavirus genome and how it is replicated may make it easier for the reader to appreciate how factors other than cellular receptors can affect viral tropism.

The genome of a picornavirus consists of single stranded plus sense RNA. The size of the genome varies from 7,200 nucleotides for rhinoviruses to 8,500 nucleotides for aphthoviruses. As illustrated in Fig. 1.1, the 3' terminus of the RNA strand is polyadenylated (called the poly A tract) and the length of the poly A region varies among members of the picornavirus family (Ahlquist & Kaesberg, 1979). It is shortest in cardioviruses (35 nucleotides) and longest in aphthoviruses (100 nucleotides).

At the 5' terminus a small protein, VPg (virion protein, genome), is covalently attached to the RNA via a tyrosine residue of the VPg protein. Among different picornaviruses, the length of VPg varies slightly, ranging from 20 to 24 amino acid residues. The function of VPg is not known (Rueckert, 1991). Between the 5' VPg and the beginning of the 3' poly A tract, the genome is further divided into a 5' nontranslated region, a protein-coding region and a 3' nontranslated region.

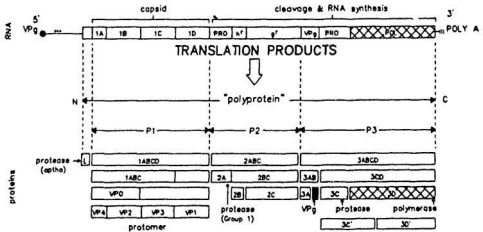


Fig. 1.1 Organization of the picornaviral genome. The three "dots" over the 5' nontranslated region indicate the points of the poly C tract. Protein synthesis is from left (N terminus) to right (C terminus). P1, P2, and P3 are the precursor proteins. (Rueckert, 1991).

The size of the 5' nontranslated region ranges from 624 (rhinoviruses) to 1,200 (aphoviruses) nucleotides (Rueckert, 1991), which is unusually long when compared to the 5' nontranslated regions of cellular RNAs which are less than 50 nucleotides. In some picornaviruses the 5' nontranslated region contains a segment, 80 to 250 nucleotides long, which is rich in cytosine residues (called the poly C tract) and lies 150 nucleotides from the 5' end in cardioviruses, and 400 nucleotides from the same end in aphoviruses (Rueckert, 1991). The function of the 5' nontranslated region in picornaviral genomes remains to be fully elucidated. However, reports suggest that this region is involved in determining some of the biological properties of picornaviruses. For instance, in a study of three strains of poliovirus type 3 which differed in neurovirulence, it was shown that these virus strains varied only at position 472 within the 5' nontranslated region; a single base change from cytosine to uracil at this position caused an increase in neurovirulence in one strain (Sabin) of the virus (Stanway *et al.*, 1984; Cann *et al.*, 1984). Other studies suggest that changes in the nucleotides near position 472 are important in the attenuation of type 1 and type 2 polioviruses (Kawamura *et al.*, 1989, Pollard *et al.*, 1989). A recent report by Philip Minor's group in England showed that the determinants of attenuation and temperature-sensitive phenotypes of the Sabin strain of type 2 poliovirus reside at position 492, within the 5' nontranslated region (Macadam *et al.*, 1991).

The protein-coding region of picornaviral RNAs encodes a long polypeptide chain called the polyprotein. The capsid proteins VP1 to VP4 are encoded at P1 (see Fig. 1.1) which is located at the 5' end of the protein-coding region. Genes at the P2 region code for:

(a) an "early" protease called 2A, whose function is to cleave the capsid proteins away from the nascent polypeptide.

(b) protein 2B, a product of the h^r gene which carries a host-range determinant. It is known that when a given human virus also grows in murine cells, this indicates that the virus has a wide host range. With this knowledge in mind, the function of the h^r gene was elucidated from studies which showed that growth of type 2 human rhinovirus in murine cells, selected for an altered h^r gene product (Lomax & Yin, 1989). How the h^r gene controls host range is yet to be determined.

(c) 2C. The function of 2C is not known, but it is thought it may be involved in the initiation of picornaviral RNA synthesis (Anderson-Sillman *et al.*, 1984; Rightsel *et al.*, 1961; Tershak, 1982).

At the 3' end of the protein-coding region are the P3 genes which code for VPg, a protease called 3C and an RNA-dependent RNA polymerase.

The nascent polyprotein is cleaved by virus-coded proteases so that up to eleven end products are generated (see Fig. 1.1). Most of the cleavages are carried out by protease 3C except those which involve the "early" protease 2A and a maturation protease whose precise identity is not yet known but functions to cleave the precursor virion protein VP0 to yield the mature capsid proteins VP2 and VP4 (Rueckert, 1991).

The 3' nontranslated region which ranges in length from 47 nucleotides for rhinoviruses to 126 nucleotides for cardioviruses, is short in comparison to the 5' nontranslated region which ranges from 624 to 1200 nucleotides. The function of the 3' nontranslated region is not known although one can speculate that it might be important at some stage in replication of the viral genome since this region would be expected to contain signals for polymerase binding.

1.1.5. Entry of viruses into cells

Virus infection of susceptible cells may be cytocidal, resulting in the host cell's death, or non cytocidal, in which case the host cells survive. The replication cycle of a virus in an infected cell can be divided into stages. First, the virus must attach to specific receptors on the cell surface, penetrate into the cell and uncoat before it can begin to replicate. Uncoating involves the

physical separation of the viral genome from the capsid or, in the case of enveloped viruses, disruption of the envelope and liberation of the nucleocapsid. Different viruses uncoat at different locations within the cell; for instance, picornaviruses and poxviruses uncoat in the cytoplasm, whereas herpesviruses uncoat at the nuclear pores. After uncoating, the viral genome is transcribed, translated and replicated and these processes occur repeatedly as new virus-specific proteins, RNA and/or DNA molecules are synthesized in an infected cell.

Some enveloped viruses, typically togaviruses, enter cells by receptor mediated endocytosis (Marsh & Helenius, 1989). In this process virus bound to receptors on the cell surface forms a complex which is moved and accumulated at specialized regions on the plasma membrane called coated pits. These pits are coated on their cytoplasmic side by a protein called clathrin. The coated pits invaginate and are internalized in the form of coated vesicles. Subsequently, the clathrin is removed from the coated vesicles and the uncoated vesicles fuse together to form endosomal vesicles whose internal pH is acidic. The drop in pH within endosomal vesicles causes glycoproteins on the surface of the virus particle to undergo conformational changes which result in the exposure of hydrophobic proteins on the surface of the virus. The hydrophobic proteins promote fusion between the lipid envelope of the virus

particle and the membrane of the endosomal vesicle, resulting in release of the nucleocapsid into the cytoplasm.

Other enveloped viruses make use of virus-specific fusion proteins to gain entry into cells. For example, paramyxoviruses do not enter cells by receptor mediated endocytosis; instead, they use a fusion glycoprotein in their lipid envelope to fuse the virus envelope with the host plasma cell membrane directly, without intervention of clathrin coated endosomal vesicles (Marsh & Helenius, 1989). Following fusion the nucleocapsid is liberated into the cytoplasm.

Adenoviruses are non-enveloped, but they too enter cells by receptor-mediated endocytosis (Pastan *et al.*, 1986). It is obvious that with non-enveloped viruses, entry cannot be by fusion of a viral lipid envelope with the plasma membrane. Pastan and coworkers (1986) have speculated that for non-enveloped viruses, the low pH within the endosome activates a protein on the surface of the virus particle which then causes lysis of the endosomal membrane releasing the virus particle into the cytoplasm.

Picornaviruses are non-enveloped and much of what we know about their entry into cells comes from work done with poliovirus (Madshus *et al.*,

1984b), human rhinovirus type 2 (Madshus *et al.*, 1984c) and EMC virus (Madshus *et al.*, 1984c). After binding to cell surface receptors, poliovirus gets internalized and delivered to endosomes. Studies have shown that a low pH of 5.5 is required for entry of poliovirus RNA into the cytosol (Madshus *et al.*, 1984a). The low pH causes exposure of polioviral hydrophobic proteins and, at physiological temperature, the low pH also causes the virus particle to undergo conformational changes (Madshus *et al.*, 1984b).

Other studies by Madshus and collaborators showed that the observations made with poliovirus do not apply to all members of the picornavirus family. Human rhinovirus type 2 (HRV-2) was found to require a low pH for entry into cells, while EMC virus required a slightly alkaline pH for entry (Madshus *et al.*, 1984c). Although a model for virus entry put forward by Lonberg-Holm & Whitely (1976) suggested that HRV-2 is taken up by endocytosis the mechanism by which picornaviruses enter cells is yet to be fully elucidated.

1.1.6. Strategy for picornavirus replication

Picornaviruses contain an RNA-dependent RNA polymerase (RNA replicase), which can copy their positive RNA strands into negative RNA strands. The requirements for replication of the positive strands to negative

strands are still being worked out. Studies on cell-free synthesis of negative strands from positive templates suggest that at least three proteins are required: a protease called 3C^{pro}, a VPg donor, and a host factor protein with a molecular weight of 67 kd (Baron & Baltimore, 1982a). It is thought that the so called VPg donor (whose identity is not yet known) donates a primer such as VPg-pUpU from which the new negative strand is elongated by protein 3D^{pro}, which is a polymerase (Baron & Baltimore, 1982b; Crawford and Baltimore, 1983). The strategy, by which picornaviruses replicate their genome in infected cells, is to use the copied negative RNA strands as templates for the synthesis of many positive strands; the latter may serve as mRNA and be translated by host ribosomes into proteins, or may be packaged to form progeny virus. A characteristic feature is that, instead of making individual mRNAs which specify single proteins, the picornaviral RNA is translated into one large protein (the polyprotein) which is subsequently cleaved by virus-coded proteases into many proteins, including four individual capsid proteins (VP1, VP2, VP3, and VP4), an RNA-dependent RNA polymerase, proteases and several other proteins (Fig. 1.1).

1.1.7. Strategies by which other viruses replicate

Like the picornaviruses, almost all other RNA containing viruses replicate in the cytoplasm of the infected cell. The only exceptions are

retroviruses and orthomyxoviruses, which require a nuclear phase during replication. Retroviruses contain single stranded plus sense RNA and they are characterized by the presence of reverse transcriptase in the virion. Following penetration of the virion into the cell, the viral genome is reverse transcribed in the cytoplasm, into viral DNA which subsequently enters the nucleus. In short, retroviruses replicate in the nucleus because the viral DNA must become integrated into the cellular DNA to form the provirus, which is used as a template by cellular RNA polymerase II for the synthesis of viral RNA.

Orthomyxoviruses contain single stranded minus sense RNA. Upon entry into cells, they uncoat at endosomal vesicles in the cytoplasm from where the virus genome migrates into the nucleus. These viruses replicate in the nucleus because the virus-coded RNA-dependent RNA polymerase is unable to initiate replication of the negative strand viral RNA into positive strand RNA, without a primer (Plotch *et al.*, 1978). Capped 5' end fragments of cellular mRNAs, which are synthesized in the nucleus, are therefore used as primer (Bouloy *et al.*, 1978; Caton & Robertson, 1980; Plotch *et al.*, 1981). Following replication in the nucleus, the mRNA is transported into the cytoplasm and translated into protein. The strategy for replication, is to build a positive strand RNA from the minus sense single stranded viral RNA genome. The positive strand, which is complementary to the viral RNA, is

used as a template to make negative strand RNA, which is packaged and released as progeny virus.

On the other hand, all DNA containing viruses, except poxviruses, replicate in the nucleus of the infected cell. For example, both poxviruses and herpesviruses contain double stranded DNA genomes which comprise a positive and a negative strand. In simple terms, these viruses replicate by transcription of the negative strand to produce positive strand RNAs (i.e. mRNA), which can be translated into protein. Herpesviruses uncoat at the nuclear pores of the infected cell where the empty capsids remain while the viral DNA is released into the nucleus and transcribed by the cellular DNA-dependent RNA polymerase II. Processing of the mRNA transcript takes place in the nucleus. But poxviruses are unique among DNA containing animal viruses because their genome is transcribed and replicated in the cytoplasm. We know that the cytoplasm of eukaryotic cells lacks the transcriptase required to transcribe DNA and as such, eukaryotic cells synthesize mRNA by transcription of DNA in the nucleus. Therefore, to be able to replicate in the cytoplasm a DNA virus must bring its own enzymes into the cell in order to transcribe the viral genome. Poxviruses are able to replicate in the cytoplasm because they contain a DNA-dependent RNA polymerase which they use to transcribe their genome (Esteban & Holowczak,

1977).

Poxvirus genomes possess other unique features. For instance, it is usual that mRNA transcribed from DNA in the nucleus by host cell DNA-dependent RNA polymerase is post-transcriptionally modified in the nucleus through capping, polyadenylation and splicing, before being transported to the cytoplasm for translation into proteins by host cell ribosomes and tRNA molecules. In the case of the poxvirus genome, the primary transcript is not spliced because the viral mRNAs have no intervening sequences (Moyer & Graves, 1981).

With regard to post transcriptional modifications, poxviruses have the capability to process their primary transcript so that the modified transcript is recognized by the host cell translation machinery present in the cytoplasm. Following transcription of viral DNA in the cytoplasm, poxviruses modify their mRNAs by capping the 5' terminus of the mRNA and polyadenylating the 3' end. Capping involves guanylation and methylation in a process whereby the 5' triphosphate end of a new RNA chain is modified and then linked to the nucleotide guanosine triphosphate (GTP), which is methylated at the guanine base. The effect of capping is that it ensures efficient translation by protecting the 5' ends of mRNAs from enzymes such as nucleases and thus provides

strong sites onto which ribosomes can bind.

In eukaryotic cells, polyadenylation occurs after the transcript has been cleaved by a specific endonuclease, which recognizes the polyadenylation signal sequence AAUAAA. Poxviruses do not have this signal sequence and the reason for this discrepancy is unknown. Perhaps poxviruses have evolved their own polyadenylation signals or perhaps the viral poly (A) polymerase simply polyadenylates the 3' ends of mRNAs without the requirement for a signal sequence.

1.2. Basic Properties of the Plasma Membrane

Cellular membranes contain three major types of lipid: phospholipids, cholesterol and glycolipids. The majority of the lipid component in membranes is cholesterol and phospholipids and, together, they make the membrane less permeable to hydrophilic molecules. The glycolipids may be present in membranes as cerebrosides (glycolipids which contain neutral sugars like glucose and galactose), or as gangliosides (glycolipids which contain sialic acid in addition to sugars). All three types of lipid are amphipathic; that is, one end of the lipid molecule has a hydrophilic head while the other end has a hydrophobic tail.

The plasma membrane consists of a phospholipid bilayer in which cholesterol and various proteins are embedded. The lipid and protein molecules are held together mainly by non-covalent interactions, and because of the fluidity of the plasma membrane, most of the molecules are able to move about in the plane of the membrane. The structure of the phospholipid bilayer is not symmetrical; in other words, the lipid and protein molecules located on the inside face of the bilayer, are different from those situated on the outside. For instance, the human erythrocyte membrane has on its inside face, phospholipids which contain choline at their hydrophilic ends (such as, phosphatidylcholine and sphingomyelin), while the outside face of the bilayer contains phospholipids with terminal amino groups at the hydrophilic end (such as, phosphatidylethanolamine and phosphatidylserine). In the human erythrocyte, this asymmetry in the phospholipid bilayer results in different charges between the two halves of the bilayer. Phosphatidylserine is negatively charged and, therefore, the charge in the interior of the bilayer becomes significantly different from the exterior.

The protein molecules of the plasma membrane have several functions. They may serve as a system for transporting molecules into and outside of the cell. For instance, the enzyme $\text{Na}^+ \text{K}^+$ ATPase which is an ion pump present in the membrane of cells, uses the energy of ATP hydrolysis to pump Na^+ out

of the cell and K^+ in. Plasma membrane proteins may also function as structural links between the membrane and either the cytoskeleton or the extracellular matrix. For example, in the red cell the cytoskeleton, which is principally spectrin, is linked to the membrane by the membrane proteins called band 3 and band 4.1; both membrane proteins bind to spectrin via the protein ankyrin. Some protein molecules of the plasma membrane are intercellular adhesion molecules which may serve to connect the plasma membrane of one cell with that of an adjacent cell or they may serve as cell surface receptors for various ligands (cell adhesion molecules are described in section 1.3.2.).

1.3. Virus-cell Interaction

1.3.1. Definition of a virus receptor

The first stage in the infectious cycle of a virus is attachment to the host cell plasma membrane. This interaction between viruses and cells involves two components: a viral attachment protein (VAP) which recognizes a cellular receptor and a receptor on the cell surface which is recognized by the VAP. In non-enveloped viruses, the capsid is the VAP while in enveloped viruses the protein of the lipoprotein coat forms the VAP.

Cell surface receptors function as binding sites for a wide variety of ligands, which include antigens, hormones, toxins and viruses. A virus receptor is the structure on the surface of a cell to which a virus binds, prior to entering the cell. By definition, virus attachment to cell surface receptors must be followed by virus infection of the cell. A virus receptor can be carbohydrate (Fried *et al.*, 1981), protein (Greve *et al.*, 1989; Staunton *et al.*, 1989) or lipid (Schlegel *et al.*, 1983; Mastromarino *et al.*, 1987) in nature. It is not known how many receptor molecules constitute a receptor site.

It is unlikely that cell surface receptors which serve as virus receptors evolved for the purpose of virus binding. Such receptors have other important biologic functions. For instance, the receptor for reovirus type 3 also serves as the beta adrenergic receptor (Co *et al.*, 1985), although this claim has been contested (Choi & Lee, 1988). Another example is the CR2 molecule which serves as a receptor for Epstein Barr virus and is found on B lymphocytes (Fingerth *et al.*, 1984). CR2 is important in generating immunity against antigens since it also functions as a receptor for the C3 complement fragment, C3d.

Virus binding to cells may occur directly via specific cell surface receptors, or indirectly via intermediate molecules. In antibody enhanced viral

infections, antiviral antibodies may facilitate viral infection of cells by cross-linking virions to cell surface Fc receptors, even when such cells do not possess specific surface receptors for the viruses present. The intermediate molecule does not have to be an antibody. It has been shown that hepatitis B virus can bind to hepatocytes via polymerized serum albumin which binds to albumin receptors on hepatocytes (Machida *et al.*, 1984).

1.3.2. Cell adhesion molecules

The receptor for human rhinovirus type 14 has been identified as a cell adhesion molecule (Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*, 1989a) while that for poliovirus is a member of the immunoglobulin superfamily (Mendelsohn *et al.*, 1989). These virus receptors are discussed in detail in section 1.3.3.

Cell adhesion molecules are cell surface proteins that participate in cell to cell interactions. The interaction may be between two cells or between a cell and extracellular matrix glycoproteins, such as fibronectin or laminin. There are several kinds of cell adhesion molecules and they are expressed on various cells. These cell surface proteins include: the adhesion molecules of the immunoglobulin superfamily, the integrins, the cadherins which are calcium-dependent cell adhesion proteins that can act as receptors or ligands,

and the LEC-CAMs which contain lectin-like domains and are involved in interactions between leucocytes and endothelial cells (Albelda & Buck, 1990). A characteristic feature of the integrins is that they are heterodimers composed of common, smaller β subunits and variable, larger α subunits which are non-covalently associated with each other (Hynes, 1987). Integrins seem to recognize specific amino acid residues, usually those which contain the common sequence, Arg-Gly-Asp (RGD). However there are integrins which bind ligands that do not contain the RGD tripeptide. For example, the integrin $\alpha_4\beta_1$ binds to a region on fibronectin which lacks an RGD sequence (Guan & Hynes, 1990).

In order to classify a protein as a member of the immunoglobulin superfamily, it must contain domains like those found in the immunoglobulin molecule. Each domain on both light and heavy chains of immunoglobulin molecules comprises approximately 110 amino acid residues and all of the domains are stabilized by intra-chain disulphide bonds. A second requirement for inclusion into the immunoglobulin superfamily is that the amino acid sequence of the domains in the molecule of interest, must be significantly similar to those of immunoglobulin or immunoglobulin-related domains. Although the presence of carbohydrate structures is not a criterion for inclusion into the family, it is a characteristic feature for members of the

immunoglobulin superfamily. Neural cell adhesion molecule (NCAM) and lymphocyte function associated antigen type 3 (LFA-3) are just two examples of cell adhesion molecules which belong to the immunoglobulin superfamily. NCAM mediates contact among neurons or between neurons and muscle cells (Rutishauser & Jessel, 1988) while LFA-3 mediates the initial contact between T lymphocytes and antigen presenting cells or target cells, prior to antigen-specific recognition (Springer *et al.*, 1987).

Intercellular adhesion molecule-1 (ICAM-1) is a cell surface glycoprotein which was identified in 1986 (Hogg *et al.*, 1991). It has a molecular weight of 80-110 kd (Hogg *et al.*, 1991) and is expressed on a variety of cell types including endothelial cells, fibroblasts, monocytes and, B and T lymphocytes (Hogg *et al.*, 1991). ICAM-1 does not contain the tripeptide (RGD) recognition sequence used by some integrins and, unlike the integrins, it is not made up of α and β protein subunits (Hogg *et al.*, 1991). ICAM-1 has a rod-like structure and contains five immunoglobulin-like extracellular domains at its amino terminal, a single transmembrane domain and a short C-terminal cytoplasmic domain (Staunton *et al.*, 1990). The extracellular domain contains many potential N-linked glycosylation sites and each of the five extracellular domains shows significant amino acid sequence similarity with other members of the immunoglobulin family (Staunton *et al.*, 1990). Based on

the size of the proteins recognized by antibodies specific for ICAM-1, it has been shown that rat and mouse ICAM-1 are similar but not identical to human ICAM-1 (Horley *et al.*, 1989). At the protein level, murine ICAM-1 shares 50% homology with human ICAM-1 (Horley *et al.*, 1989; Ballantyne *et al.*, 1989).

1.3.3. Cell surface receptors for picornaviruses

So far, human rhinovirus type 14 is the only picornavirus whose cell surface receptor has been unambiguously identified (Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*, 1989a). For others such as poliovirus, coxsackie virus or foot-and-mouth disease virus only the general nature of the cell surface receptor is known.

1.3.3.1. The rhinovirus receptor

Studies on the cell surface receptor for rhinoviruses can be traced back almost three decades, to when it was first realized that different rhinoviruses use different receptors to bind to cells (Haff *et al.*, 1966). Haff and collaborators found that both human rhinovirus type 2 (HRV-2) and HRV-14 attached to the human cell line, WI-26, but only HRV-2 could bind to African green monkey kidney cells. Later, studies showed that trypsin treatment prevented rhinovirus from binding to cells (Stott & Heath, 1970). Biochemical

studies of rhinovirus attachment to cells further showed that exposure of the cells to the lectin concanavalin A, which recognizes the sugars mannose and glucose, prevented virus binding to cells. These findings suggested that the receptor is a cell surface glycoprotein (Lonberg-Holm, 1975). Subsequent studies revealed that a monoclonal antibody made against HeLa cell surface molecules blocked rhinovirus binding to HeLa cells and prevented certain serotypes of the virus from infecting the cells. Colonno and associates (1986) reported that, out of about 110 distinct serotypes of human rhinovirus, close to 90% bound to a single receptor on HeLa cells. This major group of rhinoviruses is represented by HRV-14 while the prototype virus for the remaining 10% (the minor group) is HRV-2. It was not until 1989, that a glycoprotein with an apparent molecular weight of 90 kd was isolated as the cell surface receptor for the major group of rhinoviruses (Tomassini *et al.*, 1989a). This glycoprotein has been identified as human ICAM-1 (Greve *et al.*, 1989; Staunton *et al.*, 1989). Interestingly, HRV-14 does not bind to murine ICAM-1 (Staunton *et al.*, 1990).

Very little is known about the receptor for the minor group of rhinoviruses. It is possibly a sialylated glycoprotein because binding of HRV-2 to cells is prevented by concanavalin A, which recognizes mannose and glucose, and by wheat germ lectin which recognizes N-acetylneuraminic acid

and N-acetylglucosamine (Tomassini *et al.*, 1989b). Other reports have suggested that the minor group receptor has a molecular weight of about 450 kd (Mischak *et al.*, 1988). Cell surface expression of the minor group rhinovirus receptor is not affected by treating cells with cytokines such as interleukin-1, γ -interferon and tumour necrosis factor (Tomassini *et al.*, 1989a).

Evidence that human ICAM-1 is the cell surface receptor for HRV-14 was derived mainly from experiments which showed that HeLa cells transfected with cDNA encoding human ICAM-1, expressed ICAM-1 on their cell surface and bound radiolabelled HRV-14 whereas non-transfected cells which did not express ICAM failed to bind HRV-14 (Tomassini *et al.*, 1989a). When ICAM-1 was purified and adsorbed to a plastic plate, 40% of the input virus bound to the molecule, within 90 minutes. No significant increase was observed in HRV-14 binding to bovine serum albumin similarly adsorbed to a plastic plate, during the same period. Furthermore, using cytopathic effect to determine virus infectivity, it was found that exposure of the cells to anti-ICAM-1 monoclonal antibodies prevented HRV-14 infection of HeLa cells, whereas a control monoclonal antibody did not prevent HRV-14 infection of the same cells (Colonno *et al.*, 1986; Colonno *et al.*, 1988). The anti-ICAM-1 monoclonal antibodies which blocked infection of HRV-14, did not prevent

other viruses from infecting the same cells, indicating specific blocking of the receptor for HRV-14. In addition, the amino acid sequence of the HRV-14 receptor matched the predicted amino acid sequence for ICAM-1, deduced from the cDNA sequence (Greve *et al.*, 1989; Staunton *et al.*, 1989).

1.3.3.2. The poliovirus receptor

Initial research on poliovirus-cell interaction studied the bond between polioviruses and cells, and the effects of ions and temperature on poliovirus attachment. It was found that binding of poliovirus to monkey kidney cells and human HeLa cells occurred electrostatically, the virus binding being salt-dependent and temperature-independent (Youngner, 1955; Fogh, 1955; Bachtold *et al.*, 1957). Later, it was shown that poliovirus replicated in cultured cells of human or monkey origin but not in cells from species such as mouse, dog, rabbit or swine (McLaren *et al.*, 1959). However, even within infectable primates, poliovirus replication occurred only in specific cells and tissues. The virus was found to attach to human and monkey cells from the central nervous system and intestine, but not to human cells from the lung, heart or skin. By the middle 1950s, it was becoming clear that there was an association between cell surface receptors and, pathogenicity and tissue tropism of polioviruses (Kaplan 1955; McLaren *et al.*, 1959).

In 1974, the gene encoding the cell surface receptor for poliovirus was mapped to chromosome 19. In that study, it was shown that in a population of somatic cell hybrids between human and mouse cells, cells susceptible to poliovirus infection contained chromosome 19, whereas cells which were resistant to poliovirus infection lacked chromosome 19 (Miller *et al.*, 1974). Other studies revealed that by using whole Hep2C or HeLa cells as immunogen, monoclonal antibodies could be produced which protected the cells from infection by all three poliovirus serotypes (Minor *et al.*, 1984; Nobis *et al.*, 1985). In 1986, using a procedure called DNA-mediated gene transfer (described in section 1.3.9.), Mendelsohn and associates were able to transfer the poliovirus receptor gene into previously receptor-negative mouse cells, so that the mouse cells expressed the poliovirus receptor and became susceptible to poliovirus infection (Mendelsohn *et al.*, 1986). Later, Mendelsohn's group isolated two cDNA clones which encoded functional receptors for poliovirus (Mendelsohn *et al.*, 1989). The predicted molecular weights of the two receptor proteins were reported to be 43 kd and 45 kd, and the poliovirus receptor was identified as a new member of the immunoglobulin superfamily (Mendelsohn *et al.*, 1989). Later, mutant poliovirus receptor cDNAs were constructed so that the domains of the receptor molecule which are required for binding could be identified. Koike and associates reported that the poliovirus binding site is located at the N-terminal domain, which is called

domain 1, and they claimed that the cytoplasmic domain is not essential for virus infection (Koike *et al.*, 1991). Other researchers have found that while domain 3 and the extreme carboxyl terminus are not required for viral receptor function, the two N-terminal domains (domains 1 and 2) play a role in the initiation of poliovirus infection (Freistadt & Racaniello, 1991).

There is however a complication; another research group reported that the cell surface receptor for poliovirus is a 100 kd protein (Shepley *et al.*, 1988). This group used a non-genetic approach; they isolated the poliovirus receptor by immunoaffinity, using a monoclonal antibody which was generated by immunizing mice with HeLa cells. According to Shepley and associates, the monoclonal antibody they produced, specifically inhibited radiolabelled poliovirus binding to HeLa cells. When it was used to probe blots containing membrane preparations, the antibody detected a 100 kd protein only in those cells and tissues which are susceptible to poliovirus infection (Shepley *et al.*, 1988). They also found, that the 100 kd protein was detected in human cells from the spinal cord but not in any murine tissue, which is consistent with the observed tropism for poliovirus. The 100 kd protein is not encoded by the receptor gene cloned previously by Mendelsohn and associates (Shepley 1992).

These two contradictory findings suggest that the cell surface receptor

for poliovirus is either a 43-45 kd protein or a 100 kd protein. Alternatively, assuming that the 43-45 kd protein is the true receptor, the 100 kd protein may be closely associated with the poliovirus receptor site but not encoded by the poliovirus receptor cDNA clones isolated by Mendelsohn and collaborators. If the latter is true, then perhaps the monoclonal antibodies which detected the 100 kd protein were able to block poliovirus infection by steric hindrance.

1.3.3.3. Cell surface receptors for coxsackievirus

The coxsackieviruses have been divided into two groups based on the type of tissue damage produced after inoculation into suckling mice. Group A coxsackieviruses (CVA) affect the skeletal muscle cells of newborn mice causing flaccid paralysis, whereas group B coxsackieviruses (CVB) usually cause lesions in the central nervous system and induce spastic paralysis.

About three decades ago, McLaren and associates (1960) reported that groups A and B coxsackieviruses attach to different receptors on the surfaces of human cells. The report also showed that coxsackievirus types B1, B3 and B5 attached to a continuous human cell line whereas coxsackievirus type A9 did not attach to these cells, although the A9 virus could infect the same cells if its RNA was transfected into them. Further studies showed that the six

serotypes of CVB attached to a common receptor on HeLa cells which is distinct from the receptor used by group A coxsackieviruses (Longberg-Holm *et al.*, 1976).

Cell surface receptors for CVB have been shown to be integral membrane proteins which are regenerated by synthesis of new receptors (Zajac & Crowell, 1965a; Zajac & Crowell, 1965b; Levitt & Crowell, 1967). While trying to isolate the CVB receptor, Krah and Crowell (1985) showed that the prototype for this group of viruses, CVB type 3 (CVB-3), binds to sodium deoxycholate solubilized HeLa cell membranes and forms a virus-receptor complex. Virus binding to the receptor was shown to be sensitive to treatment with glycosidases and lectins, suggesting that the receptor is a glycoprotein. The CVB-3 receptor on HeLa cells was later isolated as a 49 kd protein (Mapoles *et al.*, 1985). Monoclonal antibodies raised against the HeLa cell surface were shown to protect HeLa cells from infection with CVB types 1, 3 and 5, and CVA type 21 but not HRV-14, although previous competition experiments showed that both CVA-21 and HRV-14 attached to the same receptors (Crowell *et al.*, 1986).

Reports from more recent studies suggest that CVB could employ more than one receptor on the cell surface (Lee-Hsu *et al.*, 1990). Human

rhabdomyosarcoma (RD) cells lack receptors for CVB and therefore RD cells do not support CVB growth. Through blind passage of CVB-3 into RD cells, a variant of CVB-3 (called CVB3-RD) was generated which could grow in RD cells (Reagan *et al.*, 1984). Further studies showed that CVB3-RD attached to a 60 kd protein on RD cells. However, on HeLa cells CVB3-RD attached to a 60 kd protein and to a 49 kd protein, similar to the putative receptor protein previously identified for the prototype virus CBV-3 (Lee-Hsu *et al.*, 1990). These receptor proteins have not yet been identified but it would be interesting to know whether the CVB receptor is also a member of the immunoglobulin superfamily.

1.3.3.4. Cell surface receptors for other picornaviruses

Compared to receptors for HRV-14 and poliovirus, very little is known about receptors for other picornaviruses. Studies on the foot-and-mouth disease (FMD) virus particle have revealed that the virus attachment site in all seven serotypes contains the Arg-Gly-Asp (RGD) sequence (Fox *et al.*, 1989). This is the tripeptide sequence commonly found in the integrins (described in section 1.3.2.). Since FMD virus binding to receptors on BHK cells was blocked by peptides containing the RGD sequence, it was suggested that the gene for the FMD virus receptor might belong to the integrin supergene family (Fox *et al.*, 1989).

Recent reports suggest that the receptor for echovirus type 1 is also a member of the integrin family (Bergelson *et al.*, 1992). According to Bergelson and associates (1992), their monoclonal anti-receptor antibody protected susceptible cells from infection and recognized both protein subunits of the integrin VLA-2. Human rhabdomyosarcoma (RD) cells express low amounts of VLA-2 and bind only minimal amounts of echovirus-1 (Bergelson *et al.*, 1992). The investigators reported that following transfection with cDNA encoding the VLA-2 gene, the RD cells expressed cell surface VLA-2 and gained the ability to bind echovirus-1 (Bergelson *et al.*, 1992).

The picornavirus that our laboratory is involved with, namely EMC virus, attaches to human erythrocytes, although the biological significance of its interaction with red blood cells is not known. It has been suggested that virus-erythrocyte interactions probably augment the host's immune response by aiding virus clearance and by presenting viruses more effectively as antigens to immunocompetent cells (McClintock *et al.*, 1980). Although EMC virus attachment to human and murine nucleated cells has been investigated (McClintock *et al.*, 1980), most of what is known so far about the EMC virus receptor was derived from studies on human erythrocytes, which do not support virus growth. Reports from our laboratory show that EMC virus binds to glycophorin A on human erythrocytes (Allaway & Burness, 1986).

Glycophorin A is a member of a family of red cell sialoglycoproteins called glycophorins A to E (Anstee, 1990; Cartron *et al.*, 1990). About 85% of the glycophorins on human erythrocytes is glycophorin A while approximately 10% of the glycophorins on these cells is glycophorin B. Glycophorin A contains 131 amino acid residues and, residues 1 to 72 form the amino-terminal extracellular domain. Glycophorin A exists in two antigenic forms known as glycophorin A^M with serine and glycine in positions 1 and 5, respectively, and glycophorin A^N with leucine and glutamic acid in these positions. The glycophorin A molecule contains 15 oligosaccharides O-linked to serine and threonine residues and one N-glycosidically linked to asparagine (Marchesi *et al.*, 1976). The extracellular domains of glycophorin A and glycophorin B contain notable differences. The first 26 amino acids, including glycosylation sites, are identical in glycophorin A^N and in glycophorin B. Amino acids 27 to 56 in glycophorin A have no equivalent residues in glycophorin B. Residues 57 to 72 in glycophorin A show significant homology with residues 27 to 43 in glycophorin B (Anstee, 1990; Cartron *et al.*, 1990).

The conclusion that glycophorin A is the binding protein for EMC virus on human erythrocytes was based on the inhibition of virus attachment to erythrocytes pretreated with *Vibrio cholerae* neuraminidase (Angel & Burness, 1977), or with proteases such as papain and ficin (Allaway & Burness, 1986),

and on the inability of virus to attach to En (a-) red cells (Allaway & Burness, 1986), which are otherwise normal cells that lack glycophorin A but contain glycophorin B (Taliano *et al.*, 1980). Burness and Allaway (1986) also reported that the EMC virus binding site on human red cells is located at amino acid residues 27 to 56, which are unique to glycophorin A.

1.3.4. Cell surface receptors for other viruses

So far, the best characterized viral receptors belong to four RNA containing viruses. Two of these, rhinovirus and poliovirus are non-enveloped picornaviruses and their receptors have been described (see sections 1.3.3.1. and 1.3.3.2., respectively). The other two, influenza virus and human immunodeficiency virus type 1 (HIV-1) are enveloped. The receptor for influenza virus has been identified as sialic acid (Paulson, 1979) while that for HIV-1 is the CD4 molecule of T lymphocytes (Dangleish *et al.*, 1984; Klatzman *et al.*, 1984). The CD4 molecule is a 60 kd glycoprotein which functions as a cell adhesion molecule by binding to class II major histocompatibility complex (MHC) molecules on B lymphocytes and antigen-presenting cells. The process by which the CD4 molecule was identified as a receptor for HIV-1 was very thorough. First, the virus was shown to infect cells that express the CD4 molecule. Then it was shown that monoclonal antibodies directed against the CD4 molecule blocked HIV-1 binding or

infection of CD4-positive cells (Dangleish *et al.*, 1984; Klatzman *et al.*, 1984). Also, HIV-1 binding to cells blocked binding of the monoclonal antibodies to the CD4 molecule (McDougal *et al.*, 1986a). Using lymphocytes infected *in vitro*, immunoprecipitation of HIV-infected cells with HIV antibodies was shown to precipitate a complex of the HIV envelope glycoprotein (gp120) and the CD4 molecule (MacDougal *et al.*, 1986b). Further, human cells that lacked the CD4 molecule and were not infectable by HIV, acquired the ability to bind virus and become infectable following transfection with cDNA encoding the CD4 protein (Maddon *et al.*, 1986).

Although the evidence that HIV-1 infects CD4-positive T lymphocytes (Dangleish *et al.*, 1984; Klatzman *et al.*, 1984) and other CD4-bearing cells such as monocytes and macrophages (Gartner *et al.*, 1986; Ho *et al.*, 1986) is strong, a number of studies have also shown that cells that do not express demonstrable amounts of CD4 can be infected with HIV-1 (Cheng-Mayer *et al.*, 1987; Dewhurst *et al.*, 1987; Cao *et al.*, 1990; Kanopka *et al.*, 1991). Other studies showed that infection of cells by HIV-1 is not always prevented by anti-CD4 antibodies or neutralizing doses of recombinant soluble CD4 (Clapham *et al.*, 1989; Weiss *et al.*, 1989; Weber *et al.*, 1989) suggesting that receptors other than CD4 are involved in the susceptibility of cells to HIV-1 infection.

Evidence for the identity of receptors for other viruses is not as strong as that for HIV-1, rhinovirus or poliovirus. However, the receptor for reovirus type 3 on susceptible cells is suggested to be the β -adrenergic hormone receptor (Co *et al.*, 1985) although this claim has been contested (Choi & Lee, 1988). The cell surface binding molecule for rabies virus may be the acetylcholine receptor (Burrage *et al.*, 1985) or gangliosides (Superti *et al.*, 1986). The sendai virus receptor on susceptible cells is also suggested to be a ganglioside (Markwell *et al.*, 1984). Vesicular stomatitis virus may bind to phosphatidylserine or phosphatidylinositol (Schlegel *et al.*, 1983; Mastromarino *et al.*, 1987). Among DNA containing viruses, hepatitis B virus binds to hepatocytes via polymerized serum albumin which binds to albumin receptors on hepatocytes (Machida *et al.*, 1984). The class I MHC molecule has been suggested as receptors for human adenovirus (Chatterjee & Miazal, 1984) and for simian virus 40 (Atwood & Norkin, 1989), while the CR2 molecule found on B lymphocytes serves as a receptor for Epstein-Barr virus (Fingerroth *et al.*, 1984).

1.3.5. Virus attachment proteins of picornaviruses

Although Colonno and collaborators (1986) showed that almost 90% of rhinovirus serotypes shared a single receptor (described in section 1.3.3.1.), further studies revealed that there was no cross-immunity between members

of this major group of rhinoviruses. This observation suggested that the virus attachment site for the major rhinovirus group is not immunogenic. To explain this phenomenon, Rossmann postulated the canyon hypothesis which suggests that the virus attachment site of the virion comprises a cleft, the inside of which is likely to contain conserved amino acid residues among similar strains of virus (Rossmann *et al.*, 1985). The hypothesis further suggests that such a depression on the virus is protected from the host's antibody response because it would be too narrow for an antibody Fab fragment with a diameter of 35 Å to penetrate. According to the hypothesis, the region surrounding the cleft would contain hypervariable amino acid residues which could represent binding sites for neutralizing antibodies and tolerate mutations so as to escape the host's immune response (Rossmann *et al.*, 1985).

Using X-ray crystallographic studies to determine the atomic structures of viral capsids, it has been shown that the virus attachment proteins of human rhinovirus type 14 (Rossmann *et al.*, 1985; Arnold & Rossmann, 1990), poliovirus type 1 (Hogle *et al.*, 1985) and mengo virus (Luo *et al.*, 1987) contain depressions which have been variously referred to as "canyon", "cleft" or "pit". In rhinovirus, the cleft is 25 Å deep, has a diameter tapering from 30 Å at the rim to 12 Å at the bottom and is lined by amino acid residues from VP1 and VP2 (Rossmann *et al.*, 1985). Mengo virus contains a 20 Å deep cleft

on its surface, which is lined primarily by amino acid residues from VP1 and VP2 and a segment of VP3 (Luo *et al.*, 1987). Amino acid changes in the VP1 region of the Lansing strain of poliovirus type 2 have been shown to significantly affect neurovirulence of the virus in mice suggesting that the attachment site involves VP1 (Moss & Racaniello, 1991). These depressions have also been found on viruses other than picornaviruses. For instance, the haemagglutinin glycoprotein of influenza virus which projects 130 Å from the viral membrane, contains a cleft which is a more shallow depression than those found on some picornaviruses. The cleft on the haemagglutinin of influenza virus measures about 10 Å across and 5 Å deep (White & Littman, 1989).

In support of the canyon hypothesis, it has been reported that the cleft found at the influenza virus attachment site binds sialic acid, contains conserved amino acid residues and is surrounded by amino acids that vary between influenza epidemics (Wiley *et al.*, 1981; Weis *et al.*, 1988). Among picornaviruses, it has been reported that the immunogenic sites for HRV-14 are located at the hypervariable region surrounding the cleft, within the virus attachment site (Sherry *et al.*, 1986). Site-directed alteration of the amino acid residues inside the cleft was shown to affect HRV-14 binding to isolated cellular receptors (Colonna *et al.*, 1988). When drugs were used to selectively

alter the topography of the floor in the cleft, it was found that attachment of HRV-14 to HeLa cells and to isolated receptors was blocked (Pevear *et al.*, 1989).

In contradiction to the canyon hypothesis, there are reports which indicate that even among picornaviruses the cleft phenomenon is not universal. For instance, the attachment site of FMD virus does not have a cleft. Rather, the domain of FMD virus which binds the receptor is located at the surface of a prominent, antigenic loop, which contains amino acid residues from VP1 (Acharya *et al.*, 1989). This region contains a conserved arginine-glycine-aspartic acid (RGD) sequence which is surrounded by residues that vary from strain to strain (Fox *et al.*, 1989).

With coxsackie viruses, Beatrice and coworkers (1980) have suggested that VP2 serves as the virus attachment protein since coxsackie virus B3 is neutralized by antibodies to VP2. In Theiler's murine encephalomyelitis virus, VP1 has been shown to be important in determining virus persistence and disease in mice (Zurbriggen *et al.*, 1991). Depending on the virus, reports vary as to which capsid protein is primarily involved in virus attachment to cells. This makes one to wonder whether a single capsid protein functions as the viral attachment site. It is conceivable that interactions between the three

exposed capsid proteins VP1, VP2 and VP3, may result in a unique conformational state which allows binding of virus to cell surface receptors.

1.3.6. Virus attachment proteins of other viruses

Adenoviruses possess apical projections called pentons which have been identified as the site of attachment to cells (Morgan *et al.*, 1969; Boulanger and Lonberg-Holm, 1981). The evidence for this conclusion is that antibodies against the pentons inhibited adenovirus attachment to susceptible cells and virus binding was competitively blocked by excess of purified penton protein. On the other hand, reoviruses have an outer capsid which is composed of three polypeptides: $\mu 1C$, $\sigma 3$, and $\sigma 1$ (Smith *et al.*, 1969). The $\sigma 1$ polypeptide which makes up 1 - 2% of the outer capsid and is located at the vertices of the icosahedral structure is the major determinant of reovirus interactions with cells (Lee *et al.*, 1981).

The virus attachment proteins of enveloped viruses such as herpesviruses, togaviruses, poxviruses, orthomyxoviruses, paramyxoviruses and rhabdoviruses consist of glycoproteins which are specific to a particular group of viruses. For example, in herpes simplex virus the gB glycoprotein appears in a dimeric form in the viral envelope and is essential for penetration and for infectivity of the virus (Person *et al.*, 1982). The J12 mutant of herpesvirus

which lacks gB glycoprotein is able to attach but not penetrate susceptible cells suggesting that gB is essential for infectivity of herpesvirus at the level of penetration (Little *et al.*, 1981). Respiratory syncytial virus, a member of the paramyxovirus family, has two surface glycoproteins, GP90 and GP70. GP90 is reported to serve as the attachment protein (Ferne and Gerin, 1982). On the surface of some togaviruses there are three glycoproteins, E1, E2 and E3; E1 and E2 are reported to serve as virus attachment proteins (Garoff *et al.*, 1982). Only one surface glycoprotein in rhabdoviruses called G glycoprotein is reported to serve as the attachment protein (Wagner *et al.*, 1983).

Orthomyxoviruses have two surface glycoproteins referred to as haemagglutinin and neuraminidase but only the haemagglutinin serves as the attachment protein (Klenk *et al.*, 1975). Poxviruses have about seven surface glycoproteins but it is not known which glycoprotein is involved in virus attachment to cells. Human immunodeficiency virus type 1 uses an envelope glycoprotein (gp120) to bind to the CD4 molecule (Landau *et al.*, 1988). It has been reported that a segment of the HIV-1 gp120 which interacts with the CD4 molecule contains regions which are highly conserved among various HIV-1 isolates as well as in HIV-2 and simian immunodeficiency virus, which also bind to CD4 (Lasky *et al.*, 1987).

1.3.7. Factors which influence virus attachment to cells

Virus attachment to cells *in vitro*, is influenced by physical conditions such as temperature, pH, ionic strength and cell concentration (Lonberg-Holm and Philipson, 1974). For instance, the rate at which rhinoviruses attach to cells is reduced at temperatures below 37°C, probably due to the need for receptors to diffuse laterally through the membrane (Dimmock, 1982). Binding of EMC virus to L929 and Friend cells has been shown to be reduced as the temperature was raised, possibly due to an increase in the rate of dissociation. In contrast, EMC virus binding to HeLa cells was unaffected by temperature; similarly, the attachment of Mengo virus to L929 cells was independent of temperature (McClintock, *et al.*, 1980).

The interaction between viruses and their receptors is mainly electrostatic and it is therefore strongly influenced by the charge distribution on both the ligand and the receptor (Perutz, 1978; Bailey *et al.*, 1984). Binding of virus does not occur in the absence of ions (Holland and McLaren, 1959). Some rhinoviruses, coxsackievirus A9 and FMD virus require divalent cations, while some group B coxsackie viruses and echoviruses are strongly influenced by the pH of the environment (Fiala and Kenny, 1967; McLaren *et al.*, 1960).

Binding of viruses like influenza to the cell surface can result in

destruction of the receptor itself. The surface of the influenza virus is covered with tightly packed haemagglutinin and neuraminidase glycoproteins and it is the haemagglutinin which binds virus to sialic acid-containing cell surface receptors, since antibody to the haemagglutinin but not to neuraminidase, neutralizes infectivity and prevents agglutination of erythrocytes. It has been reported that the neuraminidase of influenza virus destroys sialic acid containing cell surface receptors (Collins and Knight, 1978). If this is true, it is a puzzle that a virus would contain on its surface, the mechanism for destroying cell surface receptors, which it needs to infect cells. However, according to Merz and associates (1981), haemagglutinin and neuraminidase activities can be regulated by environmental conditions such as chloride concentration and pH. High concentrations of halide ion enhance haemagglutinating activity while neuraminidase activity is inhibited at high concentrations of these ions (Merz et al., 1981).

Changes in cell concentration, which affect the number of receptors available, also affect attachment in a directly proportional manner. Virus concentrations too can have an effect upon attachment because saturation occurs as the upper limit is approached. A reduction in the rate at which virus attaches to cells could occur as a result of competition for receptor sites on the cell surface. Other factors which influence the rate of virus attachment

to cells include reagents such as sucrose that increase viscosity, which then decreases attachment rates (Crowell and Siak, 1978).

Sulphydryl groups on the surface of some picornaviruses have an influence on virus attachment. For example, echoviruses are profoundly affected by the presence of reagents that block sulphydryl groups whereas polioviruses are less influenced (Philipson and Choppin, 1962).

Cell surface molecules which by themselves do not serve as virus receptors but which are located close to a virus receptor may influence virus attachment to cells. Because HIV-1 attaches to mouse cells which have been transfected with the gene encoding the CD4 molecule without infecting the cells, it has been suggested that HIV-1 probably also binds to the MHC class II molecule HLA-DR, which may be in close proximity to the CD4 molecule (Mann *et al.*, 1988). Other factors which may affect the attachment of viruses to cells include natural or synthetic molecules (such as antibodies or peptides) which resemble structurally or conformationally the binding site on the virus or the binding domain on the receptor. These molecules, if present, could competitively inhibit binding of viruses to receptors on susceptible cells.

1.3.8. Factors which could affect viral tropism

Expression of cell surface receptors for viruses is not the only factor that determines viral tropism. The inability of viruses to infect cells can be overcome by transfecting the cells with viral DNA, and resistance to virus infection may be due to a block in virus entry, to uncoating, or to non expression of specific cellular proteins required for viral replication.

There are several examples which illustrate cases where factors other than cellular receptors may determine whether or not cells become infected. One such case is the cell surface receptor for HRV-14, human ICAM-1, which has been shown to be expressed on a variety of human cell types including cells which are resistant to rhinovirus infection (Hogg *et al.*, 1991). The fact that the receptors are found on a variety of cell types, including cells which are resistant to infection, suggests that factors other than cellular receptors may be involved in rhinovirus tissue tropism. Similarly, polioviruses have been shown to bind to insect cells which express the poliovirus receptor on their surface, without leading to a productive infection suggesting that the defect may be at the virus entry stage or later (Racaniello, 1992).

Another example where viral tropism has been shown to be affected by factors other than cell surface receptors occurs in retroviruses. The retroviral

life cycle consists of the processes of viral entry, reverse transcription of the RNA genome, integration of viral DNA, transcription, translation, post translational modification including protein processing, and assembly of the virus particle. It has been reported that intracellular levels of deoxyribonucleotides, the substrates for reverse transcription, can vary between host cells suggesting that the rate of reverse transcription can be a factor affecting retroviral growth (Hao *et al.*, 1988). Also, retroviral genes contain nucleotide sequences called long terminal repeats (LTR). These repeat sequences (LTRs) carry multiple signals such as promoters and enhancers. There are reports which suggest that susceptibility of lymphocytes to retroviral infection is linked to the influence of LTRs on gene expression (Garcia *et al.*, 1987). Because the LTRs interact with a number of cellular factors whose expression is modulated, it is suggested that susceptibility of lymphoid cells to retroviral infections may be influenced by a regulatory protein(s) which is produced only in susceptible cells and acts on the LTR to enhance expression of a gene which is required for virus replication (Garcia *et al.*, 1987). Alternatively, it is conceivable that in cells which are resistant to retroviral infection, expression of a specific protein(s) suppresses LTR function and therefore, virus replication also gets suppressed.

Viral tropism is influenced by features of the internal environment of

an organism which may affect the behaviour of a virus. For example, the attachment protein on the virus particle can affect host range and tissue tropism because it is that part on the virus which must attach to specific cell surface receptors prior to viral infection. Although specific receptors for a virus may be expressed on a cell, the virus may not bind to the receptors if a viral surface protein required for virus-cell interaction is altered, perhaps by digestion with enzymes.

1.3.9. Strategies used to isolate virus receptors

A variety of approaches has been used in numerous attempts to isolate virus receptors from whole cells or from plasma membranes. Several attempts to isolate receptors containing lipids employed chloroform-methanol extraction techniques as described by Kritchevsky and Shapiro (1967). Procedures used to isolate glycoprotein receptors included the use of hot phenol (Howe *et al.*, 1972), or phenol in combination with either lithium diiodosalicylate (LIS) (Marchetti and Andrews, 1971), or sodium deoxycholate (Segrest *et al.*, 1979), or Triton X-100 (Fukuda and Osawa, 1973). Other methods for isolating glycoprotein receptors employed aqueous pyridine (Blumenfeld and Zvilichovsky, 1972), chloroform-methanol-sodium dodecyl sulphate (Hamaguchi and Cleve, 1972), or Triton-Wheat germ agglutinin (WGA) affinity chromatography (Adair and Kornfeld, 1974). The advantage of the LIS

method for RBC is its high yield (approximately 35 mg of receptor material per gram of membrane protein). The disadvantages are that up to 10 moles of LIS per mole of receptor material is retained (Segrest *et al.*, 1979), and the LIS method does not completely remove pigments such as haem from the preparation (Segrest *et al.*, 1979). The sodium deoxycholate procedure, while resulting in a relatively purer product, gives a lower yield (Segrest *et al.*, 1979). Hot phenol has been shown to cause chemical modification of the receptor material (Segrest *et al.*, 1979).

More recently, other strategies have been used for isolation of cell surface receptors. For instance, the receptor for coxsackievirus B3 on HeLa cells was isolated by extraction of the coxsackievirus-receptor complex from sodium deoxycholate solubilized cell membranes (Mapoles *et al.*, 1985). Even though this technique yielded biologically active material that was purified and in sufficient amount for biochemical characterization, this approach may not be of general application because many virus-receptor complexes are unstable during purification. Co and associates (1985) used polyclonal anti-idiotypic antibodies to reovirus $\sigma 1$ protein, to isolate the reovirus type 3 receptor from rat neuroblastoma, human lymphoma and monkey kidney cell lines. They reported that these antibodies, whose binding sites resemble viral surface structures, effectively bound to a 67 kd cell surface protein which also bound

virus. The technique of producing anti-idiotypic antibodies is based on the fact that the binding of both a ligand (for example, EMC virus) to its receptor and of the same virus to an antibody, depends on protein-protein interactions and, involves complementary conformations. The strategy is to produce antibodies against appropriate determinants on the surface of a ligand that recognize the ligand in much the same way that a receptor does. The combining site of these receptor-mimicking antibodies would have structural features which are similar to those of the receptor's own ligand-binding site. If a second set of antibodies is produced against the antigen-combining site of the first antibody, the combining sites of these anti-idiotypic antibodies should mimic the determinants on the ligand (virus) that mediate binding to its receptor. The anti-idiotypic antibody approach has several advantages. Anti-receptor antibodies can be produced without having to use the receptor as immunogen. Antisera containing anti-idiotypic antibodies to cellular or viral proteins can be generated and stored in large quantities for a long time whereas viruses lose their activity over a short period of time. Binding of anti-idiotypic antibody is specific whereas viruses have been known to bind non-specifically to inanimate surfaces such as glass, nitrocellulose and carbon. Also, from a safety point of view, it is easier to work with antibodies than with viruses.

Employing a different strategy, Tomassini and Colonno (1986) used

anti-receptor monoclonal antibodies to isolate and purify a protein which was later identified as the receptor for human rhinovirus type 14 (HRV-14) on HeLa cells (Greve *et al.*, 1989; Staunton *et al.*, 1989). A major disadvantage of the approach used by Tomassini and coworkers is the low frequency at which monoclonal antibodies are generated; these investigators reported that out of thousands of assays, only one hybridoma cell culture supernatant was identified which could protect cells from HRV-14 infection (Tomassini and Colonno, 1986).

The most exciting approach so far, employed a technique called DNA-mediated gene transfer (Mendelsohn *et al.*, 1986). Using this method, cells which were previously receptor-negative and resistant to virus infection, were made receptor-positive and susceptible to poliovirus infection. Poliovirus infects HeLa cells which express receptors on their surface whereas the mouse fibroblast cell line, L cells, do not have receptors for poliovirus and are resistant to infection. The strategy was to transfect tk⁻ L cells with HeLa cell DNA and plasmid DNA containing the thymidine kinase gene. Tk⁺ L cells (L cell transformants) were selected in HAT (hypoxanthine, aminopterin and thymidine) medium and screened for sensitivity to poliovirus infection. Mendelsohn and coworkers were able to isolate L cell transformants which were sensitive to poliovirus infection whereas untransformed L cells were

resistant to infection with the same virus.

Mendelsohn and associates (1986) then raised monoclonal antibodies directed against the poliovirus receptor on HeLa cells by immunizing mice with viable HeLa cells. From experiments based on competition for the receptor between radiolabelled poliovirus and the monoclonal anti-receptor antibodies it was concluded that the monoclonal antibodies were specific for the poliovirus cell surface receptor on HeLa cells. To determine whether the L cell transformants expressed receptors for poliovirus, the transformants were screened by a rosette assay in which the cells were first treated with the mouse monoclonal anti-receptor antibodies and then with human erythrocytes coated with goat anti-mouse antibodies. The results showed that the transformed L cells contained receptors for poliovirus.

Since confluent monolayers of transformed L cells contained cells that express the poliovirus receptor and those that do not express the receptor, Mendelsohn and coworkers (1986) isolated the receptor-positive cells using a panning technique. Polystyrene petri dishes were coated with goat anti-mouse antibodies and washed to remove unbound antibodies. The anti-receptor monoclonal antibodies were then added to the dish and following incubation the transformed L cells were added. The rationale is that mouse

monoclonal antibodies will be recognized by the anti-mouse antibodies attached to the dish and the monoclonal antibodies will in turn recognize poliovirus receptors on the transformed cells. The investigators found that receptor-positive cells adhered strongly to the petri dish even after several washes while receptor-negative cells did not attach and were washed away. Individual receptor-positive cells were cloned and used to maintain a cell line.

Using the receptor-positive cell line, Mendelsohn and associates (1986) subsequently carried out tissue culture experiments and showed that poliovirus replicated in the transformed mouse L cells as well as it did in HeLa cells. In contrast, the untransformed mouse L cells showed no evidence of being infected by poliovirus. These experiments showed very strongly that in this case susceptibility of mouse L cells to poliovirus infection depended on the presence of viral receptors. To confirm that the "susceptibility gene" was the human poliovirus receptor gene which was introduced into mouse L cells from HeLa cells, the human receptor gene was identified based on its linkage to human Alu sequences. These are short (~ 300 bp) repetitive sequences which are scattered throughout the human genome and they contain a target site for the Alu restriction endonuclease (hence their name). They are used as markers when identifying human DNA, in non-human cells which have been transfected with human DNA. In the experiments by Mendelsohn and

associates (1986), DNA from receptor-positive L cell transformants was digested with a restriction endonuclease and the fragments generated were separated on an agarose gel by electrophoresis and then transferred to nitrocellulose. The nitrocellulose which contains the fragments of DNA, was probed with radiolabelled Alu DNA and subsequently exposed to X-ray film. The results showed that the probe hybridized to fragments of DNA from mouse L cell transformants as well as to HeLa cell DNA but not to DNA from untransformed mouse L cells, showing that the "susceptibility gene" in the mouse cells was derived from the HeLa cells.

Although this approach is very impressive and the technique is attractive, it could not be used in this project, because EMC-D virus was found to infect all the human and mouse cell lines tested. In other words, there were no receptor-negative cells to be used.

1.4. Insulin-dependent Diabetes Mellitus (IDDM)

Insulin-dependent diabetes mellitus, otherwise called type 1 diabetes or juvenile onset diabetes, results from destruction of the insulin-producing β -cells which are located in the pancreatic islets of Langerhans. The process of β -cell destruction occurs over a long and variable period, to the point where

insulin in the pancreas is no longer able to regulate the concentration of glucose in the blood. The onset of the disease is characterized by infiltration of the islets of Langerhans by mononuclear cells of the immune system such as T-lymphocytes (a process called insulinitis), and selective destruction of the β -cells. The appearance of circulating autoantibodies to β -cell components often precedes overt symptoms and is used as a serological marker for IDDM (Neufeld *et al.*, 1980; Baekkeskov *et al.*, 1987). Destruction of a significant number of the insulin-producing β -cells results in an increase in glucagon, which is produced by the pancreatic α -cells. Whereas insulin secretion lowers the level of glucose in the blood, the secretion of glucagon raises it. IDDM results from the metabolic effects of insufficient insulin due to β -cell destruction, and excess glucagon. Several factors have been implicated in the aetiology of IDDM. They include genetic and environmental factors, and possibly, the breakdown of immunological tolerance to self antigens.

1.4.1. Role of genetic factors in IDDM

Studies have shown that the concordance rate for developing IDDM in identical twins is 36% (Olmos *et al.*, 1988), compared to an average 6% risk for other siblings (Thomson *et al.*, 1988). This finding suggests that genetic as well as other factors play a role in the development of the disease. Other studies employed animal models such as the non obese diabetic (NOD) mouse

which spontaneously develops IDDM similar to human type 1 diabetes. Some of the characteristic features of human IDDM which are shared by NOD mice include the appearance of autoantibodies to β -cell components (Baekkeskov *et al.*, 1982), autoimmune islet-cell destruction (Baekkeskov *et al.*, 1982), and the presence of susceptibility genes in the MHC (Todd *et al.*, 1987; Wicker *et al.*, 1987). [The strain origin of NOD mice is given in the appendix].

Perhaps the most impressive experimental results on IDDM are those derived from immunogenetic studies of NOD mice. MHC class II molecules are glycoprotein in nature and consist of two polypeptide chains, α and β , which are non-covalently associated with each other. The major function of these molecules is to bind fragments of foreign antigens and present them to helper T lymphocytes which are involved in controlling the immune system. Studies have shown that the amino acid residue at position 57 of the class II β chain is important in determining susceptibility to IDDM. An aspartic acid residue at position 57 was found to be protective against IDDM, whereas neutral residues such as serine and threonine permitted development of the disease. The NOD mouse possesses a serine residue at position 57 in the I-A β chain (Acha-Orbea & McDevitt, 1987). Similar observations have been made in humans, where the amino acid at position 57 in the HLA-DQ β chain was shown to play a critical role in determining predisposition to IDDM

(Todd *et al.*, 1987). However, studies using transgenic mice which expressed the wild type I-A^k or a mutated I-A^k molecule with a serine residue at position 57 on the β chain, have shown that the presence of serine at this position of the I-A beta chain is not sufficient to permit development of diabetes. The study showed that in both instances the presence of new I-A^k molecules, with or without a serine residue at position 57, prevented development of the disease (Slattery *et al.*, 1990; Miyazaki *et al.*, 1990).

1.4.2. Role of environmental factors in IDDM

Environmental factors which could play a role in the aetiology of IDDM include diet, chemicals and viruses. These may serve as agents which directly destroy the insulin-producing β -cells of the pancreas or they may initiate processes, such as autoimmunity, which ultimately lead to destruction of the pancreatic β -cells.

Very little is known about the role of diet in the causation of IDDM. The incidence of the disease is known to vary according to geographic location; in Japan the incidence is about 0.8/100,000 people per year while in Canada and Finland its 9/100,000 people per year and 28.6/100,000 people per year, respectively (LaPorte *et al.*, 1985). Further, the incidence of IDDM in certain populations (e.g. the Polynesians of Western Samoa) increases as a

result of changes in diet following migration to other parts of the world (LaPorte *et al.*, 1985). Several studies on the effect of diet on the incidence of IDDM in the BB rat model have suggested that a diet of intact proteins such as skim milk or soy as opposed to a mixture of amino acids, increases the probability of developing the disease (Elliott & Martin, 1984). Other reports suggested that BB rats on low vitamin E diets are more likely to develop IDDM than those on a high vitamin E diet (Scott *et al.*, 1988). Although it is generally believed that among susceptible individuals diet is only a contributory factor rather than a trigger for IDDM, a recent report suggests that bovine albumin in milk may be a culprit. This report showed that anti-albumin antibodies can initiate β -cell destruction by cross-reacting with β -cell molecules (Karjalainen *et al.*, 1992).

Chemicals such as streptozotocin, alloxan and vacor can cause specific destruction of the pancreatic β -cells, resulting in IDDM. Streptozotocin is a broad spectrum antibiotic and it has been reported that just one dose of the drug is sufficient to cause type 1 diabetes-like symptoms in mice, rats, hamsters and guinea pigs (Brodsky & Logothetopoulos, 1968). Studies involving immunologically incompetent athymic (nude) mice have shown them to be resistant to streptozotocin-induced diabetes (Paik *et al.*, 1982) suggesting that the ability of the drug to induce diabetes is somehow linked with the cell-

mediated immune status of the host. Alloxan (pyrimidinetetrone) when injected into rats, rabbits or dogs produces diabetes which resembles human type 1 diabetes (Dunn & McLetchie, 1943; Bailey & Bailey, 1943; Goldner & Gomori, 1943). The mechanism by which alloxan acts is unknown; it has been suggested that alloxan probably restricts insulin secretion by inhibiting calcium uptake by the islet cell endoplasmic reticulum (Pershasingh *et al.*, 1980). Vacor is a drug, and observations made in various parts of the world revealed that individuals who took it, subsequently developed IDDM (Johnson *et al.*, 1980). Vacor destroys only the beta cells of the islets of Langerhans although how it damages these cells is still a puzzle. It is thought that vacor may be just toxic to pancreatic β -cells or perhaps it somehow induces peripheral resistance to the action of insulin.

1.4.2.1. Virus-induced IDDM

Certain viruses cause IDDM to develop. This could be by direct destruction of the pancreatic β -cells by a cytotoxic virus; alternatively, β -cell destruction might be caused by cellular and/or humoral autoimmune mechanisms which may be triggered in the host by a virus. There are several lines of evidence, derived from observations in humans and in experimental laboratory animals, which link viruses with IDDM:

i. Several serologic studies have reported the presence of viral antigens which correlate with histopathologic findings that show significant β -cell destruction in the pancreatic islets of some patients with systemic viral infections (Yoon *et al.*, 1979; Jenson *et al.*, 1980; Patterson *et al.*, 1981).

For instance, a 10-year-old boy admitted to hospital following an influenza like illness, died about seven days later. Coxsackievirus type B4 was isolated from the boy's pancreas and histopathologic examination of pancreatic tissue obtained at autopsy, showed that the boy's pancreatic β -cells were significantly destroyed, with lymphocytic infiltration of the islets of Langerhans (Yoon *et al.*, 1979). Similarly, coxsackievirus B4 has been isolated from throat washings and stool samples obtained from a 5-year-old girl who was diagnosed as having IDDM, while coxsackievirus B5 was isolated from the same sources, in a 16-month-old child who later developed type 1 diabetes (Champsaur *et al.*, 1982).

Viral antibodies have been detected in sera obtained from patients diagnosed with IDDM. Yoon and associates (1979) showed that serum taken from the 10-year-old boy described above contained rising titres of neutralizing antibodies against coxsackievirus B4, measured from the second day of admission to the day the patient died. Also, antibodies to

coxsackievirus B4 were found in the serum of the 5-year-old girl who developed IDDM.

Other findings, both in humans and in laboratory animals, have prompted suggestions that infections with mumps virus (Prince *et al.*, 1978), rubella virus (Menser *et al.*, 1978), some variants of coxsackievirus (Yoon *et al.*, 1978; Yoon *et al.*, 1979; Champsaur *et al.*, 1982), reovirus type 3 (Yoon *et al.*, 1981) and lymphocytic choriomenengitis (LCM) virus (Oldstone *et al.*, 1984) cause pancreatic β -cell damage, or at the very least, a reduction in insulin production.

ii. Consistent with Koch's postulate, mice inoculated with virus isolated from IDDM patients developed symptoms characteristic of type 1 diabetes. When Yoon and associates (1976) injected several inbred strains of mice with the virus isolated from the 10-year-old boy, SJL/J mice developed diabetes while C57BL/6J mice failed to develop the disease. Champsaur and coworkers (1982) also showed that the coxsackievirus B5 which was isolated from the 16-month-old child who later developed diabetes, caused abnormal glucose tolerance tests when inoculated into mice.

These findings do not suggest that all cases of IDDM are caused by

coxsackieviruses, since less than 20% of IDDM cases have been associated with coxsackie B virus infections.

iii. Another line of evidence linking viruses to IDDM has to do with autoantibodies, which may be used to initiate β -cell destruction. A number of studies have reported the presence of autoantibodies which cross-react with insulin and other non viral molecules, in or on islet cells, in the serum of virus-infected IDDM patients. According to Pak *et al.*, (1988), up to 15% of newly diagnosed IDDM patients infected with cytomegalovirus, also produced islet cell autoantibodies in their sera. Other studies have shown that about 21% of IDDM patients infected with rubella virus produced antibodies against antigens on the surface of islet cells while 13% of patients infected with rubella virus produced anti-insulin autoantibodies (Ginsberg-Fellner *et al.*, 1984). In contrast, only 1% of non diabetic, non virally infected subjects were found to produce islet cell or anti-insulin autoantibodies.

iv. Studies using animal models of IDDM have found that the pancreatic β -cells of NOD mice expressed endogenous retrovirus particles and, expression of the virus particles correlated with the development of insulinitis and type 1 diabetes in these mice. It has been observed that administration of the immunosuppressant, cyclophosphamide, to NOD mice

hastened the development of IDDM (Harada & Makino, 1982) while removal of macrophages by silica treatment prior to administering cyclophosphamide, prevented the development of IDDM in NOD mice (Lee *et al.*, 1988). These studies further showed that sections of islet cells from NOD mice which received silica prior to administration of cyclophosphamide contained retrovirus particles whereas sections from mice which received only silica did not contain these virus particles. The particles were not found in α or δ cells of the islets of Langerhans nor were they found in any other organs, either in cyclophosphamide-treated or -untreated NOD mice. How beta cell specific expression of endogenous retrovirus leads to development of IDDM in NOD mice is not yet known.

v. Further evidence linking viruses to IDDM, is the finding that the diabetogenic variant of EMC virus, EMC-D virus, infects mouse pancreatic β -cells and may induce a type 1 diabetes-like syndrome (Yoon *et al.*, 1980). EMC-D virus induces diabetes in male SJL/J mice but not in female SJL mice or, in male or female C57BL/6J mice (Yoon *et al.*, 1980; Yoon & Notkins, 1983). The non-diabetogenic variant, EMC-B virus, does not cause diabetes in either SJL/J or C57BL/6J mice (Yoon *et al.*, 1980) and it was found to be serologically indistinguishable from EMC-D virus. At the genomic level, it has been shown that EMC-D differs from EMC-B by only 14 nucleotides (Bae *et*

al., 1989). The differences between the two virus variants include two deletions of five nucleotides, one base insertion and eight point mutations (Bae *et al.*, 1989). The first deletion of three nucleotides and the second deletion of two nucleotides are located at the 5' poly (C) tract and the 3'-end polyadenylation site of EMC-B virus, respectively. The single base insertion in EMC-B virus occurs at the 5' noncoding region whereas the eight point mutations are located in the polyprotein coding region. Two of these point mutations are "silent" while the remaining six result in changes in amino acid residues. One of the six "productive" mutations is located on the L gene which codes for the leader protein whereas the other five are located on the VP1 gene (Bae *et al.*, 1989; Bae *et al.*, 1990). Since EMC-B virus is a better inducer of interferon than EMC-D virus, it was suggested that interferon produced during EMC-B virus infection protects pancreatic β -cells from viral destruction (Yoon *et al.*, 1980; Cohen *et al.*, 1983). More recent findings suggest that two amino acids on the viral polyprotein, one located on the leader peptide and the other on the virion protein VP1, may have a role in the diabetogenic activity of EMC-D virus (Bae *et al.*, 1990). Such reports suggest that the observed induction of interferon may be a secondary manifestation and does not account fully for the non diabetogenicity of EMC-B virus.

1.4.2.2. Induction of autoimmunity in virus-induced IDDM

The aim of this section is is not to provide a comprehensive review of immunological mechanisms, or for that matter, of the immunology of IDDM. Rather, the objective of this portion of the thesis is to provide an outline of possible immunological mechanisms that may be involved in the initiation of β -cell destruction.

Class II MHC molecules are expressed on the surfaces of macrophages, B lymphocytes and activated T lymphocytes. Put simply, helper T lymphocytes become activated when their receptors bind the complex of fragments of foreign antigens and class II MHC molecules on the antigen presenting cells (such as macrophages). The helper T cells are both antigen-specific and MHC-restricted in that each one recognizes specific antigen only when it is associated with a certain class II MHC molecule. Once foreign antigens are recognized, helper T cells produce a range of lymphokines which may affect other cells of the immune system. For instance, during a T cell dependent immune response following contact with antigen, the T cells stimulate antigen presenting cells to secrete interleukin 1 (IL-1) which in turn activates the helper T cells themselves. The activated T cells go on to produce IL-2 and also to stimulate proliferation of activated B lymphocytes. Further stimulation of the activated B cells (via IL-5 and IL-6) subsequently transforms the B cells

into antibody producing plasma cells. Antibodies produced by such B cells are specific in that they only recognize the antigen which stimulated their production. However, there are also antigens which do not require T cell help in order to stimulate antibody production. Such antigens are usually large polymers with repeating identical antigenic determinants (for example, microbial polysaccharides). These T-cell independent antigens are effective in part because of the multiple binding of these antigens to the membrane bound molecules that serve as antigen receptors on the B cells.

Autoimmunity results from lack of tolerance to self antigens. Autoimmunity occurs when self antigens bind self class II MHC molecules, resulting in self antigen presentation to the helper T cells. One way by which the immune system may achieve tolerance is by deletion of self antigen reactive T cells (autoreactive T cells) from the T cell repertoire, during T cell education. T lymphocytes are educated very early during their development to mount an effective immune response by recognizing and responding to foreign antigens, but to remain unresponsive or tolerant of their own self antigens throughout life. Since helper T cells do not recognize antigens (foreign or self) in the absence of class II MHC molecules, tolerance occurs when antigens are presented to the immune system in a non immunogenic fashion. For instance, tolerance will occur if antigens are presented to

autoreactive T cells by cells which do not express class II MHC molecules (for example, pancreatic β -cells) as opposed to class II positive cells such as macrophages. Given this outline, ways by which viruses may subvert the immune system and possibly trigger autoimmune responses in the host, will be described next.

i. Following a virus infection, destruction of the infected cells could result in release of sequestered cellular components that are normally not exposed to the immune system and, are therefore, not immunogenic. Induction of an autoimmune response in the host could result from exposure to such cellular self antigens. Although there is no evidence to suggest that this mechanism is actually used to initiate an autoimmune response in virus-infected IDDM patients, it is a possible mechanism, especially since coronavirus-induced autoimmune demyelinating encephalomyelitis is initiated by the release of sequestered antigen, namely myelin, from damaged cells (Watanabe, 1983).

ii. Another possible mechanism for induction of autoimmune IDDM involves alteration of antigens. Experiments using mice infected with influenza virus indicate that viruses can induce alterations of host cell membrane antigens so that normal host cell antigens become immunogenic. Wylie and

Klinman (1981) have reported that following immunization with influenza virus, the mice produced monoclonal antibodies which recognized antigens on virus-infected cells, but the antigens were not present on the virion or on uninfected cells.

iii. A virus infection may lead to an autoimmune response following infection of cells of the immune system. A virus that infects lymphocytes or cells such as macrophages which are involved in presenting antigen to lymphocytes, could initiate an autoimmune response by interfering with the normal processes by which the host distinguishes between self and non self. The virus may kill the cells or modify the expression of certain cell surface molecules or affect the production of lymphokines. For example, it has been reported that in the case of Epstein Barr virus which infects B lymphocytes, the virus actually stimulates the B cells to secrete antibodies which may then react with host tissues (Fox *et al.*, 1986; Rosen *et al.*, 1977). This author is not aware of any reports about viruses which destroy pancreatic β -cells and, infect lymphocytes or antigen presenting cells in order to effect an autoimmune response.

iv. Another mechanism by which viruses may initiate an autoimmune response is via cross-reaction between host determinants and antiviral

antibodies or antibodies produced against the idiotype on an antiviral antibody (called anti-idiotypic antibodies; described in section 1.3.9.). Oldstone and Notkins (1986) have reported that demyelinating autoimmune disease in mice is induced via molecular mimicry, when antibodies against Theiler's virus cross-react with host mouse tissues. Other investigators have found that cytomegalovirus-infected IDDM patients produce antiviral antibodies which recognize a 36 kd protein on human pancreatic islet cells (Baekkeskov *et al.*, 1982; Pak *et al.*, 1988). The role of this anti-36 kd protein in the pathogenesis of IDDM is not yet clear. Anti-idiotypic antibodies made against antibodies to the haemagglutinin of reovirus type 3 have been shown to bind to reovirus receptors on the surfaces of lymphocytes and nerve cells. In fact Co and collaborators (1985) went a step further and showed that anti-idiotypic antibodies to reovirus can be used to isolate the reovirus type 3 receptor. However, despite these reports which show that anti-idiotypic antibodies can react with host cell determinants, the question of whether these antibodies can elicit an autoimmune response in the host remains to be fully elucidated.

v. Aberrant expression of class II MHC molecules has been proposed as the mechanism for induction of autoimmunity in virus-infected individuals (Bottazzo *et al.*, 1983). The idea is based on the premise that helper T lymphocytes recognize antigen only in association with class II MHC

molecules, which are expressed on only a few cell types. Consequently, cells which possess autoantigens (or foreign antigens) but normally do not express cell surface class II MHC molecules are unable to elicit an immune response. The hypothesis by Bottazzo and associates (1983) suggests that interferon produced in response to viral infections can induce expression of class II MHC molecules on cells which were previously class II negative. This aberrant expression of class II MHC molecules effects presentation of autoantigens to the helper T cells resulting in the effector functions outlined before. The B cells in cooperation with activated helper T cells produce antibodies which may react with host tissue antigens such as the cell surface autoantigens and cause tissue damage. Thus tolerance to the self antigens fails and autoimmunity develops.

This hypothesis has been tested in transgenic mice containing constructs of mouse H-2 (the MHC of mouse; HLA, which is the MHC of human) genes linked with the insulin promoter or constructs of the insulin promoter fused with interferon gamma genes (Sarvetnick *et al.*, 1988; Lo *et al.*, 1988; Bohme *et al.*, 1989). The results showed that the situation *in vivo* is more complicated than one imagined. The transgenic mice showed strong expression of both class I and class II MHC genes in their pancreatic beta cells which normally do not express class II MHC molecules. It was found that transgenic mice

which lacked I-E genes but expressed I-A class II MHC molecules on their β -cells both developed IDDM without any evidence for autoimmunity or pancreatic infiltration (insulitis) (Sarvetnick *et al.*, 1988; Lo *et al.*, 1988). On the other hand, transgenic mice which produced interferon expressed class II MHC molecules on their pancreatic beta cells, developed IDDM with insulitis but showed no evidence for autoimmunity (Sarvetnick *et al.*, 1988). In transgenic mice which contained the rat insulin promoter linked to an oncogene (SV40 T antigen) the mice had insulitis and produced autoantibodies but they did not develop type 1 diabetes.

Taken together, these experiments showed that simple expression of class II MHC molecules on the surface of β -cells is not sufficient to initiate an autoimmune response, although it may result in IDDM. Further, induction of an autoimmune response may not necessarily lead to development of IDDM, even when the pancreatic β -cells are involved. In mouse and rat models of type 1 diabetes, the disease is usually accompanied by insulitis, yet these experiments showed that diabetes can occur without insulitis. The experiments using interferon gamma genes also found that mere expression of class II MHC molecules on previously class II negative cells affected cell viability. This finding has prompted the speculation that aberrant expression of class II MHC molecules probably induces the production of endonucleases

which digest cellular nuclear DNA, and perhaps such cells die from DNA fragmentation (Sarvetnick *et al.*, 1988).

So far, there is no experimental evidence to suggest a mechanism by which virus-infected IDDM patients could induce an autoimmune response. While other immunological mechanisms may be involved in the pathogenesis of IDDM, the role of viruses in the induction of autoimmune IDDM or in the destruction of the pancreatic β -cells, which secrete insulin, remains unknown.

1.5. Objective of Study

Fairly recent reports have suggested that amino acid changes unique to virion protein VP1 of the diabetogenic variant of EMC virus may be responsible for its diabetogenicity, perhaps by increasing the binding efficiency between EMC-D virus and pancreatic β -cells (Bae *et al.*, 1990). Other reports have suggested an association between pathogenicity of EMC-D virus and the number of cell surface receptors on pancreatic β -cells available for virus binding (Chairez *et al.*, 1978).

Cell surface receptors are determinants of viral tropism because they

are required for the initiation and spread of viral infections. Very little is known about the nature of the β -cell surface receptor for EMC-D virus (Chairez *et al.*, 1978; Kaptur *et al.*, 1989). Although EMC-B and EMC-D viruses vary in pathogenicity, it has been reported that both variants compete for the same receptor on baby mouse pancreatic (BMP) cells, an SV40 transformed pancreatic β -cell line derived from newborn mice (Kaptur *et al.*, 1989). It was reported that up to six times more EMC-D than EMC-B virus attached to primary mouse pancreatic β -cells (Kaptur *et al.*, 1989) and, β -cells from susceptible *SL/J* strains bound more EMC-D virus than β -cells from resistant *C57BL/6J* mice (Chairez *et al.*, 1978).

Despite these reports, important questions regarding the number of cell surface receptors and biochemical characteristics of the receptor for EMC-D virus on pancreatic cells have remained unanswered. The objective of this project was to characterize and isolate the receptor for EMC-D virus on baby mouse pancreatic cells and, to elucidate the role of virus receptors in EMC-D virus-induced diabetes in *SL/J* mice.

Chapter Two

MATERIALS and METHODS

2.1. BMP cells and EMC-D virus

Baby mouse pancreatic (BMP) cells, an SV40 transformed pancreatic β -cell line derived from newborn mice (Kaptur *et al.*, 1989), and EMC-D virus, were a generous gift from Dr. J.W. Yoon (Faculty of Medicine, University of Calgary, Canada). The cells were grown in RPMI 1640 medium supplemented with 5% foetal calf serum and 100 units/ml of penicillin and 100 μ g/ml of streptomycin at 37°C in a 5% CO₂ atmosphere. Confluent monolayers of BMP cells were trypsinized to detach them from culture dishes except when it was critical to retain cell surface receptors, in which case BMP cells were removed by scraping with a rubber policeman.

EMC-D virus was grown in suspension cultures of freshly harvested Krebs mouse ascites tumour cells in Earle's medium and purified as described in section 2.4. Radiolabelled virus was prepared by adding 5 μ Ci/ml of a ³H-labelled leucine to the medium three hours post infection.

2.2. Harvesting of Krebs ascites tumour cells

These cells were established from a carcinoma arising from the inguinal region of a mouse (Klein and Klein, 1951). Subcutaneous injection of the original solid tumour into similar mice produced haemorrhagic exudates which when reinjected into the peritoneum of mice yielded ascites tumours rather than solid tumours (Klein and Klein, 1951). In our laboratory, Krebs ascites cells were grown in the peritoneal cavity of heterogeneous albino CD1 mice.

Materials:

- (a). Phosphate buffered saline (PBS), pH 7.4: Solution A consists of 40.0 g NaCl, 1.0 g KCl, 5.75 g Na_2HPO_4 , and 1.0 g KH_2PO_4 in 4 L of deionised water. Solution B consists of 0.5 g CaCl_2 and 0.5 g MgCl_2 in 1 L of deionised water. The two solutions were sterilized separately and then combined, with vigorous stirring.
- (b). Ice-cold calcium and magnesium free PBS (Ca^{++} - Mg^{++} free PBS) contains just solution A above, made up to 5 L with deionised water.
- (c). 0.1% Trypan blue in Ca^{++} - Mg^{++} PBS.

(d). 70% ethanol.

Method:

CD1 mice weighing about 20-30 g were inoculated intraperitoneally with 0.1 ml of a suspension of 10^8 cells per ml of $\text{Ca}^{++}\text{-Mg}^{++}$ free PBS. Mice bearing seven day tumours were killed by cervical dislocation. Their abdomens were washed with 70% ethanol and a small incision was made into the sterilized area with a pair of scissors. With the mouse held over a funnel in a centrifuge bottle containing about 50 ml $\text{Ca}^{++}\text{-Mg}^{++}$ free PBS, the skin was pulled open to reveal the abdominal wall. The wall was punctured to allow cells to drain into the funnel and the cells were flushed from the cavity with $\text{Ca}^{++}\text{-Mg}^{++}$ free PBS.

The freshly harvested cells were washed three times in cold $\text{Ca}^{++}\text{-Mg}^{++}$ free PBS by centrifugation at 1000 g for 5 minutes to remove red blood cells and ascitic fluid. After centrifugation, the cells were suspended in 1 ml of $\text{Ca}^{++}\text{-Mg}^{++}$ free PBS and about 10 μl of the suspension was diluted 1:10 in PBS. The suspension of cells diluted in PBS was again diluted 1:10 in 0.1% Trypan blue and counted. Based on the counts obtained, the undiluted suspension of cells was adjusted to a final concentration of 10^8 unstained cells/ml. Krebs cells used to grow virus were distributed into 100 ml quantities

in 1 L Erlenmeyer flasks, and maintained in Earle's medium at 37°C while swirling at 80 rpm.

2.3. EMC-D virus grown in Krebs cells

Materials:

(a). Earle's saline growth medium. Consists of 10X Earle's balanced salt solution (Gibco laboratories, N.Y., U.S.A.) supplemented with 10 ml of inactivated horse serum, 100 units/ml of penicillin, 10 µg/ml of streptomycin, and 50 ml of 4.4% NaHCO₃ which has been presaturated with CO₂. The volume was made up to 1 L with sterile deionised water.

(b). 0.1% Trypan blue in PBS.

(c). EMC-D virus.

Method:

i. Krebs cells (10⁸ cells/ml) were infected with EMC-D virus at a multiplicity of infection of 3 p.f.u./cell and the suspension was maintained at 4°C for 30 minutes to allow for adsorption and encourage synchronous infection of the cells.

ii. The cell suspension was then diluted to 10^7 cells/ml with prewarmed (37°C) Earle's medium and distributed into 100 ml quantities in 1 litre Erlenmeyer flasks (i.e. 10% of the flask volume occupied by liquid to permit adequate gaseous exchange).

iii. The flasks were sealed with screw caps and set swirling at 80 rpm at 37°C overnight. An uninfected control was prepared in a similar way.

iv. About 12 to 18 hours later, the flasks were removed from swirlers. Cells (100 μl) were taken from the culture, stained by diluting 1:10 with 900 μl of 0.1% trypan blue in a test tube and counted to check for cell death.

v. When 80% or more of the infected cells were stained, virus growth was considered complete. The number of stained uninfected control cells must remain below 10%. When cells were not at least 80% stained the cultures were returned to the swirlers and cell death checked at one hour intervals until over 80% of the cells were stained.

vi. Virus yield was estimated by the haemagglutination test and crude virus preparations, which comprise suspensions of virus plus cells and cellular debris, were stored at -20°C . When required, radiolabelled virus was prepared

by adding 5 $\mu\text{Ci/ml}$ of ^3H -labelled leucine to the medium 3 hours post infection.

2.4. Purification of EMC-D virus

EMC-D virus was purified as described by Ziola and Scraba (1974).

Materials:

- (a). Cold (-25°C) methanol.
- (b). Ice-cold 0.2 M sodium phosphate buffer, pH 7.4.
- (c). Ice-cold 0.2 M sodium pyrophosphate, pH 8.0.
- (d). 30% and 15% sucrose solutions made in sodium phosphate buffer
- (e). Cesium chloride, trypsin solution (10 mg/ml in PBS) and a Dounce homogenizer.

Method:

- i. Crude virus preparations were thawed and clarified in 250 ml bottles by centrifugation in a Beckman centrifuge, model J21B, at 10,000 rpm for 15

minutes using a Beckman JA-20 rotor. The supernatants were collected in a large beaker on ice and the combined volume was estimated.

ii. Cold methanol was measured to approximately half the volume of the combined supernatants. The methanol was slowly added to the supernatants from a funnel while stirring in an ice-bath. Precipitation was allowed to occur for at least 24 h at -20°C after which the precipitate was collected by centrifugation at 7,200 rpm for 30 minutes at 4°C , using a Beckman J-14 rotor.

iii. The supernatants were discarded and the pellets were combined and resuspended in a 125 ml flask containing about 10 ml phosphate buffer, 3 ml trypsin, and 30 ml of sodium pyrophosphate, and incubated for 30 minutes at 37°C while stirring.

iv. The enzyme-treated pellet was then subjected to differential centrifugation. First, the sample was centrifuged at 16,000 rpm for 20 minutes at 4°C in a JA-21 rotor and the pellet was discarded. The supernatant was then centrifuged at 30,000 rpm for 60 minutes at 4°C using a SW40 rotor.

v. The pellet was resuspended in 1.2 ml of 0.1 M sodium phosphate

buffer, homogenized with 5-10 strokes of the Dounce homogenizer and centrifuged through a sucrose gradient which comprised 2 ml of 30% sucrose (at the bottom) and 2 ml of 15% sucrose, layered with 1.2 ml of the resuspended virus pellet. Centrifugation through sucrose was performed at 4°C for 2.7 h at 50,000 rpm in a SW50.1 rotor.

vi. The pellet was resuspended in 2 ml of 0.1 M sodium phosphate buffer. Cesium chloride (2.2g) was added to the resuspended pellet, mixed gently, and made up to about 5.5 ml with phosphate buffer to bring the density to approximately 1.33. The sample was then centrifuged at 4°C for 24 h at 40,000 rpm in a SW50.1 rotor.

vii. The virus bands seen after centrifugation were recovered and layered onto a Sephadex G25 column. The column was eluted with 0.1 M sodium phosphate buffer and 1 ml fractions were collected. Fractions with optical densities above 0.3 were combined and stored at 4°C as purified virus. The amount of virus obtained was determined by plaque assay. (Note: OD_{260}/OD_{280} should be ~ 1.64 ; 1 mg virus = 7.6 OD_{260} ; 1 $OD_{260} = 9.5 \times 10^{12}$ virus particles.)

2.5. Haematoxylin and Eosin staining

Haematoxylin is commonly used as a nuclear stain preceding staining of the cytoplasm with eosin. The nucleus is stained blue to blue-black and the cytoplasm stains shades of pink.

Materials:

- (a). Formalin fixed paraffin embedded 5 μ sections of pancreas from SJL/J and C57BL/6J mice.

- (b). Mayer's haematoxylin stain: contains haematoxylin 1 g; sodium iodate 0.2 g; potassium alum 50 g; citric acid 1 g; chloral hydrate 50 g; and 1 L of distilled water. The haematoxylin, alum and sodium iodate were allowed to dissolve overnight. Citric acid and chloral hydrate were added and the mixture was brought to boil for 5 minutes after which the solution was cooled and ready for use. The chloral hydrate serves as a preservative.

- (c). A 5% stock solution of eosin prepared in distilled water. To prevent mould growth a crystal of thymol or a few drops of formalin were added.

(d). Scott's tap water substitute (S.T.W.S.): contains 2 g sodium bicarbonate and 20 g magnesium sulphate dissolved separately, combined, and made up to 1 L with distilled water. A crystal of thymol or a few drops of formalin were added to prevent mould growth.

Method:

All procedures were carried out at room temperature. Sections were deparaffinized twice in xylol each lasting 3-5 minutes, and then rehydrated by immersion for 2 minutes each in absolute, 95%, 80%, and 70% ethanol. Sections were rinsed in running tap water for 5 minutes and stained for 15 minutes with Mayer's haematoxylin. After rinsing with tap water, sections were exposed to S.T.W.S. for 2 minutes, rinsed again in running tap water and stained with 1% Eosin for 2 minutes. Sections were dehydrated in 95% ethanol and absolute alcohol, cleared in xylene, and mounted in synthetic resin for microscopic examination.

2.6. Aldehyde-Fuchsin staining

The islets of Langerhans in the pancreas contains various types of cells. The aldehyde-fuchsin stain is used to identify these different cell types in the islet. One type, the β -cells, produce insulin. Using this stain, the pancreatic β -cells stain deep purple-violet.

Materials:

- (a). Formalin fixed paraffin embedded 5 μ sections of pancreas from SJL/J and C57BL/6J mice.
- (b). Lugol's iodine: consists of 1 g iodine; 2 g potassium iodide; and 100 ml distilled water.
- (c). Aldehyde-Fuchsin stain: consists of 0.5 g Basic fuchsin; 100 ml of 70% alcohol; 1 ml of concentrated HCl; and 1 ml of paraldehyde.
- (d). Celestine Blue-Haemalum: contains 0.5 g celestine blue B; 5 g ferric ammonium sulphate; 14 ml glycerin; and 100 ml of distilled. The alum was dissolved in water without heat after which the celestine blue was added and boiled for 3 minutes. The solution was filtered after it had cooled down and the glycerin was then added.
- (e). Orange G-light green counterstain: consists of 0.2 g Light green; 1.0 g Orange G; 0.5 g phosphotungstic acid; 1 ml glacial acetic acid; and 100 ml of distilled water.
- (f). Acid alcohol: consists of 1% HCl in 70% alcohol.

Method:

Sections of mouse pancreas were deparaffinized in xylol, hydrated in decreasing concentrations of alcohol in water and oxidized with Lugol's iodine for 10 minutes. After washing in tap water and in 70% alcohol, sections were stained with aldehyde-fuchsin for 20 minutes. Sections were washed in 95% alcohol followed by water and then stained with celestine blue-haemalum for 15 minutes. After another wash in water, sections were briefly differentiated in acid alcohol, washed well in tap water, rinsed with distilled water and counterstained with orange G-light green for 45 seconds. Sections were then rinsed briefly with 0.2% acetic acid followed by 95% alcohol, dehydrated in absolute alcohol, cleared in xylene and mounted in synthetic resin.

2.7. Immunoperoxidase staining

This staining procedure is used to show the presence of insulin within the pancreatic β -cells. The insulin is stained brown. The materials listed in (c) to (g) below were obtained from Chemical Credential, ICN ImmunoBiologicals, Lisle, IL, U.S.A.

Materials:

- (a). Formalin fixed paraffin embedded 5 μ sections of pancreas from SJL/J and C57BL/6J mice.

(b). 3% H_2O_2 in distilled water.

(c). Normal rabbit serum diluted 1:100 in PBS pH 7.4.

(d). Guinea pig anti porcine insulin diluted 1:200 in PBS containing 1% BSA.

(e). Rabbit anti guinea pig IgG diluted 1:200 in PBS containing 1% BSA.

(f). Guinea pig polyclonal Peroxidase Anti-Peroxidase (PAP) diluted 1:500 in PBS containing 1% BSA.

(g). 3-Amino 9-Ethyl Carbazole (AEC).

(h). Mayer's haematoxylin stain (section 2.5.).

Method:

All the procedures described below were carried out at room temperature. Sections of mouse pancreas were deparaffinized in xylol and hydrated in decreasing concentrations of alcohol in water. Then, endogenous

peroxidase was blocked by immersing the sections in 3% H_2O_2 for 20 minutes. The sections were rinsed in PBS and, to further minimize non-specific binding, sections were incubated with normal rabbit serum (1:100) for 15 minutes. The serum was drained off and sections were then exposed to the 1:200 guinea pig anti porcine insulin for 1 h. After rinsing in PBS, sections were incubated with the 1:200 dilution of rabbit anti guinea pig IgG for 20 minutes. Sections were rinsed in PBS before incubating with guinea pig PAP (1:500) for 20 minutes. After another rinse in PBS, sections were stained with AEC for 15 minutes, rinsed in distilled water and counterstained for 10 minutes in Mayer's haematoxylin. Following a rinse in tap water, sections were dehydrated in 95% ethanol and absolute alcohol, cleared in xylene, and mounted in synthetic resin for microscopic examination.

2.8. Growth of EMC-D virus in BMP cells

Monolayers of BMP cells grown to confluency (approx. 3×10^6 cells) on 35 mm tissue culture dishes (Corning, New York) were washed twice in ice-cold Ca^{++} and Mg^{++} free phosphate buffered saline (PBS) containing 1% bovine serum albumin (PBS-BSA) before infection with EMC-D virus at a multiplicity of infection of 3 p.f.u./ cell. After addition of virus, cells were kept at 4°C for 30 min to minimize virus internalization and to synchronize virus growth on different cells. Unadsorbed virus was removed from the cells

by washing in PBS prior to the addition of prewarmed RPMI 1640 medium and incubation at 37°C in a 5% CO₂ atmosphere. At various times, dishes of infected cells were frozen and thawed twice and the amount of virus present was determined by plaque assay.

2.9. Virus attachment assay

Materials:

(a). Aquasol-2 and ³H-labelled leucine were from New England Nuclear (Mississauga, Ont., Canada).

(b). Triton X-100 was from Eastman Kodak.

Method:

Purified, ³H-labelled EMC-D virus in PBS-BSA was added (100 µl containing approx. 60,000 c.p.m./dish) to washed, confluent BMP cells grown on 35 mm dishes and attachment was allowed to proceed at 4°C. After specified periods, the cells were washed three times (1 ml/wash) with ice-cold PBS-BSA to remove unattached virus. Cells with virus attached were lysed with 1 ml of 1% Triton X-100. The washes and lysate were each added to 10 ml of Aquasol-2 and the percentage of cell-associated virus was determined after liquid scintillation counting.

2.10. Enzyme treatment of BMP cells

Materials:

(a). Trypsin, chymotrypsin, papain, phospholipase C, and phospholipase D were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.)

(b). Endoglycosidase F, glycopeptidase F and O-glycosidase were from Boehringer Mannheim (Laval, Que., Canada).

(c). *Vibrio cholerae* and *Arthrobacter Ureafaciens* neuraminidases were from Calbiochem-Bering (La Jolla, Calif., U.S.A.).

Method:

Baby mouse pancreatic cells grown to confluency in 35 mm dishes were incubated with 20 milli-units (mU) of *V. cholerae* or *A. ureafaciens* neuraminidase or with 100 μ l of prewarmed Hank's balanced salt solution (HBSS) containing 5 mg/ml of one of the other enzymes specified in Table 1, for 1 h at 37°C. In other instances, cells were treated with 600 mU of Endoglycosidase F or glycopeptidase F at 37°C overnight, or with neuraminidase prior to treatment with 5mU of O-glycosidase at 37°C, overnight. Following enzyme treatment, the cells were washed three times in ice-cold PBS-BSA and virus attachment was measured as described in section

2.9. Where enzymes caused cells to detach from culture dishes, treatment was done on cells in suspension.

In a similar set of experiments, cells were treated with lectins or sugars instead of enzymes, followed by measurement of virus attachment. The amount of lectin or sugar and the conditions used are given in tables 3.3 and 3.4. Wheat germ (*Triticum vulgaris*), horse gram (*Dolichos biflorus*) and horseshoe crab (*Limulus polyphemus*) lectins were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Concanavalin A (*Jack bean*) was supplied by Pharmacia (Baie d'Urfe, Que., Canada). The sugars: N-acetyl glucosamine, N-acetyl-D-galactosamine, α -methyl-D-glucoside, α -methyl-D-mannoside, α -lactose, D-mannose, maltose and galactose were also obtained from Sigma.

In another set of experiments, cells were treated with cycloheximide or tunicamycin after treatment with trypsin, followed by measurement of virus attachment. Tunicamycin and cycloheximide were purchased from Sigma.

2.11. Identification of sialic acids by HPLC

Materials:

(a). N-acetyl and N-glycolyl neuraminic acid standards were from

BioCarb Chemicals (Lund, Sweden).

- (b). An Aminex A-29 anion-exchange column (40 X 4.6 mm) preppacked for high-pressure liquid chromatography (HPLC) was purchased from Bio-Rad (Mississauga, Ont., Canada).

Method:

BMP cells were scraped from culture dishes, pooled and washed in cold PBS-BSA. Sialic acids were released from 3×10^6 cells either by hydrolysis at 80°C for 1 h in 200 μl of 0.1 N H_2SO_4 or by treatment with 20 mU of *V. Cholerae* or *A. ureafaciens* neuraminidase in 200 μl of 0.02 M sodium phosphate buffer, pH 5.0, for 1 h at 37°C . After incubation, cells were pelleted in an Eppendorf microcentrifuge for 0.5 min at maximum speed and 20 μl of the supernatant was injected into an Aminex A-29 anion-exchange column and analyzed on a Beckman (model 344 CRT) HPLC system. Elution was carried out at ambient temperature in 0.75 mM Na_2SO_4 , pH 7.0, at a flow rate of 0.3 ml/min. The effluent was monitored for UV absorbance at 195 nm. Sialic acids from BMP cells were identified by comparing their elution times with those of known sialic acid standards or by coinjection of BMP sialic acids with standards.

2.12. Purification of anti-EMC virus antiserum

Antiserum against EMC virus (AEMC) was produced by injecting purified EMC virus into New Zealand white rabbits and purified as described below. Before using the anti-EMC virus antibody to produce anti-EMC virus anti-idiotypic antibodies, its biological activity and specificity for EMC virus were determined by gel immunodiffusion, by enzyme-linked immunosorbent assay (ELISA) and by the haemagglutination inhibition (HAI) test.

Materials:

- (a). Globulin fraction of anti-EMC serum prepared by ammonium sulphate precipitation of whole serum as described by Hudson and Hay (1980).
- (b). Virus-Sepharose column.
- (c). 0.02 M sodium phosphate buffer pH 8.0.
- (d). 0.17 M glycine-HCl buffer pH 2.3 containing 0.5 M NaCl.
- (e). 0.1% Triton X-100 in 0.02 M phosphate buffer.

Method:

This procedure is a modification of the method described by Hudson and Hay (1980). A virus affinity column was prepared by coupling 1 mg of purified EMC virus to 1 g of CNBr-activated Sepharose 4B as described previously. The globulin fraction (1 ml) of anti-EMC virus immune serum (HAI titre 2×10^3) was added to the virus-Sepharose in a 50 ml centrifuge tube and mixed for 1 hour at room temperature before leaving it overnight to mix at 4°C. The virus-Sepharose plus adsorbed antibodies, was packed into a column and washed successively with 20 ml phosphate buffer; with 20 ml glycine-HCl containing NaCl; and with 20 ml Triton X-100 in phosphate buffer. One ml fractions were collected and ELISA tests using EMC virus attached to polyvinyl microtitre plates were performed on samples from each fraction in order to determine the biological activity and specificity of the purified antibodies. The protein content of each wash was measured by absorbance at 280 nm. The degree of purification was assessed by comparing ELISA activity with protein content before and after purification.

2.13. Production of anti-EMC virus anti-idiotypic antibodies**Materials:**

- (a). Affinity purified anti-EMC virus IgG antibodies (i.e. purified AEMC).

(b). Complete and incomplete Freund's adjuvant.

(c). Two New Zealand white rabbits supplied by the Animal Care Facility, in this faculty. These rabbits were from the same breed of rabbits as those used to produce anti-EMC virus antibodies.

Method:

i. Affinity purified anti-EMC virus antibodies were determined to be biologically active and specific for EMC virus using the ELISA and haemagglutination inhibition tests. About 40 ml of preimmune blood was taken from the ear veins of two New Zealand white rabbits before they were injected subcutaneously with 100 μ g of affinity purified AEMC antibodies emulsified in an equal volume of complete Freund's adjuvant.

ii. Three weeks later, 2 ml of blood was taken from the rabbits and the serum was immediately screened for the presence of anti-EMC virus anti-idiotypic antibodies by the haemagglutination test. Then, an intramuscular booster of 30 μ g of purified AEMC IgG in Freund's incomplete adjuvant was administered to the rabbits and they were allowed to rest for another 3 weeks. Blood samples (2 ml) were taken from each rabbit every week and screened for anti-idiotypic antibodies. Two intramuscular boosters of 30 μ g of IgG each

in Freund's incomplete adjuvant were given on weeks six and nine. Ten days after the ninth week, the rabbits were anesthetized by intramuscular injection of 100 mg of Ketamine and bled to death by cardiac puncture. Sera obtained from the blood collected were stored at -20°C .

2.14. Polyacrylamide gel electrophoresis (Laemmli)

Materials:

- (a). 4X Upper Tris (0.5 M) Buffer, pH 6.8: contains 0.1 g SDS and 3 g Tris in 50 ml deionised water. The solution was adjusted to pH 6.8 with 1 M HCl.
- (b). 4X Lower Tris (1.5 M) Buffer, pH 8.8: contains 0.2 g SDS and 18.15 g Tris in 100 ml deionised water. The solution was adjusted to pH 8.8 with 1 M HCl.
- (c). 2X Sample Buffer: consists of 4 ml deionised water, 1 ml of the 4X Upper Tris, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2-mercaptoethanol and 0.2 ml of 0.05% bromophenol blue.
- (d). 30% acrylamide containing 0.8% Bis [w/w] (acrylamide-Bis).

(e). Freshly prepared ammonium persulphate (100 mg/ml) in deionised water.

(f). Tetramethylethylenediamine (TEMED).

(g). Electrophoresis Buffer: contains 3 g Tris, 14.4 g glycine and 1.0 g SDS, dissolved in 1 litre of deionised water.

Method:

The slab gel was made in a Bio-Rad apparatus which holds plates measuring 16 cm X 18 cm. The separating (lower) gel was prepared by mixing acrylamide-Bis (9.9 ml), lower Tris buffer (7.5 ml), deionised water (12.6 ml), ammonium persulphate (99 μ l) and TEMED (24.0 μ l). The gel mixture was degassed for 1-2 minutes at room temperature, transferred to the plates already set to hold the gel and layered with 1-2 ml of butanol. When polymerization was complete (about 30 minutes at room temperature), the top of the gel was rinsed three times with deionised water and then overlaid with the upper gel mixture, which contains 30% acrylamide-Bis (1.3 ml), upper Tris buffer (2.6 ml), deionised water (6.1 ml), ammonium persulphate (50 μ l) and TEMED (10 μ l). An electrophoresis comb was inserted into the upper gel and the gel was allowed to set at room temperature (about 30 minutes). The gel

was transferred to an electrophoresis tank containing electrophoresis buffer and samples were prepared for electrophoresis by adding to them, an equal volume of the sample buffer, followed by boiling for 5 minutes. Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at constant voltage, overnight (30 volts for 18 h).

2.15. Coomassie blue staining

After electrophoresis, separate gels were stained for protein with Coomassie blue and for carbohydrate by the periodic acid-Schiff (PAS) procedure.

Materials:

- (a). Fixative: 25% isopropylalcohol in 10% acetic acid.
- (b). Staining solution: 0.05% Coomassie brilliant blue (R250).
- (c). Destaining solution: 10% methanol in 10% acetic acid.

Method:

Gels were fixed for 2 hours at room temperature and then stained in 0.05% Coomassie blue for 2 hours at room temperature. After staining the

gels were decolorized by washing at 37°C , while shaking, for several hours in different changes of destaining solution until the background became clear. Stained gels were stored at 4°C in destaining solution.

2.16. PAS staining

Materials:

- (a). Fixative: 25% isopropylalcohol (also called 2-propanol), 10% acetic acid.
- (b). 0.5% periodic acid.
- (c). 0.5% sodium arsenate in 5% acetic acid.
- (d). 0.1% sodium arsenate in 5% acetic acid.
- (e). 5% acetic acid.
- (f). 0.1% sodium metabisulphite in 0.01 M HCl ($\text{Na}_2\text{S}_2\text{O}_5/\text{HCl}$).
- (g). Schiff's reagent was prepared by dissolving 2.5 g of Basic Fuchsin in 500 ml of deionised water. Then, 5 g of sodium metabisulphite was

added to the solution, followed by 50 ml of 1 M HCl. The solution was stirred for several hours at room temperature after which 2 g of activated charcoal (Fisher brand, also called NORIT-A) was added, while stirring, to decolorize the solution. The reagent was then filtered and stored at 4°C.

Method:

After electrophoresis, gels were removed from the plates and soaked in fixative for 2 hours at room temperature. Following fixation the gels were immersed in 0.5% periodic acid for 2 hours; washed in 0.5% sodium arsenate-acetic acid for 30-60 minutes; washed in 0.1% sodium arsenate-acetic acid for 20 minutes (twice); and washed in 5% acetic acid for 10-20 minutes. The gels were then transferred to Schiff's reagent and left to stain overnight at 4°C. After staining the gels were put in $\text{Na}_2\text{S}_2\text{O}_5/\text{HCl}$ and washed for several hours, until the rinse solution failed to turn pink upon addition of formaldehyde. The stained gels were stored at 4°C in $\text{Na}_2\text{S}_2\text{O}_5/\text{HCl}$.

2.17. Preparation of membranes from BMP cells

Confluent monolayer BMP cells were scraped from 150 mm tissue culture petri dishes (Becton Dickinson, New Jersey), pooled and washed in

cold PBS. Cells were suspended in hypotonic buffer (5 mM Na_2HPO_4 containing 2 mM of the protease inhibitor phenylmethylsulphonyl fluoride [PMSF], adjusted to pH 8.0) and left to swell for 1 h at 4°C , after which the cells were disrupted with 20 strokes in a Dounce homogenizer. Nuclei were removed by centrifugation at 1,000 X g for 5 min and the supernatant was collected and recentrifuged at 100,000 X g for 1 h. The crude membrane pellet was resuspended in cold PBS containing 2 mM PMSF and stored at -70°C . For use, membrane preparations were solubilized by incubating in 6 mM sodium deoxycholate containing 2 mM PMSF (final concentrations) for 1 h at 4°C .

2.18. Immunoblotting

After electrophoresis of membrane preparations, gels were transferred to nitrocellulose in transfer buffer containing 48 mM Tris, 39 mM glycine and 20% methanol for 6 h at 60 V, using a Bio-Rad Trans-Blot transfer apparatus. The nitrocellulose was then blocked with a 5% solution of milk powder (Carnation) in PBS-Tween (0.05% Tween 20) overnight at 4°C . All other incubations were performed at ambient temperature. After blocking, the nitrocellulose was incubated with 25 μg of purified EMC-D virus diluted in 5-10 ml of PBS-Tween, for 2 h, in a sealed bag on a rotator. The nitrocellulose was removed from the bag, washed three times in PBS-Tween and incubated

for 2 h with rabbit anti-EMC virus polyclonal antiserum diluted 1:200 in PBS-Tween. The nitrocellulose was washed again in PBS-Tween before it was incubated for 2 h with approximately 10^6 c.p.m. of ^{125}I -labelled goat anti-rabbit IgG antibody, in 10 ml of PBS-Tween, in a sealed bag. Then the nitrocellulose was washed three times in PBS-Tween, air dried, and exposed to Kodak-AR X-ray film at -70°C , for autoradiography.

2.19. Preparation of a virus-Sepharose 4B affinity column

Materials:

- (a). 1 mM HCl.
- (b). Coupling buffer pH 8.3: consists of 0.1 M NaHCO_3 containing 0.5 M NaCl.
- (c). Blocking buffer pH 8.0: consists of 0.2 M glycine.
- (d). Acetate buffer pH 4.0: consists of 0.1 M sodium acetate containing 0.5 M NaCl, adjusted to pH 4.0 with acetic acid.
- (e). 1 mg of Purified EMC-D virus.

(f). Cyanogen bromide (CNBr) activated CH-Sepharose 4B supplied by Pharmacia (Baie d'Urfe, Que., Canada).

Method:

i. 20 ml of 1 mM HCl was added to 1 g of activated Sepharose in a 50 ml beaker, and allowed to swell for approximately 15 minutes at room temperature. The swollen suspension was emptied into a funnel with a sintered glass filter and washed with 200 ml of 1 mM HCl under vacuum.

ii. 1 mg of purified EMC-D virus (i.e. the ligand) was dissolved in 10 ml of coupling buffer in a capped 50 ml centrifuge tube. The washed, activated Sepharose was then transferred from the sintered glass funnel into the tube containing the ligand. The mixture was rotated end-over-end at 6 rpm for 1 hour at room temperature using a multi purpose rotator (Scientific Industries Inc., U.S.A.). The slurry was then emptied into the sintered glass funnel and excess ligand was washed off with 20 ml of coupling buffer.

iii. The Sepharose with virus now bound, was transferred to the capped centrifuge tube and any remaining ligand attachment sites (active ester groups) were blocked by addition of 10 ml of 0.2 M glycine, pH 8.0, and rotating end-over-end for 1 hour at room temperature. The product was then

washed three times; each time, with 10 ml of acetate buffer, pH 4.0, followed by a 10 ml wash with coupling buffer pH 8.3. The final product, that is EMC-D virus bound to Sepharose, was stored at 4°C in 0.02 M phosphate buffer pH 8.0, containing 0.1% NaN₃ as a preservative.

iv. To estimate the amount of virus bound to Sepharose, a similar preparation was made using ³H-labelled EMC-D virus and the radioactivity in the entire preparation, rather than portions of it, was measured in a Beckman LS 3801 liquid scintillation counter.

2.20. Affinity chromatography

A virus-Sepharose affinity column was prepared as described in section 2.19. Solubilized membranes from BMP cells were prepared as described in section 2.17., and applied to the column in 0.02 M sodium phosphate buffer pH 8.0 which was also used to wash the column until all the unbound material was removed. Bound material was eluted with phosphate buffer containing either 0.2 M NaCl or 0.1% Triton X-100. The absorbance of the eluate was detected at 280 nm using an LKB 2138 Uvicord S UV monitor and recorder. Peak fractions were combined, dialysed against deionised water at 4°C overnight, lyophilized and analyzed by SDS-PAGE on 10% Laemmli gels.

2.21. Chromatofocusing of a putative receptor sialoglycoprotein

Materials:

- (a). PBE 94 ion-exchange resin suspended in ethanol as a preservative and supplied by Pharmacia (Baie d'Urfe, Que., Canada).
- (b). Starting buffer: consists of 0.025 M imidazole adjusted to pH 7.4 with 0.1 M HCl. Imidazole was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).
- (c). Eluting buffer: consists of polybuffer 74 diluted 1:8 with distilled water and then adjusted to pH 4.0 with 0.1 M HCl. Polybuffer 74 was obtained from Sigma Chemical Co.
- (d). ^{125}I -labelled putative EMC virus receptor obtained by affinity chromatography on EMC-D virus-Sepharose columns.
- (e). *Vibrio cholerae* and *Arthrobacter ureafaciens* neuraminidases supplied by Calbiochem-Bering (La Jolla, Calif., U.S.A.).

Method:

- (i). Chromatofocusing was performed at ambient temperature.

Approximately 10 ml of the ion-exchange resin PBE 94 was transferred to an equal volume of starting buffer and degassed for about 5 minutes. The slurry was then transferred to a 22 X 0.9 cm column and equilibrated at a flow rate of 1 ml/min with approximately 80 ml of starting buffer, or until the pH of the eluent equalled that of the starting buffer (pH 7.4).

(ii). The iodinated putative receptor sialoglycoprotein was added to the column (approx. 2.6×10^6 c.p.m.) in about 1 ml of starting buffer and allowed to adsorb for 5 minutes. The column was washed with polybuffer at a flow rate of 0.4 ml/min and 1 ml fractions were collected. The pH of each fraction was measured with a combination electrode using a Beckman Expandomatic SS-2 pH meter and the radioactivity in each fraction was detected with an LKB 1277 (Gammamaster) gamma counter. Each fraction was plotted against its pH and against the amount of radioactivity (in c.p.m.) it contained.

(iii). In a another run, the iodinated putative receptor sialoglycoprotein (approx. 2.6×10^6 c.p.m.) was desialylated by incubation with 20 mU of *Vibrio cholerae* and *Arthrobacter ureafaciens* neuraminidase for 1 h at 37°C, before adding the receptor material to the chromatofocusing column.

2.22. Iodination of glycophorin

Glycophorin was prepared from human erythrocytes by the method of Marchesi and Andrews (1971) and iodinated according to the method described by Markwell and Fox (1978).

Materials:

- (a). Glycophorin in solution.
- (b). ^{125}I was supplied by New England Nuclear, Mississauga, Ont., Canada.
- (c). 5% bovine albumin solution in 0.02 M sodium phosphate buffer pH 8.0 (BSA solution).
- (d). Disposable Sephadex G-25 columns (internal diameter 1.5 cm, bed height 5 cm, bed volume 9.1 ml) obtained from Pharmacia, Baie d'Urfe, Que., Canada.
- (e). Glass test tubes coated with Iodogen. These were prepared as follows: 200 μg of chloroglycoluril (available as Iodogen from Pierce Chemical Co., U.S.A.) was dissolved in 400 μl of chloroform. To each

of a number of glass tubes was transferred 20 μ l (i.e. 10 μ g) of the Iodogen solution. The solution was plated on the surface of the tubes by drying under a stream of N₂ gas at room temperature. Tubes coated with Iodogen were stored in a desiccator at room temperature.

Method:

A Sephadex G-25 desalting column was set up and equilibrated with 1 ml BSA solution followed by a 10 ml wash with 0.02 M sodium phosphate buffer. A glass tube coated with 10 μ g of Iodogen was rinsed with sodium phosphate buffer, pH 8.0, immediately before use to remove any loose flakes of Iodogen. Approximately 100 μ g of glycoporphin in solution was transferred to the coated tube and, working in a fume hood, 200 μ Ci of ¹²⁵I was added to the glycoporphin. After incubating the tubes for 10 minutes at room temperature, 1 ml of 0.02 M sodium phosphate buffer was added to the mixture and then applied to the Sephadex column. The column was washed with 5 ml of 0.02 M sodium phosphate buffer. 1 ml fractions were collected and the radioactivity in each fraction was detected with an LKB 1277 (Gammamaster) gamma counter.

2.23. Cell surface iodination of pancreatic cells

Baby mouse pancreatic (BMP) cells were surface labelled using a

modified procedure of the lactoperoxidase technique described by Meuer and associates (1983).

Materials:

- (a). ^{125}I was obtained from New England Nuclear, Mississauga, Ont., Canada.
- (b). Baby mouse pancreatic cells (4×10^7 cells/ml).
- (c). Ice-cold phosphate buffered saline (PBS, pH 7.4) containing 10 mM NaI. Ice-cold PBS without NaI, is also required.
- (d). Lysis buffer: consists of PBS containing 1% Triton X-100, 2 $\mu\text{g/ml}$ trypsin inhibitor and 1mM Phenylmethylsulphonylfluoride (PMSF).
- (e). Lactoperoxidase (2 mg/ml).
- (f). 0.03% H_2O_2 (v/v).

Method:

Baby mouse pancreatic cells were suspended in PBS at a concentration

of 4×10^7 cells/ml and 20 μ l of the lactoperoxidase was added together with 1 mCi of ^{125}I . The iodination was carried out by adding 10, 15, and 20 μ l of the H_2O_2 at time zero, 5 minutes and 10 minutes, respectively. After the last addition of H_2O_2 the reaction was allowed to proceed for an additional 10 minutes and stopped by transferring the cell suspension to 5 ml of ice-cold PBS containing NaI. The cells were washed three times in ice-cold PBS (without NaI) and then lysed for 30 minutes on ice at 4×10^7 cells/ml in lysis buffer. The lysate was stored at 4°C .

2.24. Immunoprecipitation of receptor from iodinated membranes

Materials:

- (a). Sepharose-protein A (obtained from Pharmacia, Baie d'Urfe, Que., Canada, and packaged as a 1 mg/ml suspension in 0.9% NaCl)
- (b). Heat inactivated (56°C , 1 h) EMC virus antiserum (AEMC) made in rabbits. Also, preimmune rabbit serum.
- (c). Wash buffer: contains 0.25% Nonidet, 0.5% Triton X-100 in 0.02 M phosphate buffer, pH 8.0.
- (d). ^{14}C -labelled protein markers.

Method:

Iodinated BMP cell lysates were solubilized by incubating them in 6 mM sodium deoxycholate containing 2 mM PMSF (final concentrations) for 1 h at 4°C. Solubilized lysates were then centrifuged in an Eppendorf microcentrifuge for 0.5 minutes at maximum speed. To a portion of the supernatant (2 X 10⁵ cpm) was added 200 µg of purified EMC-D virus and the mixture was incubated for 1 h at 37°C. After incubation, 50 µl of a 1:200 dilution of the EMC virus antiserum was added and incubated for 1 h at 37°C. The mixture was then incubated with 50 µl of the Sepharose protein A suspension for 1 h at 37°C. The beads were collected by centrifugation in the microcentrifuge for 0.5 minutes and washed twice in wash buffer and then once in PBS. Then the beads were mixed with an equal volume of Laemmli sample buffer and boiled for 5 minutes. The supernatant was collected and 1 µl of it was counted in a gamma counter while the rest was analyzed by polyacrylamide gel electrophoresis. After electrophoresis the gel was dried and exposed to Kodak X-Omat RP film at -70°C for at least 18 hours. Controls, in which virus was excluded from the protocol or rabbit preimmune serum was substituted for AEMC, were prepared in a similar manner.

2.25. Production of anti-receptor monoclonal antibodies in rats

Method:

(i). Twenty non-immunized Lou rats (7-8 weeks old), purchased from Harlan Sprague Dawley Inc., were bled by cardiac puncture. Preimmune serum was collected and stored at -20°C to be used as control serum. The rats were allowed to recover for 3 days from any trauma caused by the cardiac puncture.

(ii). While the rats were resting, BMP cells were grown in tissue culture petri dishes (Falcon, 150 X 25 mm) at 37°C in RPMI 1640 medium in a 5% CO_2 atmosphere. The cells were subsequently recovered from the dishes by scraping with a rubber policeman (trypsin would destroy receptors for the virus), washed twice with ice-cold PBS (Ca^{++} & Mg^{++} free) and resuspended at 2×10^7 cells/ml in PBS. Each rat was immunized intraperitoneally with 0.5 ml of the cell suspension, without adjuvant.

(iii). Three weeks later, each rat received a second intraperitoneal injection (no adjuvant) of 0.5 ml crude BMP membranes preparations. Crude membranes were made as described in section 2.17. but without solubilization in sodium deoxycholate.

(iv). Three weeks after the second injection the rats were divided into 3 groups. Group 1 received 0.5 ml of 10^7 BMP cells in PBS intraperitoneally, without adjuvant. Group 2 was given an intraperitoneal injection (without adjuvant) with 0.5 ml of crude membrane in PBS while rats in group 3 were administered a booster intrasplenically, without adjuvant, with 0.2 ml of crude membrane in PBS.

(v). Three days later blood was collected from the tail vein of each rat. The sera were heat inactivated and tested in the MTT/INT cell protection assay (described in section 2.26. below) to determine if any of the rats had made antibodies against BMP cell surface proteins which function as virus receptors. Rats whose sera tested positive in the MTT/INT assay had their spleens removed and the spleen cells were fused with YB2/O rat myeloma cells (obtained from ATCC; YB2/O is a hybrid originating from a fusion between the LOU rat myeloma Y3/Ag 1.2.3. and AO rat spleen cells) to generate hybridoma cultures.

2.26. The MTT/INT cell protection assay

This assay uses a colour reaction to detect viable cells. MTT, is a tetrazolium salt and the presence of active dehydrogenase enzymes in cells, results in cleavage of the tetrazolium ring in active mitochondria. The reaction

occurs only in living cells; not in dead cells or in tissue culture medium. Live cells metabolize the two dyes, MTT and INT, forming a deep-violet cytoplasmic precipitate and the colour produced is read on a multiscan spectrophotometer. For example, when BMP cells become infected with EMC virus the dead cells will produce no colour in the presence of MTT/INT. However, if the BMP cells were treated with antibodies which have the ability to protect them from EMC virus infection, the cells which survive subsequent infections with the virus will produce a colour in the presence of MTT/INT. The assay is simple and quantitative (when serum/hybridoma culture is diluted), and because there are only very few washing steps one can screen a large number of samples, in a relatively short period.

Materials:

- (a). MTT which is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
- (b). INT which is p-iodonitrotetrazolium violet. Both MTT and INT were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

The MTT/INT solution was prepared fresh, every week. 10 mg of INT was dissolved in 50 ml of PBS preheated to 90°C (final concentration is 200 µg/ml PBS). Then, 15 µl of glacial acetic acid was

added to the solution and the mixture was left to cool in the dark at room temperature after which 150 mg of MTT (final concentration, 3 mg/ml PBS) was added and allowed to dissolve. The solution was kept in a dark brown bottle and stored at 4°C.

Method:

(i). Baby mouse pancreatic (BMP) cells or HeLa cells (as a control) were grown to confluency (approx. 10^5 cells/well) in 96-well flat bottomed tissue culture plates (Linbro, Flow Laboratories).

(ii). Various samples of hybridoma culture presumed to contain monoclonal antibodies raised in Lou rats against the putative EMC-D virus receptor on BMP cells or heat inactivated (56°C for 1 hour) rat serum, were serially diluted in RPMI 1640 medium, added (50 µl/well) to BMP cells and incubated for 2 hours at 37°C in a 5% CO₂ atmosphere. When large numbers of hybridomas cultures were screened, supernatants were used at a single concentration and protection or non-protection was recorded.

(iii). The following controls were included:

(a). BMP cells plus hybridoma growth medium which does not contain antibodies (or heat inactivated rat preimmune serum), minus EMC-D virus.

(b). BMP cells in RPMI 1640 growth medium, minus hybridoma culture (which contains antibodies) and minus EMC-D virus.

(c). BMP cells in RPMI 1640 growth medium (no hybridoma culture), plus EMC-D virus at a multiplicity of infection of 3 p.f.u./cell.

(d). HeLa cells plus hybridoma culture which contains antibodies (or rat immune serum), plus EMC-D virus at a multiplicity of infection of 3 p.f.u./cell.

(iv). Without removing the serum or hybridoma culture from the wells, EMC virus was added to each well at a multiplicity of infection of 3 p.f.u./cell and the plates were incubated for 1 h at 37°C in a 5% CO₂ atmosphere.

(v). Without removing the virus, 100 µl of prewarmed (37°C) RPMI 1640 medium was added to each well and the plates were incubated for 18 to 20 h at 37°C in a CO₂ atmosphere. The next day the medium was removed from each well, replaced with the MTT/INT solution (50 µl/well), and the plates were incubated for 2 h at 37°C.

(vi). Then, the MTT/INT solution was removed and 200 µl of glacial acetic acid was added to each well. The plates were left to stand at room temperature for at least 30 minutes after which they were read on a multiscan ELISA plate reader (Flow Laboratories) at 415 nm. (The colour produced

remained stable for at least 5 hours). Wells showing high optical densities, compared to controls, in more than one assay were considered positive. Hybridomas scoring positive are subcloned twice.

2.27. Preparation of a primary culture of β -cells

Primary β -cells were isolated from the pancreas of SJL/J and C57BL/6J mice using a previously described procedure (Kaptur *et al.*, 1989).

Method:

(i). Mice pancreases were aseptically removed from 5 mice at a time, and washed two to three times in ice-cold Ca^{++} - Mg^{++} free PBS containing penicillin (500 U/ml) and streptomycin (1000 $\mu\text{g/ml}$).

(ii). The tissues were minced using a pair of scissors and washed in cold PBS. The supernatant was discarded and the small pieces of pancreatic tissue were transferred to a flask containing about 15 ml of prewarmed collagenase solution (3 mg/ml in PBS) and incubated at 37°C for 15-20 minutes while stirring vigorously.

(iii). The fragments were allowed to settle and the supernatant was collected and temporarily kept at 4°C . More collagenase solution was added

to the remaining tissues and the previous step was repeated until the pancreatic tissues were completely digested.

(iv). The supernatants were combined, diluted with cold PBS and centrifuged at 500 rpm for 5 minutes. The supernatant was discarded while the pellet which contains islets, acinar cells fibroblasts and other cells was saved and washed three times with cold PBS.

(v). The pellet was resuspended in cold PBS and transferred to petri dishes where single islets were collected using a Pasteur pipette and a microscope. The islets were then disrupted by stirring very gently in a prewarmed collagenase solution for 5 minutes at 37°C.

(vi). After centrifugation at 500 rpm for 5 minutes, the cells were resuspended in cold PBS and filtered through two layers of sterile gauze to eliminate any large aggregates. Then, the cells were resuspended in prewarmed RPMI 1640 medium containing 5% foetal calf serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Approximately 12-15 h later, non adherent β-cells were decanted and recultured in RPMI 1640 medium.

2.28. Dual labelling of primary β -cells for FACS analysis

Materials listed in (a) to (c) were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Materials:

- (a). Guinea pig anti-insulin serum diluted 1:40,000 in PBS.
- (b). FITC-conjugated goat anti-guinea (IgG) serum diluted 1:20 in PBS.
- (c). Goat anti-rabbit IgG R-Phycoerythrin conjugate diluted 1:20 in PBS.
- (d). Purified EMC-D virus (concentration = 2.1 mg/ml).
- (e). Heat inactivated (56°C , 1 h) antiserum against EMC virus, diluted 1:200 in PBS.
- (f). Washing buffer (PBS-NHS): contains 90 ml of PBS, 10 ml of heat inactivated normal horse serum, 2 μl of Triton X-100, and 0.1 g of sodium azide.
- (g). 5% paraformaldehyde in PBS.

Method:

(i). Primary mouse pancreatic β -cell cultures were isolated as described in section 2.27. Approximately 1×10^6 of these β -cells were fixed in 1 ml of cold (-20°C) 90% methanol in PBS, at -20°C for 15 minutes, primarily to permeabilize the plasma membrane. After fixation, cells were immediately stored at -20°C until required for use.

(ii). The fixed cells were washed once in PBS-NHS and then stained for 1 h at 37°C with 100 μl of guinea pig anti-insulin serum. After washing three times in PBS-NHS, the cells were stained with 100 μl of FITC-conjugated anti-guinea pig serum for 1 h at 37°C .

(iii). The FITC-stained cells were washed three times in PBS-NHS and 100 μl ($\sim 200 \mu\text{g}$) of the purified EMC-D virus preparation was added to the cells and incubated for 30 minutes at 4°C , to minimize internalization. The cells were washed three times in PBS-NHS to remove excess, unbound virus, followed by addition of 100 μl of anti-EMC virus serum (or preimmune serum as a control) for 30 minutes at 4°C .

(iv). The cells were washed again three times in PBS-NHS and then incubated with 100 μl of the phycoerythrin for 30 minutes at 4°C . After

washing three times in cold PBS, the cells were fixed in 1% paraformaldehyde in PBS for at least 15 minutes, sieved through non-absorbent cotton wool to remove clumps of cells and analyzed in an Epics C Coulter fluorescent activated cell sorter (FACS) or in a FACS 440 (Bectin Dickenson, Palo Alto, CA). The FACS was programmed to use right angle light scatter to exclude fluorescence due to debris. Viability of the cells in each sample was monitored by propidium iodide staining (0.5 $\mu\text{g/ml}$).

Chapter Three

RESULTS

3.1. Histopathology

The experiments carried out for this thesis made use of EMC-D virus which was obtained from Dr. J.W. Yoon, University of Calgary, Canada (see section 2.1). Before attempting to study the virus receptor it was necessary to show that this variant of EMC virus was indeed diabetogenic in SJL/J mice and not in C57BL/6J mice.

The experiments involved ten SJL/J and ten C57BL/6J mice which were infected intraperitoneally with 5×10^5 p.f.u. of EMC-D virus per mouse. Between five and twenty days after virus infection, about 200 μ l of blood was obtained from the tail vein of each mouse and analyzed for insulin and glucose content. Serum insulin levels in both virus-infected and non-infected mice were determined by radioimmunoassay using a kit supplied by Pharmacia (d'Urfe, Que., Canada) and blood glucose levels were measured using Ames dextrostix strips (Miles Laboratories, Etobicoke, Ont., Canada) and read on a glucometer. Influenza virus-infected SJL/J and C57BL/6J mice and non-virus-infected SJL/J and C57BL/6J mice were used as controls.

The results in Table 3.1 show that EMC-D virus-infected SJL mice had elevated levels of blood glucose and reduced amounts of serum insulin at 15 days post infection, while influenza virus-infected and non-infected SJL mice showed no evidence of hyperglycaemia 15 days after infection. Based on the criteria used in this thesis to identify the diabetic mice (see Table 3.1), up to 80% of SJL mice infected with EMC-D virus were regarded as diabetic. In contrast, EMC-D virus-infected C57BL mice and influenza virus-infected SJL mice were not diabetic.

To further show the diabetogenic effect of EMC-D virus in SJL mice, sections of pancreas from virus-infected and non-virus-infected SJL and C57BL mice were stained and examined under the microscope.

Sections from the controls and from EMC-D virus-infected C57BL/6J mice stained by haematoxylin and eosin (see section 2.5) showed normal, intact islet cells surrounded by acinar cells (Fig. 3.1). In contrast, sections from EMC-D virus-infected SJL/J mice showed a disordered architecture of the islet with evidence of cellular infiltration (Fig. 3.2).

Table 3.1 Blood glucose and serum insulin levels of mice before infection and 15 days after infection with EMC-D virus.

Results are expressed as the mean \pm standard deviation. Blood glucose levels exceeding the average of uninfected mice by a five-fold standard deviation were regarded as hyperglycaemic. Mice which remained hyperglycaemic up to day 20 post-infection were defined as diabetic.

SJL and C57BL mice used in this study were 8-10 weeks old.

Glucose measurements were performed on heparinized blood from non-fasting mice.

	No. of Mice	Blood Glucose (mmol/L)		Serum insulin (mU/ml)		Percent Diabetic
		Before Infection	15 days After Infection	Before Infection	15 days After Infection	
EMC-D virus-infected	SJL mice	9.9 ± 0.39	23.9 ± 2.56	124 ± 18	9 ± 3	80
	C57BL mice	9.5 ± 0.50	9.4 ± 0.44	119 ± 26	110 ± 21	0
Influenza virus-infected	SJL mice	9.3 ± 0.44	9.6 ± 0.28	130 ± 17	121 ± 23	0
	C57BL mice	9.7 ± 0.33	10.1 ± 0.22	124 ± 24	132 ± 17	0
Non-infected	SJL mice	9.6 ± 0.33	10.2 ± 0.39	129 ± 18	121 ± 18	0
	C57BL mice	10.4 ± 0.17	10.1 ± 0.5	125 ± 15	126 ± 10	0

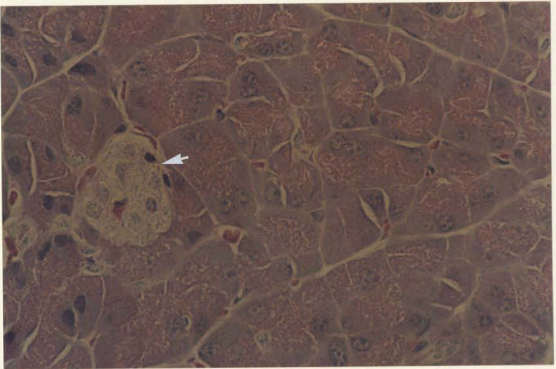


Fig. 3.1 Haematoxylin and eosin stained pancreatic section from an EMC-D virus-infected C57BL/6J mouse. Section shows a normal, intact islet of Langerhans surrounded by acinar cells. The section was processed as described in *Materials and Methods*.

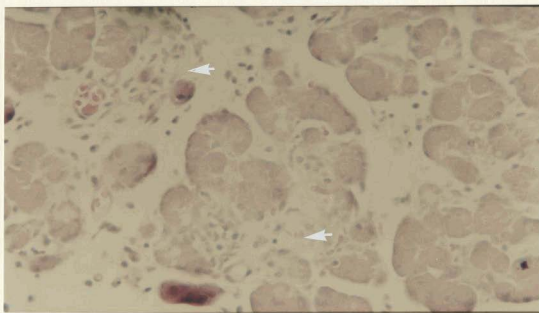


Fig. 3.2 Haematoxylin and eosin stained pancreatic section from an EMC-D virus-infected SJL/J mouse. Section shows islets with disordered architecture. The section was processed as described in *Materials and Methods*.

To identify the beta cells within the islets, sections were stained by aldehyde-fuchsin (see section 2.6) which selectively stains beta cell granules purple. Sections from EMC-D virus-infected SJL/J mice showed beta cell degranulation, indicated by little or no purple stain (Fig. 3.3), whereas sections from control mice or from virus-infected C57BL/6J mice showed intense purple staining of the beta cells (Fig. 3.4).

Since the pancreatic beta cells contained in the islet of Langerhans produce insulin, their destruction should show either a reduction in the amount of insulin or total absence of insulin in the islets. Immunoperoxidase staining (see section 2.7) with anti-insulin showed a significant amount of insulin in sections obtained from control mice or from virus-infected C57BL/6J mice (Fig. 3.5). In contrast, sections of pancreas obtained from EMC-D virus-infected SJL/J mice showed evidence of beta cell destruction as indicated by a reduction in the amount of insulin and by degeneration of the islets (Fig. 3.6).

Similar experiments were performed using the non-diabetogenic variant of EMC virus and the same number of mice. Neither EMC-B virus-infected SJL/J mice nor EMC-B virus-infected C57BL/6J mice developed diabetes-like symptoms or showed any signs of β -cell destruction (result not shown).

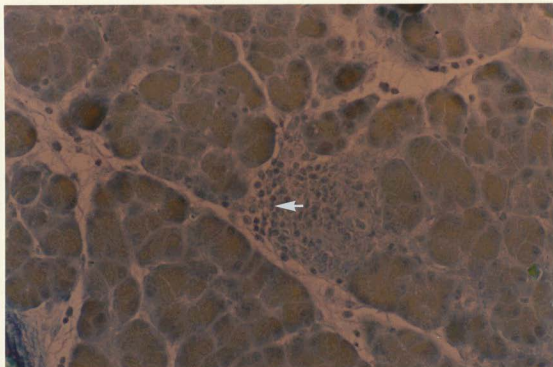


Fig. 3.3 Aldehyde-fuchsin stained pancreatic section from an EMC-D virus-infected SJL/J mouse. The decrease in β -cell granules (purple stain) can be seen. The section was processed as described in *Materials and Methods*.

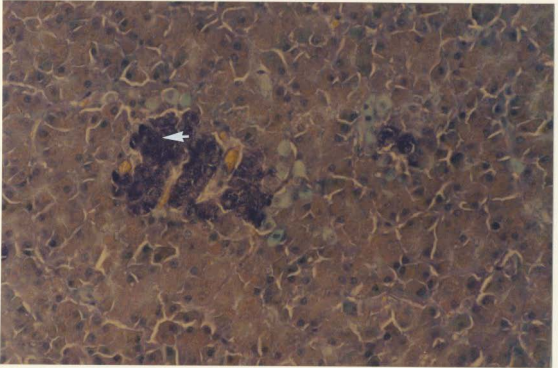


Fig. 3.4 Aldehyde-fuchsin stained pancreatic section from an EMC-D virus-infected C57BL mouse. Section shows evidence of ample β -cell granules (purple stain). The section was processed as described in *Materials and Methods*.

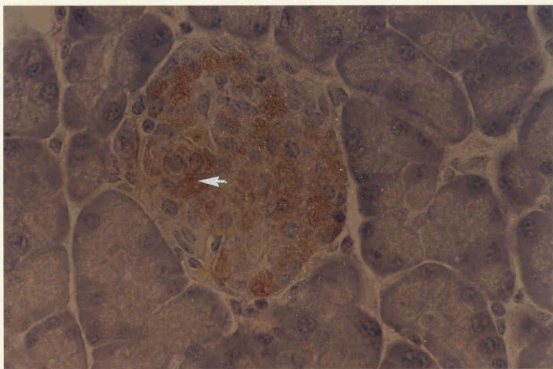


Fig. 3.5 Immunoperoxidase staining, using anti-insulin antibody, of a pancreatic section from an EMC-D virus-infected C57BL mouse. Section shows ample insulin the β -cells of an islet (brown stain). The section was processed as described in *Materials and Methods*.

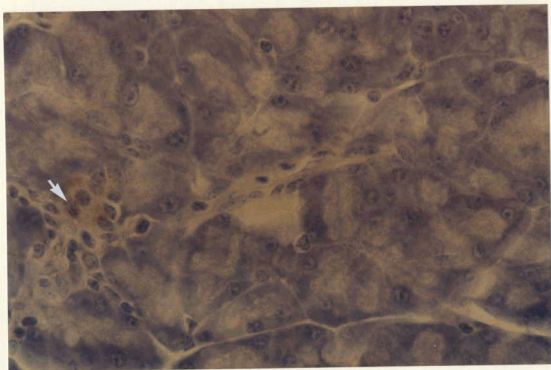


Fig. 3.6 Immunoperoxidase staining, using anti-insulin antibody, of a pancreatic section from an EMC-D virus-infected SJL mouse. Section shows evidence of reduced insulin content (brown stain) following β -cell destruction. The section was processed as described in *Materials and Methods*.

Taken together, these experiments confirmed that the EMC-D virus used in this study is diabetogenic in SJL/J mice but not in C57BL mice.

3.2. Biochemical Characterization of the Virus Receptor

The plan of the following experiments was to study the EMC-D virus receptor, by a variety of methods, in a continuous line of baby mouse pancreatic (BMP) cells. After biochemical characterization the EMC-D virus receptor on BMP cells would be isolated. These studies would culminate in production of antibodies to the receptor molecule. Such antibodies would then be used in experiments on primary beta cells isolated from the pancreas of SJL/J and C57BL/6J mice, to discover if the same receptor molecule could be found on them. This approach would alleviate the common problem, during β -cell isolation, of contamination with other cell types and more importantly, the purity of the β -cells could be ascertained using a fluorescent activated cell sorter (FACS). Details of the cell line and its handling are given in *Materials and Methods*, section 2.1.

As a basis for all future experiments, it was necessary first to study the behaviour of the virus in BMP cells. In the first three sections, data are given on virus growth, the time course of virus attachment and the saturability. With those data in hand then follow reports on a series of analytical experiments

aimed at elucidating the nature of the virus receptor.

3.2.1 Virus growth in BMP cells

The objective of these experiments was to establish a one-step growth curve to determine if EMC-D and EMC-B viruses can grow in BMP cells. The design was to infect a confluent monolayer of the cells with either virus and to measure, by plaque assay, the amount of virus present in the cells at various times. Details of the procedure are given in see section 2.8.

The results in Fig. 3.7 show that during a 2 to 4 h eclipse period, the infectious titre for EMC-D virus decreased to almost 50% of the amount of virus initially bound to cells. Five hours after infection, the infectious titre began to increase and at 18 h reached a maximum of 1.4×10^9 p.f.u./ml. Similarly, after going through an eclipse period, the infectious titre of EMC-B virus increased from 2.1×10^6 at 5 h to a maximum of 5.4×10^8 p.f.u./ml at 18 h. The peak titre for EMC-B virus in BMP cells was approximately 5-fold lower than that for EMC-D virus. These results showed that attachment of both EMC-D and EMC-B viruses to BMP cells results in productive infection.

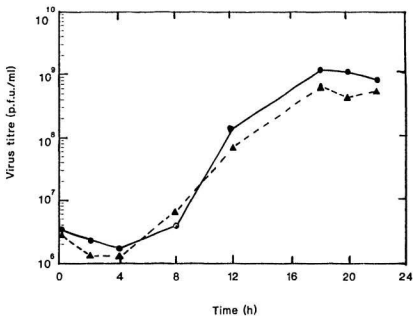


Fig. 3.7 Growth curve of EMC-B and EMC-D viruses in BMP cells. Confluent monolayers of cells (approx. 3×10^6 cells) were infected with EMC-B or EMC-D virus at a multiplicity of infection of 3 p.f.u./cell. The virus titre in the cells was measured by plaque assay at the times indicated.

▲-----▲ EMC-B virus ; EMC-D virus ●-----●

3.2.2. Time course

A study of the time taken for virus to attach to BMP cells was necessary so that in later analytical experiments the minimum time needed for optimal binding could be used. The rate at which EMC-D virus attaches to BMP cells was determined by adding ^3H -labelled virus (approx. 60,000 c.p.m.) to 3×10^6 cells (at 4°C to minimize internalization), for various lengths of time.

The results of this experiment (Fig. 3.8), showed that EMC-D virus attached to BMP cells, with binding reaching a maximum 2 h after addition of virus. Further, prolonged incubation of the cells with virus, at 4°C , did not result in a significant increase in virus binding to BMP cells.

3.2.3. Saturability

The objective of these experiments was to determine whether the surface receptors for EMC-D virus on BMP cells are saturable and if they are, to measure the number of binding sites for the virus on these cells. If the surface receptors are saturable, then increasing the concentration of the virus should cause a proportional decrease in the percentage of virus bound.

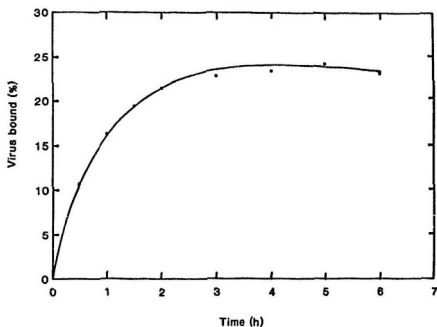


Fig. 3.8 Time curve of EMC-D virus binding to BMP cells. ^3H -labelled virus (approx. 60,000 c.p.m.) was added to confluent monolayers of cells (approx. 3×10^6 cells) at 4°C . At the times indicated, the cells were washed and the amount of virus attached to them was measured.

Cells grown to confluency were incubated for 3 h at 4°C with increasing amounts of ^3H -labelled virus. Then the cells were washed and the amount of virus bound or unbound was measured as described in section 2.9. Data derived from such saturation binding experiments were plotted as bound virus particles versus the number of unbound virus particles.

The results of this experiment are shown in Fig. 3.9. It can be seen from the shape of the curve obtained that binding of EMC-D virus to BMP cells is saturable. Competition binding experiments in which ^3H -labelled virus (approx. 60,000 c.p.m.) was mixed with increasing concentrations of non-radiolabelled purified EMC-D virus before measuring attachment to BMP cells showed that at least 80% of the ^3H -labelled EMC-D virus which bound to cells was competed for by unlabelled virus (data not shown). These experiments indicate that binding of EMC-D virus to BMP cells is specific and saturable.

3.2.3.1. Number of binding sites per cell

With data derived from saturation binding studies in hand, the number of binding sites for EMC-D virus on BMP cells was determined. Analysis of the saturation binding data was performed on a computer using the programme "Ligand", version 3.0, 1986, as described previously (Munson &

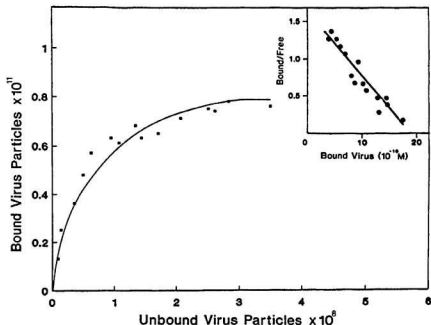


Fig. 3.9 Binding of EMC-D virus to BMP cells. Increasing amounts of ^3H -labelled virus were added to confluent monolayers of cells (approx. 3×10^6 cells) and incubated for 3 h at 4°C . The cells were washed three times in ice-cold PBS-BSA. The amount of virus bound to the cells and the amount unbound (in wash) were then measured.

Inset: A Scatchard plot of ^3H -labelled EMC-D virus binding to BMP cells obtained by analysis of the binding data.

Rodbard, 1980). From the Scatchard plot obtained, the dissociation constant (K_d), which is a measure of the affinity of the receptor for its ligand, was indicated as 1.2 nM. From B_{max} , the value of which was also given by the programme and which is defined as the maximum density of binding sites, the number of EMC-D virus binding sites was calculated to be 4×10^5 per cell (see appendix). The Hill coefficient was given by the programme as 0.5812555. The linearity of the Scatchard plot suggests that BMP cells contain a homogenous group of receptors for EMC-D virus (Inset, Fig. 3.9). However, the fact that the Hill coefficient is less than unity strongly suggests that the cells contain more than one homogenous group of virus binding sites.

3.2.4. Effect of proteases and glycosidases

In order to gain some insight into the biochemical nature of the receptor for EMC-D virus, BMP cells were treated with enzymes which either remove sialic acid molecules or other specifically linked carbohydrates, or act on proteins or phospholipids.

Following enzyme treatment, radiolabelled virus was added to the cells and incubated for 2 h at 4°C . The cells were then washed and virus attachment was measured (see section 2.9). Details of the conditions used in pre-treating the cells with enzymes are given under Table 3.2.

Since the different enzymes were used at various pH values, it was necessary first to establish the effect of varying the pH of the medium containing the cells, on virus binding. The design was to expose the cells to growth medium at a particular pH, overnight, and then to use the cells to measure virus attachment as described in section 2.9.

In preliminary experiments, it was observed that varying the pH of the growth medium in the range 4 to 10 caused 15% and 35% reduction in virus binding at pH values below 5 and above 8 respectively (data not shown). The optimum pH for virus binding was 7.0. Therefore, following enzyme treatment the pH of the medium was raised or lowered (depending on the enzyme used; see Table 3.2) to pH 7.0 prior to measuring virus attachment.

The results of pretreatment with enzyme are shown in Table 3.2. The results indicate that, compared to control non-enzyme treated BMP cells, treatment with trypsin, chymotrypsin and papain resulted in 80 to 90% reduction in virus binding, suggesting that the receptor for EMC-D virus on BMP cells is protein in nature. Incubation of the BMP cells with *V. cholerae* or *A. ureafaciens* neuraminidase which removes sialic acid residues, reduced virus binding by 45%. Treatment with phospholipases C and D, which hydrolyse phosphodiester bonds of phospholipids, or with endoglycosidase F

Table 3.2 Effect of pre-treating BMP cells with enzymes on EMC-D virus binding

Enzyme	Virus bound (%)
None (control)	100
<u>Glycosidases</u>	
Neuraminidase (<i>V. cholerae</i>) [†]	57
Neuraminidase (<i>A. ureafaciens</i>) [†]	54
Endoglycosidase F [‡]	105
Glycopeptidase F [§]	94
O-glycosidase	52
<u>Proteases</u>	
Trypsin	11
Papain	14
Chymotrypsin	13
<u>Phospholipases</u>	
Phospholipase C	90
Phospholipase D	95

* Used confluent monolayers (about 3×10^6 cells) and about 60,000 c.p.m. of ^3H -labelled virus for each enzyme treatment.

† 20 mU of neuraminidase, pH 5.4, 37°C , 1 h.

‡ 600 mU of endoglycosidase F, pH 5.0, 37°C , overnight.

§ 600 mU of glycopeptidase F, pH 7.3, 37°C , overnight.

- || Cells were treated with neuraminidase prior to treatment with 5 mU of O-glycosidase, pH 6.0, overnight. Increasing the concentration of O-glycosidase to 20 mU did not increase the reduction in virus binding.
- ¶ Used 100 μ l volumes containing 500 μ g of proteases or phospholipases, pH 7.0, 37°C, 1 h. With proteases, cells were treated in suspension.

Note: Virus binding to BMP cells treated with a mixture of V. cholerae and A. ureafaciens neuraminidases was not significantly different from virus binding to BMP cells treated with neuraminidase from either V. cholerae or A. ureafaciens alone.

Each enzyme had a control (i.e. cells untreated with enzyme) which was exposed to the same pH and temperature, for the specified period. Cells not treated with glycosidase but kept at 37°C, at the appropriate pH, overnight, remained viable as determined by trypan blue staining. The amount of radiolabelled virus bound to the control was taken as 100% and is compared with virus binding to enzyme treated cells. The results represent the mean of duplicate experiments.

or glycopeptidase F, enzymes which specifically remove N-linked carbohydrates, resulted in less than 10% reduction in EMC-D virus binding to BMP cells. Desialylation of the BMP cell surface with neuraminidase, followed by treatment with O-glycosidase which removes O-linked carbohydrates, resulted in 48% reduction in virus binding whereas treatment of cells with O-glycosidase alone had no effect on virus binding. Treatment of cells with sodium periodate (0.1 to 1.0 mM), which oxidizes sugars or the exocyclic carbon atoms of sialic acids to aldehydes, reduced virus binding by 7% at the most. These results suggest that the receptor for EMC-D virus on BMP cells is a sialylated glycoprotein.

3.2.5. Effect of cycloheximide and tunicamycin on receptor recovery

Initial experiments in this series were designed to determine how long it takes for BMP cells to regenerate EMC-D virus receptors. Cells were treated with trypsin, to destroy the virus receptors, and then grown in RPMI 1640 medium at 37°C. At various times the medium was removed, cells were washed and virus attachment was measured as described in section 2.9. Details of this experiment are given under figure 3.10.

The data given in Fig. 3.10 show that trypsin treatment reduced virus binding to 25% and, a full six hours were required, with the cells in growth

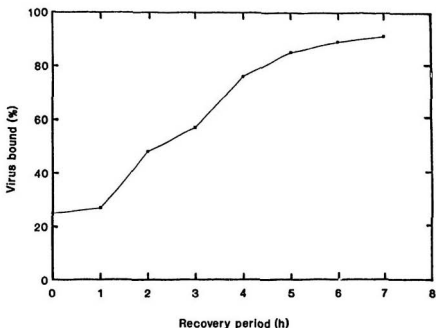


Fig. 3.10 Recovery of receptor activity after pretreating BMP cells with trypsin. Approximately 3×10^6 cells suspended in 100 μ l of HBSS were incubated for 1 h at 37°C in 100 μ l of prewarmed HBSS containing 500 μ g of trypsin. After incubation, the cells were washed in HBSS and allowed to recover in growth medium at 37°C . At the times indicated the growth medium was removed and attachment of ^3H -labelled virus (approx. 60,000 c.p.m.) was measured.

medium, for some 90% of the virus binding activity to be recovered. The results shown in Fig 3.10 indicated the need for more than six hours incubation, for studies of receptor regeneration on BMP cells.

The aim of the following experiments was to determine if EMC-D virus receptors are replaced from a pool or regenerated by synthesis of new ones, and whether EMC-D virus requires N-linked sugars to bind to BMP cells. The effect of 2-deoxy-D-glucose, an antimetabolite of glucose, on receptor regeneration, was not studied.

Cells treated with trypsin were seeded into RPMI 1640 medium containing increasing concentrations of either cycloheximide or tunicamycin. The cells were incubated overnight at 37°C until they became confluent and then the medium was removed, the cells were washed and virus attachment was measured (see section 2.9). Viability of cells grown in media containing either of the drugs was confirmed by trypan blue staining.

The data in Fig. 3.11 show that at a concentration of only 2 µg/ml of cycloheximide, virus binding was reduced by 70%, whereas incorporation of tunicamycin in the growth medium at a concentration of 12 µg/ml resulted in only a 6% reduction in virus binding. These experiments indicated that the

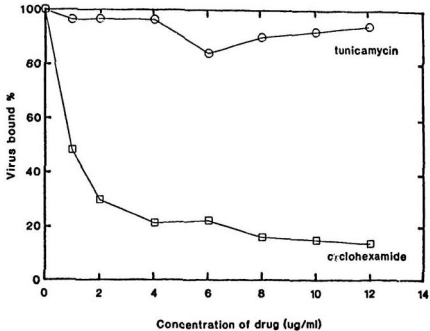


Fig. 3.11 Effect of tunicamycin or cycloheximide on recovery of receptor activity. Approximately 3×10^6 BMP cells suspended in 100 μ l of HBSS were incubated for 1 h at 37°C in 100 μ l of prewarmed HBSS containing 500 μ g of trypsin. Following incubation, the cells were washed in HBSS, seeded onto 35 mm tissue culture dishes and allowed to recover in growth medium containing tunicamycin or cycloheximide at 37°C, overnight. Attachment of EMC-D virus (approx. 60,000 c.p.m.) was measured when the cells became confluent.

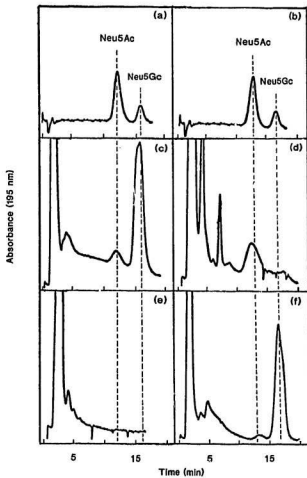
cell surface molecules to which the virus binds prior to infecting the cell, are regenerated by synthesis of new receptors. The experiments also suggested that N-linked sugars may not be required for EMC-D virus binding to BMP cells.

3.2.6. Sialic acids released from BMP cells

Since pretreatment of cells with neuraminidase (see Table 3.2) suggested that sialic acids are required for EMC-D virus binding to BMP cells, this experiment was set up to identify the type of sialic acid present on BMP cells. Sialic acids were released from BMP cells either by acid hydrolysis (total) or by neuraminidase treatment, and were analyzed by HPLC using an Aminex A-29 anion exchange column. Details of the conditions used are given in section 2.11. Cellular sialic acids were identified by comparing their elution times with those of known sialic acid standards or by coinjection of BMP cell sialic acids with standards (Fig. 3.12 *a* and *b*). A standard curve was made after injecting various amounts of sialic acid standards into the column, from which total and neuraminidase-releasable sialic acids in BMP cells were estimated.

It was estimated that both *V. cholerae* and *A. ureafaciens* neuraminidases released only 30% of the total sialic acid in BMP cells (data not shown). As

Fig. 3.12 Analysis of sialic acids by HPLC. Sialic acids were released from cells by acid hydrolysis or by neuraminidase treatment and identified on an Aminex A-29 anion exchange column. (a) and (b) are sialic acid standards showing the elution times for N-acetyl neuraminic acid (Neu5Ac) and N-glycolyl neuraminic acid (Neu5Gc); (c) sialic acids released from BMP cells by acid hydrolysis; (d) sialic acids released from BMP cells by neuraminidase treatment; (e) control: cells incubated for 1 h at 80°C without acid or for 1 h at 37°C without neuraminidase; (f) sialic acid released by acid hydrolysis from cells previously treated with neuraminidase.



shown in Fig. 3.12 *d*, the neuraminidase-releasable sialic acid comprised predominantly N-acetyl neuraminic acid. Subsequent acid hydrolysis of neuraminidase treated cells showed a major peak identified as N-glycolyl neuraminic acid (Fig. 3.12 *f*). No N-acetyl or N-glycolyl neuraminic acids were detected in control experiments where cells were kept at 80°C for 1 h without acid or at 37°C for 1 h without neuraminidase (Fig. 3.12 *e*).

3.2.7. Effect of lectins and sugars on virus binding

The effect of lectins on virus binding was investigated to determine if indeed sialic acids are involved in EMC-D virus binding to BMP cells. Cells were incubated with various lectins (see Table 3.3) for 30 min at ambient temperature, after which they were washed and virus attachment was measured as described in section 2.9. As a control, cells were incubated with PBS. The concentrations and sugar specificities of the lectins used are given in Table 3.3.

The results in Table 3.3 show that pretreatment of cells with horseshoe crab (*Limulus polyphemus*) and wheat germ (*Triticum vulgaris*) reduced virus binding by 79% and 38% respectively. Virus binding to cells pretreated with horse gram (*Dolichos biflorus*) or concanavalin A (*Jack bean*) was reduced less

Table 3.3 Effect of pre-treating BMP cells with lectins on EMC-D virus binding

Lectin	Sugar specificity	Virus bound (%)
None (control)	-	100
Horseshoe crab (<i>Limulus polyphemus</i>)	Sialic acid (Neu5Ac)	21
Wheat germ (<i>Triticum vulgaris</i>)	Sialic acid (Neu5Ac) & N-acetyl-D-glucosamine	62
Horse gram (<i>Dolichos biflorus</i>)	N-acetyl-D-galactosamine	92
Concanavalin A (Jack bean)	α -D-Mannose	94

Confluent monolayers (about 3×10^6 cells) were incubated with a 1 mg/ml solution of each lectin (1 ml/dish), 30 min, ambient temperature. Used approx. 60,000 c.p.m. of ^3H -labelled virus to measure virus attachment, after each treatment.

Note: The effect of pre-treating cells with sialoglycoproteins, such as fetuin and mucin, on virus binding, was not studied since pre-treatment of the cells with lectins, combined with other results (Table 3.2; Fig. 3.19; Fig. 3.22) showed that sialoglycoproteins are involved in EMC-D virus binding to BMP cells.

Both lectin-treated and non lectin-treated cells (control) kept at ambient temperature for 30 min remained viable as determined by trypan blue staining (not shown). The amount of radiolabelled virus bound to the control was taken as 100% and is compared with virus binding to lectin treated cells. The results represent the mean of duplicate experiments.

than 10% when compared with controls. These results (Table 3.3) showed that sialic acids are involved in the binding of EMC-D virus to BMP cells.

Since certain sugars have been shown to block the ability of EMC virus to haemagglutinate human erythrocytes (Kunin, 1967), an experiment was performed to determine whether EMC-D virus utilizes sugars other than sialic acids to bind to BMP cells. Cells were incubated with the sugars specified in Table 3.4 for 1 h at ambient temperature after which they were washed and virus binding was measured (see section 2.9). Details of the conditions used are given in Table 3.4.

The results show that none of the sugars tested inhibited EMC-D virus binding to BMP cells (Table 3.4), suggesting that none of these sugars are required for EMC-D virus binding to the cells.

3.3. Receptor Isolation

The following experiments were designed to isolate the EMC-D virus receptor from BMP cells and to determine whether the isolated receptor is able to bind to the virus. The first set of experiments provide results on the stability of the column at the temperature and pH values used to prepare the column and, on the specificity of the column used to isolate the receptor.

Table 3.4 Effect of pre-treating BMP cells with sugars on EMC-D virus binding

Sugar	Virus bound (%)
None (control)	100
D-Galactose	91
D-Mannose	94
D-Maltose	104
D-Lactose	87
N-Acetyl-D-galactosamine	93
N-Acetyl-D-glucosamine	89
α -Methyl-D-mannoside	94
α -Methyl-D-glucoside	92

Confluent monolayers (about 3×10^6 cells) were incubated with a 0.5 M solution of each sugar (0.5 ml/dish), 1 h, ambient temperature. Used approx. 60,000 c.p.m. of ^3H -labelled virus to measure virus attachment, after each treatment. Note: Treated and untreated cells (control) remained viable (not shown) after 1 h at ambient temperature. The amount of radiolabelled virus bound to the control was taken as 100% and is compared with virus binding to treated cells. The results represent the mean of duplicate experiments.

Results on the molecular weight of the isolated receptor are also given. Subsequent experiments provide data on the ability of EMC-D virus to bind to the isolated receptor.

3.3.1. Properties of the virus-Sepharose column

The aim of these experiments was to prepare a virus-Sepharose column to be used in isolation of the virus receptor. Since EMC virus disintegrates in the presence of halide ions between pH 5 and 7, and because the procedure used to couple EMC virus to Sepharose involved several alternate washes in NaHCO_3 buffer pH 8.3 and acetate buffer pH 4.0 at ambient temperature, it was necessary first to show that virus linked to Sepharose was not inactivated and that its ability to bind receptor material was not adversely affected by the pH or temperature at which the column was prepared or stored. It was also necessary to show that binding of a receptor to the virus-Sepharose is specific. To address these issues, the following experiments were performed.

A virus-Sepharose column was made for eventual isolation of the virus receptor. Non radioactive ("cold") EMC-D virus was covalently linked to cyanogen bromide activated Sepharose by mixing the two, blocking unattached sites with glycine and washing with buffers at pH 8.3 and 4.0. Details of the preparation of an EMC virus-Sepharose column for affinity chromatography

are given in section 2.19.

3.3.1.1. Amount of virus on the column

To estimate how much of the virus attached to the Sepharose, a similar preparation was made using ^3H -labelled EMC virus after which the entire radiolabelled virus-Sepharose conjugate was transferred to vials containing scintillation fluid and counted for radioactivity. Using this procedure it was determined that about 85% of the ^3H -labelled virus added, remained covalently linked to the Sepharose even after washing with NaHCO_3 buffer pH 8.3 and acetate buffer pH 4.0 containing 0.5 M NaCl (data not shown).

3.3.1.2. Void volume of the column

To estimate the void volume, the column was packed by washing it with 10 to 15 ml of phosphate buffer. Then 1 ml of 0.2 mg/ml of calf thymus DNA (Sigma) in phosphate buffer or 1 ml of a solution containing 0.1 mg of the DNA and 1 mg of adenosine in phosphate buffer was applied to the column. Without allowing any time for adsorption to take place, 1 ml fractions of the eluate were collected and the absorbance of each fraction was measured at 260 nm. Using this procedure it was determined that the void volume of the column was 3 ml (data not shown).

3.3.1.3. Stability of the column

To determine the stability of the column at various pH values, a ^3H -labelled EMC virus-Sepharose column containing 10,000 c.p.m. was washed with 0.02 M phosphate buffer pH 6.0 at a flow rate of 0.3 ml/min at ambient temperature and five 1 ml fractions were collected and counted for radioactivity. Subsequently, the column was washed with phosphate adjusted to pH 5.0, 4.0, 3.0, 2.0, 1.0 and 8.0, respectively, and fractions (five 1 ml fractions for each pH) were collected and measured for radioactivity. Then the experiment was repeated at 4°C .

The results show that the virus remained covalently linked to the Sepharose in the pH range 1.0 to 8.0 and the virus was not released at 4°C or at 20°C (Fig. 3.13). When the column was tested for its ability to bind ^3H -labelled glycoporphin, compared to a similar virus-Sepharose column which was not subjected to variable pH washes, no major difference was observed in percent binding (not shown).

3.3.1.4. Specificity of the column

Glycophorin A is the cell surface binding protein for EMC virus on human erythrocytes. In this experiment, glycophorin was extracted from human erythrocytes and used as a control, to determine if binding of

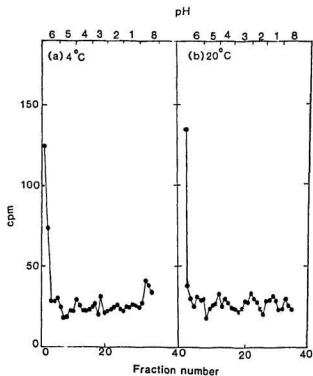


Fig. 3.13 Effect of varying pH or temperature on EMC virus coupled to Sepharose 4B. A ^3H -labelled virus-Sepharose column was washed with phosphate buffer at various pHs and virus released was detected by measuring radioactivity in the fractions collected.

receptor proteins to the EMC virus-Sepharose column was specific. Radiolabelled glycophorin (2 μ g of protein) was applied to either a Sepharose column without virus, a BSA-Sepharose column or to an EMC virus-Sepharose column. If the virus column were specific, then the last would be the only one of the three to bind glycophorin.

Each column containing ^3H -labelled glycophorin was washed with phosphate buffer until unbound material was fully removed. Then the column was eluted with phosphate buffer containing NaCl followed by the same buffer containing 0.1% (1.6 mM) Triton X-100. Fractions of 1 ml were collected for radioactivity measurements.

The amount of NaCl required to break the glycophorin-virus bond had been determined earlier by eluting glycophorin bound to virus on the Sepharose column with a salt gradient of 0 to 0.5 M NaCl in 0.02 M sodium phosphate buffer pH 8.0. The concentration of NaCl was determined from a standard curve of known NaCl molarity measured on a conductivity bridge (Model 31, Yellow Springs Instruments Co., U.S.A.). Conductivity readings obtained with phosphate buffer without any NaCl were subtracted from the results of phosphate buffer containing NaCl.

It was found that on average, 78% of the total radioactivity added to the columns was recovered. The results in Fig. 3.14 show that ^3H -labelled glycoporphin added to the virus-Sepharose column was eluted in three peaks. It can be seen from Fig. 3.14 that about 54% (peak 2) of the glycoporphin bound to the virus-Sepharose column, whereas approximately 25% (peak 1) was not retained on the column, and about 21% (peak 3) came off only when the column was washed with phosphate buffer containing 0.1% Triton X-100. In contrast, only 2% and 2.5% of ^3H -glycoporphin bound to the Sepharose and BSA-Sepharose columns respectively (not shown).

The possibility that the retained material (peaks 2 and 3; Fig. 3.14) was held back non specifically perhaps because separation occurred on the column according to size, was discounted by applying ^3H -labelled glycoporphin to the virus-Sepharose column and washing extensively with phosphate buffer. It was found that prolonged washing with phosphate buffer alone did not effect elution of the retained material (data not shown).

3.3.1.5. Saturability of the virus column

To test the possibility that material in peak 1, (Fig. 3.14) was not retained because the column was saturated, competition experiments were performed. The design of these experiments was to mix various amounts (50 -

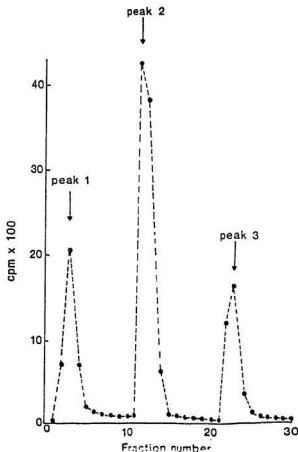


Fig. 3.14 Affinity chromatography of glycophorin on an EMC virus-Sepharose column. Two μg of ^3H -labelled glycophorin in 0.02 M phosphate buffer were added to the column and, following adsorption the column was washed with 0.02 M buffer containing 0.2 M NaCl, and then finally buffer containing 0.1% Triton X-100. The radioactivity in each 1 ml fraction was measured.

200 μg) of "cold" glycophorin with 2 μg of ^3H -labelled glycophorin and then to add each mixture (comprising a specific amount of "cold" glycophorin plus 2 μg of radiolabelled glycophorin) to the virus-Sepharose column. After adsorption for 30 minutes, the column was washed with phosphate buffer or with buffer containing NaCl or Triton X-100. The column was regarded as saturated with glycophorin when it failed to retain most of the radioactive glycophorin.

Using this procedure, it can be seen that the virus-Sepharose column is able to accommodate at least 100 μg of receptor protein (Fig. 3.15). Since previous experiments utilized only 2 μg of ^3H -labelled glycophorin (see Fig. 3.14), the result in Fig. 3.15 indicates that the three peaks observed in Fig. 3.14 are not due to saturation.

These results, when taken together, showed that EMC virus linked to cyanogen bromide-activated Sepharose 4B is capable of binding specifically to a receptor protein.

3.3.2. Properties of the affinity purified putative virus receptor

Having determined the amount (i.e. protein concentration) of receptor material required to saturate the virus column, and the stability and specificity

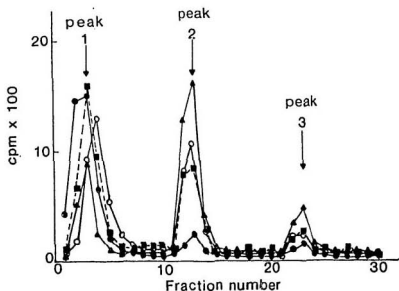


Fig. 3.15 Saturability of the virus-Sepharose column. The column was saturated with various amounts of non-radioactive glycoporphin ("cold"), mixed with 2 μg of ^3H -labelled glycoporphin and regarded as saturated when no more ^3H -labelled glycoporphin bound to the column.

— 2 μg ^3H -labelled glycoporphin plus either: ○—○ 50 μg , or ■—■ 100 μg , or ●—● 200 μg of cold glycoporphin.

of the column under conditions used for receptor isolation, the stage was set for isolating the virus receptor. The objective of the next set of experiments was to isolate the BMP cell surface receptor for EMC-D virus by affinity chromatography, and to determine if the isolated receptor would bind the virus. Data are provided on the molecular weights of two putative receptor proteins and there is evidence to show that the putative receptor is a cell surface protein.

3.3.2.1. Receptor isolation

The design of the experiment for isolating the receptor was as follows. Crude membranes were prepared from BMP cells as described in *Materials & Methods* (see section 2.17). Then the membranes were solubilized in 6 mM sodium deoxycholate and applied to the EMC-D virus-Sepharose column (see section 2.20). Material bound to the column (the putative EMC-D virus receptor) was eluted with sodium phosphate buffer containing NaCl, desalted by dialysis and concentrated by lyophilization (see section 2.20). The putative EMC-D virus receptor was reconstituted in 100 μ l of PBS and analyzed by electrophoresis on 10% SDS polyacrylamide gels [SDS-PAGE] (see section 2.14). The gels were stained with Coomassie blue as described in section 2.15. It was not possible to include receptor-negative cells as a control because EMC-D virus attached to and/or infected all the cells tested, namely, mouse

L cells, Krebs ascites mouse cells, Mardin-Darby bovine kidney (MDBK) cells, human erythroleukaemic (K562) cells and HeLa cells.

The result of this experiment was that up to 40 µg of putative receptor protein was obtained from solubilized membranes by combining eluates from several runs. When run on SDS-PAGE, as shown in Fig. 3.16, there was evidence for at least two protein bands with relative molecular weights of about 97,000 and 70,000 daltons. It can be seen from Fig. 3.16 that the larger molecular weight band is relatively more distinct while the lower molecular weight band is faint and more diffuse, perhaps because the lower band is more glycosylated. No other bands were observed. This experiment was repeated three times and gave essentially the same result each time. The conclusion from this result is that EMC-D virus probably binds to two distinct receptor proteins on BMP cells, one of which may be glycosylated.

3.3.2.2. Virus binding properties of the isolated putative receptor

To show that the column bound material was able to associate with the virus, the reconstituted putative EMC-D virus receptor was serially diluted 1:2 in PBS and analyzed on nitrocellulose in a 96-well dot blot assay. Unoccupied sites on the nitrocellulose were blocked with a redundant protein as described in *Materials & Methods* (see section 2.18). The nitrocellulose was washed twice

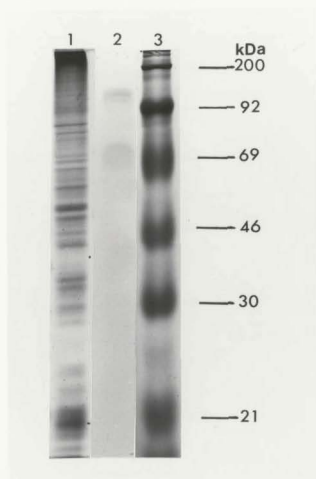


Fig. 3.16 Coomassie blue stained putative receptor proteins. BMP plasma membranes were solubilized in sodium deoxycholate and applied to an EMC-D virus-Sepharose column. Bound material was eluted with phosphate buffer containing 0.2 M NaCl. The eluate was dialysed, lyophilized, analyzed by SDS-PAGE and stained. *lane 1*, BMP membrane before application to column; *lane 2*, membrane material bound to virus on the column; *lane 3*, protein markers.

as described in section 2.18 and then incubated with 10^5 to 10^6 c.p.m. of ^{125}I -labelled EMC-D virus for 2 h at ambient temperature. After washing three times, the nitrocellulose was air dried and exposed to Kodak-AR X-ray film for autoradiography. The specificity of EMC-D virus binding to the putative receptor was investigated in a competition assay. The putative receptor bound to nitrocellulose, was exposed to saturable amounts of non-radioactive EMC-D virus before probing it with ^{125}I -labelled EMC-D virus.

The results obtained are shown in Fig. 3.17. These experiments showed that EMC-D virus bound to the putative receptor isolated from BMP membranes (Fig. 3.17 *a*). As the ratio of non radioactive EMC-D virus to putative receptor material increased (i.e. constant virus but serially diluted receptor protein) the amount of ^{125}I -labelled EMC-D virus bound to the receptor protein decreased, indicating that binding of ^{125}I -labelled EMC-D virus was specific (Fig. 3.17 *b*). Iodinated EMC-D virus did not bind if the putative receptor had been treated with trypsin prior to application to nitrocellulose (Fig. 3.17 *d*). Pre-treating the receptor protein with *V. cholerae* neuraminidase resulted in a reduction in the amount of iodinated EMC-D virus bound, suggesting that the column-derived putative receptor was sialoglycoprotein in nature (Fig. 3.17 *c*). There was no non-specific binding of ^{125}I -labelled EMC-D virus to the nitrocellulose (Fig. 3.17 *g* & *h*), and the

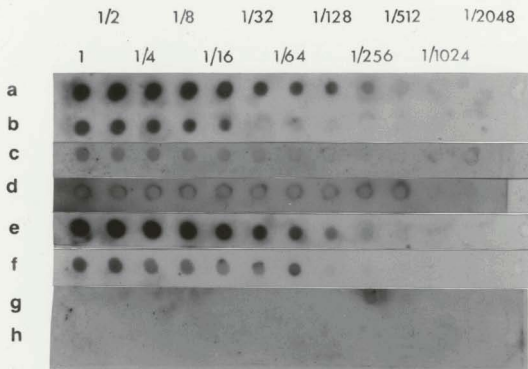


Fig. 3.17 A dot blot assay of EMC-D virus binding to the putative receptor on nitrocellulose. (a) putative receptor serially diluted and then probed with ^{125}I -labelled virus; (b) receptor exposed to unlabelled virus prior to probing with ^{125}I -labelled virus; (c) *V. cholerae* neuraminidase-treated receptor probed with ^{125}I -labelled virus; (d) trypsin-treated receptor probed with ^{125}I -labelled virus; (e) glycophorin probed with ^{125}I -labelled virus; (f) glycophorin exposed to unlabelled virus prior to probing with ^{125}I -labelled virus; (g & h) blocked nitrocellulose exposed to ^{125}I -labelled virus.

control using glycophorin behaved as predicted (e & f). Thus it appeared that the column fractionation had indeed resulted in a specific, virus-binding, protein as had been hoped.

3.3.2.3. Study of surface labelled BMP cell lysate

The aim of this experiment was to show that the putative EMC-D virus receptor is a component of the BMP cell surface. ^{125}I -labelled BMP cell lysates were prepared as described see section 2.23. Iodinated BMP cell lysates were then solubilized in 6 mM sodium deoxycholate and immunoprecipitated with non radioactive EMC-D virus as described in section 2.24. To rule out the possibility that some free ^{125}I may have been trapped on the cells and carried over in the reaction mixture, a batch of iodinated BMP cell lysates was centrifuged on sucrose density gradients and the plasma membrane band was collected, dialysed against cold PBS and concentrated by lyophilization. Lysates prepared on sucrose gradients were solubilized in sodium deoxycholate, incubated with unlabelled EMC-D virus and immunoprecipitated with anti-EMC virus antiserum and Protein A as described in section 2.24.

The SDS gel autoradiograph derived from this experiment is shown in Fig. 3.18. Although difficult to see in the photograph, several bands were

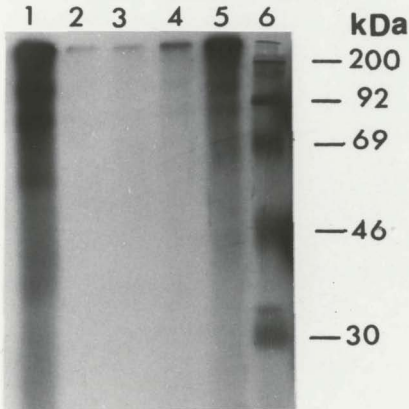


Fig. 3.18 An autoradiograph of ^{125}I surface-labelled BMP cell lysates immunoprecipitated with EMC-D virus. (1) ^{125}I -labelled lysate; (2) lysate, without virus, plus anti-EMC virus antiserum; (3) lysate, plus virus, plus preimmune serum; (4) lysate prepared on sucrose density gradients, plus virus, plus anti-serum; (5) lysate, plus virus, plus anti-serum; (6) ^{14}C -labelled protein marker.

found to coprecipitate from BMP cell lysates in the presence of EMC-D virus (Fig. 3.18 lane 5) but not in the absence of EMC-D virus (lane 2) or in the presence of preimmune rabbit serum (lane 3). Amongst these were two prominent bands, one with a molecular weight of about 97,000 and another relatively faint, and more diffuse band at about 70,000. This result has to be considered quite preliminary. The experiment was performed twice and each time there were no clear cut or sharp bands.

3.3.2.4. Chromatofocusing of the putative receptor protein

Results of previous experiments described in this thesis, such as neuraminidase treatment of whole cells, suggested that the cell surface receptor for EMC-D virus is sialylated (see Tables 3.2 & 3.3). The objective of these experiments was to determine whether the isolated putative virus receptor contains sialic acid. The design was to separate molecules of the receptor protein from each other on the basis of differences in their isoelectric points (pI), by chromatofocusing. Chromatofocusing was performed on the putative receptor protein before and after treatment with neuraminidase. Details of the procedure are given in section 2.21.

The results of these experiments are shown in Fig. 3.19. Chromatofocusing of the untreated putative receptor protein on a PBE 94

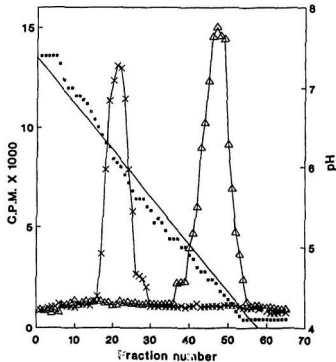


Fig. 3.19 Chromatofocusing of the affinity purified receptor protein before ($\Delta\Delta\Delta$) and after ($\times\times\times$) desialylation by treatment with neuraminidase. The shift in pH from 4.6 prior to desialylation (i.e. pH corresponding to peak in $\Delta\Delta\Delta$) to pH 6.1 after desialylation (i.e. pH corresponding to peak in $\times\times\times$), indicates that sialic acids have been released. The pH gradient ($\bullet\bullet\bullet$) was generated by elution with polybuffer as described in *Materials & Methods*. Approximately 2.6×10^6 c.p.m. were used for each run.

ion-exchange resin showed an isoelectric point (pI) of 4.6 whereas desialylation of the putative receptor protein by neuraminidase treatment, prior to chromatofocusing, revealed a pI of 6.1. The increase in pI from 4.6, prior to desialylation, to 6.1 after desialylation, confirmed that the putative virus receptor contains sialic acid.

3.3.2.5. Mass spectrometric analysis and amino acid sequencing of the putative receptor

Mass spectrometry was employed in order to determine more precisely, the molecular weights of the two putative receptor protein bands which were isolated by affinity chromatography on a virus-Sepharose column. It was also highly desirable to sequence the protein bands so as to determine, by searching through data bases, if the receptor protein(s) could be identified as known cell surface glycoprotein.

To prepare samples for mass spectrometry and amino acid sequencing, the affinity-purified putative receptor was run on SDS polyacrylamide gels and then transferred to "Immobilon", a form of nitrocellulose. Prior to SDS-PAGE, samples were boiled for three minutes in electrophoresis (Laemmli) sample buffer as described in section 2.14. The Immobilon was stained with Coomassie blue, to confirm protein transfer, destained with methanol, air

dried, covered with "saran wrap" and then sent elsewhere for analysis. To prevent N-terminal blocking that may be caused by unreacted polymerizing agents, the separating gel was cast 24 hours before running. At no point did the samples come in contact with urea. In the first instance, the samples were sent to the Biotechnology Service Centre, The Hospital for Sick Children, University of Toronto, Canada. Later, a second batch of samples was sent to the IAF BioChem International, Inc., 10900 Hamon Street, Montreal, Canada.

In both instances, attempts to sequence the putative receptor protein or to determine more precisely the molecular weights of the major protein bands by mass spectrometry were unsuccessful. Amino acid analysis performed on the proteins by both companies, prior to sequencing, showed that each of the two bands present on the Immobilon contained at least 20 picomoles of protein (correspondence from company), which, according to the companies, was more than they needed for the required analyses. No further experiments were performed to determine whether the inability to sequence the proteins was due to blockage of the N-terminus.

3.3.3. Can the receptor be isolated using radiolabelled virus?

Rather than use an EMC-D virus-Sepharose column to isolate the receptor, it is less time consuming and probably cheaper, to use radiolabelled

virus for the same purpose. In the procedure using radiolabelled virus, cell surface membrane preparations are separated on SDS polyacrylamide electrophoresis gels, transferred to nitrocellulose, blocked with an irrelevant protein and then probed with ^{125}I -labelled EMC-D virus, or with non-radioactive EMC-D virus followed by anti-EMC virus antibodies and Protein A. The nitrocellulose is then exposed to X-ray film, for autoradiography. Details of this immunoblotting procedure are given in section 2.18. Since immunoblotting is performed on nitrocellulose after SDS-PAGE of deoxycholate-solubilized membranes, and since samples are usually boiled prior to SDS-PAGE analysis, the dot blot assay was used to look at the effect of SDS and sodium deoxycholate, with and without boiling, on EMC-D virus binding to the putative receptor protein.

Such immunoblotting experiments were unsuccessful and the autoradiographs are not shown. Upon autoradiography, no protein bands were observed. The same result was obtained after innumerable attempts. Successful transfer of the proteins to nitrocellulose was confirmed by Coomassie staining of the nitrocellulose. However, it was found that boiling, or the presence of SDS or sodium deoxycholate did not prevent EMC-D virus from binding to the putative receptor protein on nitrocellulose (result not shown). The results of these experiments are puzzling, especially since ^{125}I -

labelled EMC-D virus is able to bind the putative receptor on nitrocellulose in a dot blot assay (see 3.17).

3.4. Production of Anti-receptor Antibodies

The objective of this experiment was to produce anti-receptor antibodies and to determine whether such antibodies could prevent the virus from infecting BMP cells. The plan was to use these antibodies, later on, to study the virus receptor on β -cells isolated from the pancreas of SJL/J and C57BL/6J mice.

Since the putative receptor protein was derived from mice, anti-receptor antibodies were raised in rats. Approximately 1,500 supernatants from various hybridoma cultures, presumed to contain monoclonal anti-receptor antibodies, were screened by the MTT/INT assay (see section 2.26) to see if the antibodies would protect BMP cells from EMC-D virus infection. Details of the procedure used to produce anti-receptor antibodies are given in section 2.25.

The results of this experiment are shown in Fig. 3.20. The controls show that BMP cells kept, in the absence of EMC-D virus, in hybridoma growth medium (H 3-5) or in RPMI 1640 growth medium (Fig. 3.20, H 6-8), remained

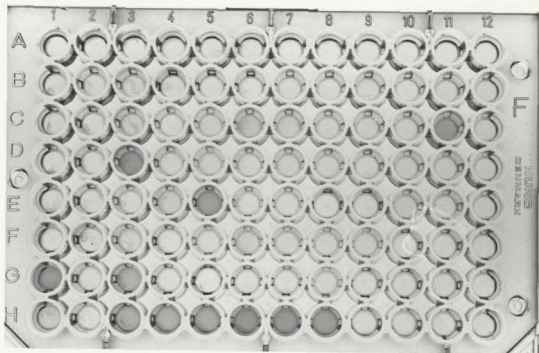


Fig. 3.20 MTT/INT colorimetric assay for detecting cell viability. Cells were infected with EMC-D virus in the presence and absence of anti-receptor antibody as described in sections 2.26 and 3.4. A1-G5 hybridoma cultures; G6-H2 BMP cells and EMC-D virus; H 3-5 BMP cells kept in hybridoma medium; H 6-8 BMP cells kept in RPMI 1640 growth medium; H 9-12 HeLa cells and EMC-D virus. C11, D3, E5, G1 and G3 are positive hybridoma cultures.

viable for the duration of the experiment. In another control, it was observed that BMP cells kept in RPMI 1640 growth medium became infected when they were exposed to the virus (Fig 3.20, G6-H2). None of the hybridoma cultures protected HeLa cells from infection with EMC-D virus (Fig. 3.20, H 9-12). However, supernatants five hybridoma cultures were able to protect BMP cells from EMC-D virus infection (Fig. 3.20, wells C11, D3, E5, G1 and G3). Confirmation that these hybridomas were truly positive was obtained through a blind assay, whereby samples (including previously positive ones) were numbered in another laboratory, prior to being sent to our laboratory for screening by the MTT/INT assay. In each instance, samples which were previously reported as positive, were found to protect BMP cells from virus infection. All of these positive hybridomas were unfortunately lost during the subsequent cloning procedure, which was necessary to ensure production of monospecific antibody. However, the results of this experiment confirmed that the putative receptor behaves as an authentic receptor, since antibodies raised against epitopes on it prevented virus infection of BMP cells. It was also shown that the mouse receptor is immunogenic for the rat.

3.5. FACS Analysis of EMC-D Receptors on β -cells

The aim of this set of experiments was to study the receptor for EMC-D virus on β -cells isolated from the pancreas of SJL/J and C57BL/6J mice by

flow cytometry, and to compare the EMC-D virus receptor on BMP cells with receptors on primary mouse β -cells.

The design of the experiments was to study binding of EMC-D virus (detected with anti-virus antibody) or anti-virus anti-idiotypic antibodies to surface receptors on cells. Both BMP cells and sorted β -cells from SJL and C57BL mice (using a fluorescent activated cell sorter (FACS)) were used.

Beta cells from SJL and C57BL mice were analyzed by a dual labelling procedure as described in section 2.28. In this procedure, the β -cells are stained first with guinea pig anti-insulin serum followed by FITC-conjugated goat anti-guinea pig antibodies (to enable sorting), prior to detection of cell surface-bound anti-virus or anti-idiotypic antibody, with anti-rabbit phycoerythrin-conjugated antibody. On the other hand, binding of anti-virus or anti-idiotypic antibody to BMP cells, which do not need to be sorted, was detected by a single labelling procedure using phycoerythrin-conjugated anti-rabbit antibody as described in section 2.28. The FACS was programmed such that the same number of cells were analyzed from each sample. As a control, cells were treated with rabbit preimmune serum and were used to set the baseline level for the FACS.

Details on how the anti-virus antibodies (AEMC) and the anti-virus anti-idiotypic antibodies were produced in rabbits are given in sections 2.12 and 2.13. Mouse pancreatic β -cells were isolated as described in section 2.27.

The results of the first set of experiments are shown in Fig. 3.21. The histograms obtained show relative cell number on a linear scale on the Y-axis and fluorescence intensity on a log scale on the X-axis. From the fluorescence intensity, it can be seen that the amount of anti-idiotypic antibody molecules bound on C57BL or SJL/J β -cells (Fig. 3.21 *e* and *f*) is not much different from the amount of virus bound to the same cells (Fig. 3.21 *b* and *c*). However, the number of binding sites for EMC-D virus on β -cells from SJL/J and C57BL mice (Fig. 3.21 *b* and *c*) were less than the number of binding sites for the virus (Fig. 3.21 *a*) or for anti-idiotypic antibodies on BMP cells (Fig. 3.21 *d*). These results showed that like EMC-D virus, the anti-idiotypic antibodies bind to BMP cells and to β -cells from SJL/J and C57BL mice. Since the number of virus binding sites on BMP cells is known (see Fig. 3.9) these results also indicated that primary β -cells from both SJL and C57BL mice contain less than 4×10^5 EMC-D virus binding sites per cell.

The results of the next set of experiments show that compared to untreated controls (Fig. 3.22 *a* and *d*), pretreating cells with trypsin reduced

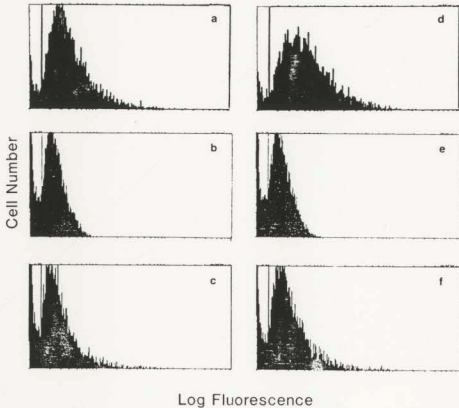


Fig. 3.21 Flow cytometric analysis of EMC-D virus binding to β -cells. Virus binding was detected using anti-virus antibody from a rabbit and phycoerythrin-conjugated anti-rabbit antibody. Positive fluorescence (see text for details) indicates binding of virus to cells. (a) - (c) BMP cells, C57BL β -cells, and SJL/J β -cells respectively, exposed to EMC-D virus. (d) - (e) BMP cells, C57BL β -cells, and SJL/J β -cells respectively, exposed to anti-idiotypic antibodies made in rabbits.

both EMC-D virus binding (Fig. 3.23 *b*) and anti-idiotypic antibody binding (Fig. 3.22 *e*) to β -cells from SJL mice. Similarly, pretreatment of cells with neuraminidase reduced the amounts of virus and anti-idiotypic antibody bound to SJL β -cells (Fig. 3.22 *c* and *f*) suggesting that both EMC-D virus and the anti-idiotypic antibodies bind to sialylated proteins.

These results are representative of similar results obtained when virus and anti-idiotypic antibody binding were studied on enzyme-treated BMP cells or enzyme-treated β -cells from C57BL mice (not shown). Also, flow cytometric analysis of binding of polyclonal rat anti-receptor antibodies to BMP cells or to β -cells from SJL and C57BL mice gave profiles similar to that seen with binding of virus or anti-idiotypic antibodies, to the same cells (not shown).

The possibility that EMC-D virus and the anti-idiotypic antibodies bind to the same site on SJL β -cells was examined by competition experiments whereby SJL β -cells were exposed to saturable amounts of EMC-D virus prior to adding the anti-idiotypic antibodies. The reduction in the amount of anti-idiotypic antibody bound to SJL β -cells after virus binding (Fig. 3.23 *c*), compared to the amount of virus (Fig. 3.23 *a*) or anti-idiotypic antibody (Fig. 3.23 *b*) attached to SJL β -cells, suggest that the two ligands recognize similar binding sites on β -cells from SJL/J mice. It was concluded from these

experiments that β -cells from C57BL mice contain approximately the same number of EMC-D virus receptors as β -cells from SJL mice.

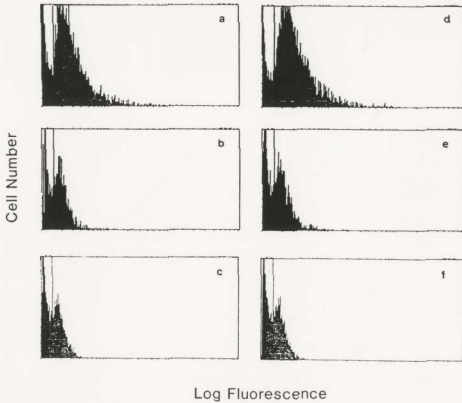


Fig. 3.22 Flow cytometric analysis of EMC-D virus binding to β -cells pretreated with enzyme. Virus binding was detected using anti-virus antibody from a rabbit and phycoerythrin-conjugated anti-rabbit antibody. Positive fluorescence (see text for details) indicates binding of virus to cells. (a) Untreated, (b) trypsin-treated, (c) *V. cholerae* neuraminidase-treated, SJL/J β -cells exposed to EMC-D virus. (d) Untreated, (e) trypsin-treated, (f) *V. cholerae* neuraminidase-treated, SJL/J β -cells exposed to anti-idiotypic antibody from a rabbit.

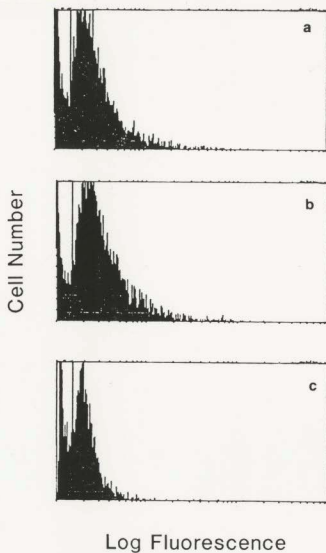


Fig. 3.23 Flow cytometric analysis of competitive binding to β -cells between EMC-D virus and the anti-idiotypic antibody. (a) SJL/J β -cells probed with EMC-D virus; (b) SJL/J β -cells probed with anti-idiotypic antibody from a rabbit; (c) SJL/J β -cells exposed to EMC-D virus prior to probing with anti-idiotypic antibody.

Chapter Four

DISCUSSION

4.1. Virus binding studies

The BMP cells used in this study are an SV40 transformed pancreatic β -cell line derived from CD1 mice (Kaptur *et al.*, 1989). The plan was that following biochemical characterization of the EMC-D virus receptor on these cells, the virus receptor would be isolated and anti-receptor antibodies would be produced in rats. Using the FACS, the anti-receptor antibodies would then be used in experiments on primary SJL/J and C57BL/6J mouse pancreatic β -cells, to see if the same receptor molecule could be found on them. This strategy was inspired by the finding that out of about 110 distinct serotypes of human rhinovirus close to 90% bound to a single receptor on HeLa cells (Colonno *et al.*, 1986), and by the observation that the major group rhinovirus receptor, ICAM-1 (Greve *et al.*, 1989; Staunton *et al.*, 1989), is expressed on different cell types from different species (Horley *et al.*, 1989; Hogg *et al.*, 1991). This approach has several advantages. Being a cell line, the amount of cells required to carry out the biochemical characterizations could be easily produced. To do this with primary pancreatic β -cells from SJL/J and C57BL/6J

mice would require hundreds of mice, since the primary β -cells grow very poorly in culture. Further, this approach alleviates the common problem, during β -cell isolation, of contamination with other cell types and, relatively pure SJL/J and C57BL/6J mouse β -cells could be produced by sorting on the FACS.

4.1.1. EMC-D virus binding sites on BMP cells

Since EMC-D virus binding to BMP cells was affected at certain pH values, virus attachment studies were performed at pH 7.0. The observation that virus binding was reduced at pH values below 5 is consistent with previous reports that cardioviruses disintegrate at acidic pH in the presence of chloride ions (Dunker & Rueckert, 1969; Dunker & Rueckert, 1971). The reduction in virus binding observed at pH values above 8 may also be due to the instability of the virus at alkaline pH, however, the reason for this effect is not clear.

The finding that EMC-D virus bound to BMP cells in a time-dependent manner at 4°C, reaching a maximum 2 h after addition of the virus, differs from a previous report which indicated that HeLa cell binding of the M-variant of EMC virus (EMC-M), from which both EMC-B and EMC-D viruses were derived (see section 1.1.2.), reached maximum three minutes after the

virus was added, at 4°C (McClintock *et al.*, 1980). Similarly, my results differ from the observation that maximum EMC-M virus binding to pancreatic β -cells from SJL/J mice occurred between 16 and 32 minutes after the virus was added (Chairez *et al.*, 1978). It should be noted that since these studies involve different virus strains and different cell types, a true comparison of these results cannot be made. Since it is known that different strains of virus show very different rates of attachment to cells (Dimmock, 1982), I speculate that the variation in binding kinetics between EMC-D virus and BMP cells, and between EMC-M virus and HeLa or β -cells, is due to differences in the amino acid sequence of the attachment proteins of the different virus strains involved. Alternatively, the variation in binding kinetics may be a function of the motility of the cell surface receptor. Fluidity of the plasma membrane is influenced by temperature; as such, the temperature at which attachment studies are performed can affect virus attachment rates. EMC-D virus binding to BMP cells was measured at 4°C, to minimize virus internalization, whereas EMC-M virus binding to HeLa and β -cells was measured at 37°C. While EMC-M virus binding to HeLa cells was unaffected by variation in temperature (McClintock *et al.*, 1980) the reduced rate of attachment of EMC-M virus to β -cells (Chairez *et al.*, 1978), compared to the rate of EMC-D virus attachment to BMP cells, may be due to lateral diffusion [and thus increased accessibility] of the virus receptors, at 37°C. Although EMC-D virus

attachment was assayed in PBS containing 1% BSA, the argument that these differences in binding kinetics are due to variation in the viscosity of the medium in which virus binding was measured (Crowell & Siak, 1978), is not tenable. EMC-M virus binding to β -cells was assayed in PBS alone (Chairez *et al.*, 1978). However, EMC-M virus binding to HeLa cells, which showed the lowest rate of attachment, was carried out in Eagle's minimal essential medium supplemented with 5% foetal bovine serum (McClintock *et al.*, 1980). Bovine serum albumin or foetal bovine serum was added to the medium used to measure attachment in order to minimize non-specific virus binding.

It is reported in this thesis that EMC-D virus binding to BMP cells was saturable, and unlabelled EMC-D virus inhibited attachment of the labelled virus to BMP cells (see section 3.2.3.). The specific activity of the radiolabelled virus and the molecular weight of the virus are given in the appendix. Accepting that a straight line in a Scatchard plot suggests the presence of a homogenous group of binding sites, the linear plot obtained by Scatchard analysis of the saturation binding data (Fig. 3.9) contradicts the electrophoresis results (Fig. 3.16) which indicate that EMC-D virus binds to two proteins with different molecular weights. While the BMP cells may indeed possess a homogenous set of high affinity binding sites for EMC-D virus, the Scatchard plot alone (Fig. 3.9, Inset) does not account for the

presence of another EMC-D virus receptor as suggested by the electrophoresis results.

Visual inspection of the points on the Scatchard plot suggests the possibility of more than one binding site; however, this approach is subjective. Apart from the difficulty of determining objectively whether a Scatchard plot comprises one or more straight lines, the Scatchard method is also limited by the fact that deviations from one-site interactions are not easily observed (Munson & Rodbard, 1980; Lee Hsu *et al.*, 1988; Titeler, 1989). So, rather than use subjective visual assessment, the computer programme "Ligand" was used to analyze the saturation binding data. This programme utilizes binding data from replicate experiments, corrects for non-specific binding, and statistically analyses the data before using a least-squares curve-fitting algorithm to objectively measure the best fit (Munson & Rodbard, 1980).

Considering that the Scatchard method works on the assumption that binding is monovalent (Munson & Rodbard, 1980; Lee Hsu *et al.*, 1988; Titeler, 1989), I suggest that the inconsistency between these two results (Figs. 3.9 & 3.16) is due to the fact that virus binding to cell surface receptors is not monovalent (Lonberg-Holm & Philipson, 1974; Lonberg-Holm *et al.*, 1976). Both reoviruses and picornaviruses (e.g. EMC-D virus) have icosahedral

structures and the concept of non-monovalent virus binding is illustrated by the reovirion particle which has twelve potential cell attachment sites (Lee *et al.*, 1981). The argument that the Scatchard method is inadequate for detecting multiple binding sites for multivalent ligands such as viruses has been made (Lee Hsu *et al.*, 1988). In fact Lee Hsu and associates (1988) had to use a different, albeit questionable, method in order to detect heterogenous binding sites for group B Coxsackieviruses on HeLa cells. Although detection of multiple binding sites by the Scatchard method using radiolabelled ligands other than viruses has been reported, particularly in pharmacologic studies (Hollenberg, 1989; Titeler 1989), ligand binding to multiple binding sites can occur in various forms. For instance, when various molecules of a radiolabelled ligand [A] bind separately to different receptors, [B] & [C], the ligand is recognizing multiple binding sites. However, it is conceivable that an icosahedral virus particle whose various attachment proteins each bind to different receptors (in effect cross-linking them) is also exhibiting multiple binding. And, perhaps the Scatchard method is able to detect the former because it is, in fact, monovalent binding although to different receptor sites, but not the latter (i.e. cross-linkage).

The Hill method is used to correct this weakness of the Scatchard method (Titeler, 1989). A Hill coefficient was obtained from the slope of a

Hill plot of the same saturation binding data that was used to produce the Scatchard plot. A Hill plot and a Hill coefficient were given by the computer programme each time a Scatchard analysis was performed. The fact that Hill analysis of the saturation binding data in Figure 3.9 revealed a Hill coefficient less than unity (see section 3.2.3.1.) strongly suggests that the interaction between EMC-D virus and BMP cells involves more than one binding site (Titeler, 1989; Hollenberg, 1989). This finding is consistent with the electrophoresis results shown in Figure 3.16. Further, this Hill coefficient indicates negative cooperativity for the radiolabelled virus. In other words, the radiolabelled virus is not only binding to multiple sites on BMP cells but there is competition for these sites (Titeler, 1989; Hollenberg, 1989). Although a Hill plot cannot be used to determine the number of binding sites or to measure the binding affinity between the virus and its receptor, the fact that the Hill coefficient is not unity indicates that the virus is likely to be binding to multiple sites with different affinities. If the Hill coefficient was equal to unity this would indicate independent binding of the radiolabelled ligand to different sites, with one affinity. On the other hand, a Hill coefficient greater than unity would indicate that the binding of a radiolabelled ligand in some way increases the affinity of another ligand for the next unoccupied site [i.e. positive cooperativity] (Titeler, 1989; Hollenberg, 1989).

In spite of the fact that the binding studies described in this thesis do not account for the second set of receptors, it could be estimated that BMP cells possess a homogenous group of about 4×10^5 EMC-D virus binding sites per cell. This number of binding sites does not exactly follow what one might expect from the saturation curve which seems to indicate saturation at about 0.7×10^{11} viruses bound per 3×10^6 cells. This calculates to be 2.3×10^4 virus binding sites per cell, and is seventeen times less than the Scatchard calculation. This difference is not readily explained. However, the Scatchard result is consistent with that obtained for the M-variant of EMC virus on HeLa cells [1.5×10^5 per cell] (McClintock *et al.*, 1980) but differs significantly from those for other picornaviruses. For example, it is estimated that all six serotypes of group B Cocksackieviruses (CVB) have about 3×10^6 binding sites per HeLa cell whereas the even-numbered serotypes of CVB have about 5×10^4 binding sites per HeLa cell (Lee Hsu *et al.*, 1988). Since these results involve different viruses and different cell types, a true comparison cannot be made. Such variation in the number of virus binding sites may be a function of differences in the surface areas of the different cells or to differences in receptor density, or a combination of both factors.

It is also possible that the variation in the number of binding sites is due to posttranscriptional and/or posttranslational events. Receptor mRNA

may not be translated into protein or the mRNA may encode a protein that cannot bind the virus because of differences in the amino acid sequence. Mendelsohn *et al.* (1989) have shown that human kidney cells contain a 3.3 kb RNA that hybridized with both coding and 3' noncoding probes derived from poliovirus receptor cDNAs. Since the kidney is not a site for poliovirus replication and the kidney cells did not contain detectable poliovirus binding activity, it was concluded that expression of poliovirus receptor mRNA is insufficient to encode functional receptor activity in this tissue (Mendelsohn *et al.*, 1989). The variation in virus binding sites may be influenced by posttranslational modifications such as the presence or absence of carbohydrate molecules, like sialic acids, at sites within the ligand binding region, or by phosphorylation or sulphation which may influence the conformation of the receptor molecule and thus the availability of the binding sites (Cunningham *et al.*, 1987; Tavakkol & Burness, 1990; Staunton *et al.*, 1990; Schultze & Herrler, 1992). For example, the activity and expression of N-CAM is influenced by sulphation of the N-linked oligosaccharides (Cunningham *et al.*, 1987), while the sulphide bonds within ICAM-1 are essential for its recognition by HRV-14 (Hogg *et al.*, 1991).

The high binding affinity ($K_d = 1.2 \times 10^{-9}$ M) between EMC-D virus and a homogenous group of binding sites on BMP cells differs significantly

from that observed between EMC-M virus and HeLa cells. EMC-M virus bound to HeLa cells with a lower affinity, $K_d = 1.3 \times 10^{-7}$ M (McClintock *et al.*, 1980). Although a true comparison is not possible because these data involve different virus strains and different cell types, one can speculate that a possible reason for this difference in binding affinities is variation in the virus attachment proteins of the different strains (Bae *et al.*, 1990). Mutations which affect virus attachment have been reported. For example, EMC-B virus has eight point mutations which are located in its polyprotein coding region and five of these mutations are located on the VP1 gene (Bae *et al.*, 1989; Bae *et al.*, 1990). These mutations result in amino acid changes on virion protein VP1 which affect the binding characteristics of the virus (Bae *et al.*, 1990).

4.1.2. Limitations of the virus binding assay

In interpreting the virus binding data, several limitations of the virus binding assay were taken into consideration. The ability to separate unbound (free) radiolabelled ligand from bound radiolabelled ligand is technically a limitation of the virus binding assay used in this study. Unbound radiolabelled EMC-D virus was separated from the bound radiolabelled virus by rapidly washing the cells several times in PBS-BSA as described in sections 2.8 and 2.9. Cells with radiolabelled virus attached were solubilized in Triton X-100 and the percentage of cell-associated virus was determined after liquid

scintillation counting. Similar methods are commonly used for getting rid of free virus and for measuring bound virus in virus attachment assays (Armstrong *et al.*, 1984; Allaway & Burness, 1986; McClintock *et al.*, 1988; Clayson & Compans 1989; Tavakkol & Burness 1990). The procedure is based on the supposition that specific radioligand binding is associated with high affinity binding ($K_d < 10^{-8}$ M), and a slow rate of dissociation, whereas nonspecifically adsorbed radioligands have low binding affinities and therefore, are easily washed off.

Distinguishing between specific binding of the radiolabelled virus to cell surface receptors and nonspecific binding of the same ligand to other surfaces is another limiting factor. In studies similar to that reported in this thesis, specific binding is usually determined by comparing virus binding to cells which lack the virus receptor, with binding to cells which express the virus receptor. In this study, this could not be done because EMC-D virus bound to all the cells tested, namely, mouse L cells, Krebs ascites mouse cells, Mardin-Darby bovine kidney cells, human erythroleukaemic (K562) cells and HeLa cells (Ingrid Pardoe, personal communication). In this discussion, specific binding refers to binding of radiolabelled virus to a cell surface receptor site for which non-radiolabelled virus with selectivity for the same site can compete. Although not as accurate as using receptor-negative cells,

this method is often used as an indicator of specific ligand binding to receptors (Munson & Rodbard, 1980; Armstrong *et al.*, 1984; Clayson & Compans, 1989; Titeler, 1989). It is based on the premise that specific receptor sites exist in small finite numbers and, as such, binding of any ligand to such sites is a saturable phenomenon. In contrast, ligand binding to nonspecific sites is nonsaturable since the sites exist in relatively large numbers and often increase linearly as the concentration of the radiolabelled ligand is increased. Based on this definition, the results shown in Figure 3.9 clearly indicate that EMC-D virus binding to BMP cells was saturable. Using these criteria, the data derived from competition binding experiments in a dot blot assay (Fig. 3.17, lane *b*) or in a virus attachment assay (section 3.2.3.) are consistent with specific EMC-D virus binding to BMP cells. However, it should be noted that although the results in Figure 3.17 show that the virus bound to the putative receptor material in a concentration-dependent manner, and that labelled EMC-D virus binding to the putative receptor was competed for by unlabelled virus, these results do not prove that specifically bound radiolabelled EMC-D virus is binding to a virus receptor.

4.1.3. Virus binds to two putative receptors on BMP cells

The virus binding data suggest that the association between EMC-D virus and BMP cells is receptor mediated. The detection of two protein bands

(M.W. 97,000 & 70,000 daltons) following Coomassie blue staining of the affinity purified putative receptor(s) is consistent with the conclusion made from analysis of the saturation binding data that EMC-D virus recognizes more than one binding site on BMP cells. EMC-D virus bound to the affinity purified protein in a dot blot assay (Fig. 3.17) just as the protein bound to the virus affinity column, suggesting that the protein-virus interaction is authentic. In the dot blot assay, the lack of virus binding observed in wells which did not contain the affinity purified proteins but which were blocked prior to addition of the virus (Fig. 3.17, *g* & *h*) indicates specific binding of the virus to these proteins. The fact that supernatants from hybridoma cultures containing antibodies made against these proteins protected BMP cells from EMC-D virus infection (Fig. 3.20) strongly suggests that one or both of these proteins is a true receptor.

Individual protein bands cut from nitrocellulose transfer blots were non-immunogenic for the rat, as determined by gel immunodiffusion tests (not shown). Therefore, it could not be determined via the use of (protein) band-specific polyclonal antibodies whether the functional EMC-D virus receptor on BMP cells consists of the two protein bands, or the 70 kd or the 97 kd protein. The idea of immunizing rats with the affinity purified material comprising both the 70 kd and the 97 kd proteins for the purpose of

producing monoclonal anti-receptor antibodies is reasonable, since antibodies will be made in the host against different epitopes on one protein and/or on different epitopes on the other. Being monoclonals, specific antibodies could be selected. The plan was that the antibodies which protected BMP cells from EMC-D virus infection would be selected and assigned to a particular protein band by an enzyme-linked immunosorbent assay (ELISA).

The possibility that the 70,000 dalton band is a proteolytic product cannot be ruled out, however, this is unlikely because of the manner in which the affinity purified protein was collected and stored. The putative receptor protein was collected at 4°C in 0.1% merthiolate and was immediately frozen or dialysed against ice-cold PBS and lyophilized. Similarly, crude BMP membranes were stored frozen in 0.02 M phosphate buffer, pH 8.0, containing merthiolate and the protease inhibitor PMSF and, were solubilized in sodium deoxycholate prior to application to the column. Besides, data derived from the saturation binding experiments argues against the existence of a single binding site.

EMC-D virus possibly recognizes two receptor molecules on BMP cells, which act in collaboration to initiate infection. One receptor, for example could require only sialic acid and might mediate the primary attachment of

the virus, while a second receptor, likely involving protein-protein interaction, may be required to complete the binding of the virus and to initiate its internalization. This hypothesis might be experimentally tested if it were possible to reassemble the two purified molecules in, for example, artificial lipid membranes or maybe electroporated erythrocytes. The binding of virus to the reassembled protein could then be tested. Although the mechanism by which picornaviruses penetrate the membrane of susceptible cells is not completely understood, I suggest that since EMC virus does not require an acidic pH for entry into cells (Madshus *et al.*, 1984c), perhaps the interaction with a second receptor is required for the exposure of an epitope which participates in virus internalization.

The concept of two receptors for a picornavirus such as EMC-D virus would not be unique. Poliovirus binds to a 43-45 kd protein (Mendelsohn *et al.*, 1989) and to a 100 kd protein (Shepley *et al.*, 1988) on susceptible cells (reviewed in section 1.3.3.2). Likewise, it has been reported that coxsackie B virus can bind to more than one receptor on the cell surface (Lee-Hsu *et al.*, 1990; reviewed in section 1.3.3.3). This does not imply that all members of the picornavirus family have to use two receptors in order to enter cells. Other picornaviruses may employ other means. For instance, increasing the binding affinity of one receptor may render the other receptor non essential. Since we

know very little about receptors for other picornaviruses, perhaps, as reported, a single receptor for FMD virus (Fox *et al.*, 1989) or echovirus type 1 (Bergelson *et al.*, 1992) is sufficient for initiation of an infection.

4.2. Nature of the putative virus receptor on BMP cells

Several studies have shown that proteins (Greve *et al.*, 1989; Staunton *et al.*, 1989), carbohydrates (Fried *et al.*, 1981) and lipids (Schlegel *et al.*, 1983; Mastromarino *et al.*, 1987) can act as virus receptors. A common method for identifying the components of a virus receptor is to determine the effect of pretreating cells with various enzymes on virus binding (Zajac & Crowell, 1965; Clayson & Compans, 1989). EMC-D virus binding studies on BMP cells treated with trypsin, chymotrypsin or papain revealed that the cell surface virus receptor comprises a protein. Pretreatment of BMP cells with endoglycosidase F or with glycopeptidase F, at their appropriate pH optima, did not affect virus binding to BMP cells. These N-glycosidases remove carbohydrates which are attached to proteins through N-linkages to asparagine residues. Endoglycosidase F cleaves the bond between GlcNAc- β (1,4) GlcNAc, leaving only a single N-acetylglucosamine (GlcNAc) residue attached to asparagine whereas the cleavage site for glycopeptidase F is at the bond which links the innermost GlcNAc to the asparagine and, as such, the entire oligosaccharide gets removed from the glycoprotein. The implication of this

result, that N-linked sugars are not required for EMC-D virus binding to BMP cells, was further investigated by growing trypsin treated cells in medium containing increasing amounts of tunicamycin, a drug which inhibits the synthesis of N-linked sugars. Cells grown in medium containing tunicamycin bound a high percentage of virus, as did cells grown in medium which lacked the drug. In contrast, virus binding to trypsin treated cells grown in medium containing cycloheximide, an inhibitor of protein synthesis, was significantly reduced. It was concluded from these results that N-linked sugars are probably not required for EMC-D virus binding to BMP cells, and that receptors destroyed by protease treatment of cells are regenerated by synthesis of new receptors, not by replacement from an intracellular pool. Similar results have been reported for Coxsackie B virus receptors on HeLa cells (Levitt & Crowell, 1967; Krah & Crowell, 1985).

EMC-D virus binding was also studied on BMP cells pretreated with O-glycosidase, which hydrolyses the N-acetylgalactosaminyl-serine/threonine bond and thereby releases the Gal- β (1,3) GalNAc disaccharide linked to serine or threonine residues of glycoproteins. For this enzyme to work, substituents such as sialic acids on either the galactosyl or N-acetylgalactosaminyl residue must be removed prior to addition of O-glycosidase and, since BMP cell desialylation alone significantly reduced the percentage of EMC-D virus

bound to them (see Table 3.2), the involvement of specific O-linked sugars in EMC-D virus binding to BMP cells could not be determined by O-glycosidase treatment of cells. However, it is concluded that the reduction in virus binding observed after treatment with O-glycosidase is most likely due to desialylation rather than O-linked deglycosylation. It is reported in this thesis that treating BMP cells with sodium periodate, which oxidizes vicinal diol sugars or the exocyclic carbon atoms of sialic acids to aldehydes, reduced virus binding by only 7 percent. The inactivity of sodium periodate in this case may be due to the presence of O-acetylated sialic acids which prevents removal of the carbon atoms and their associated hydroxyl groups by periodate oxidation-borohydride reduction (Suttajit & Winzler, 1971; McLean *et al.*, 1971); Herrler *et al.*, 1985).

4.3. Is sialic acid required for EMC-D virus binding to BMP cells?

N-acetyl neuraminic acid (Neu5Ac) is a sialic acid with an acetyl residue attached to its amino group while O-acetylated sialic acids have additional acetyl groups at O-4, O-7, O-8 and O-9. Chromatofocusing, like isoelectric focusing, is a technique used to separate molecules from each other on the basis of differences in their isoelectric points (pI). In chromatofocusing, amphoteric molecules, such as proteins, are adsorbed to an ion-exchange resin which is then washed with a pH gradient. When the pH of the gradient reaches that of the isoelectric point of a particular adsorbed molecule, the

latter becomes electrically neutral and is released. The increased isoelectric point from 4.6 to 6.1, observed when the affinity purified putative receptor was desialylated prior to chromatofocusing, clearly indicates that one (and perhaps both) of the proteins is sialylated. It is not clear why only one protein was detected by this method, especially since both proteins were detectable by Coomassie blue staining. One may speculate that perhaps the undetected band contains far less tyrosine residues than the other and therefore was not as strongly radioiodinated as the band that was detected. The alternative argument that this result indicates the presence of a single protein (hence same pI) that is differentially glycosylated cannot be ruled out.

Mouse cells contain O-acetylated sialic acids which makes them resistant to *V. cholerae* and *A. ureafaciens* neuraminidase, although N-acetyl neuraminic acid is removed by both enzymes (Corfield *et al.*, 1986; Schauer, 1987). Results from EMC-D virus binding to cells treated with lectins combined with a previous observation in this thesis that EMC-D virus binding to cells treated with *V. cholerae* or *A. ureafaciens* neuraminidase is reduced by about 50%, confirmed that Neu5Ac is involved in EMC-D virus binding to BMP cells. Although both horseshoe crab and wheat germ lectins have specificities for Neu5Ac, the results show that one was more effective than the other in reducing virus binding (see Table 3.3). The lower reduction in virus

binding following pretreatment with wheat germ lectin is possibly due to the fact that this lectin has a higher affinity for N-acetyl-D-glucosamine than for sialic acid. The observation that treatment with both *V. cholerae* or *A. ureafaciens* neuraminidases reduced virus binding to about the same level suggests that either there are no O-acetylated sialic acids on BMP cells or that these sialic acids are not required for EMC-D virus binding to BMP cells. O-acetylation of sialic acids can have a dramatic effect on the biological activity of cells. It has been reported that O-acetylation of the hydroxyl groups at different positions alter the size, hydrophobicity, net charge, neuraminidase susceptibility, and thus the biological activity, of sialic acids (Schauer, 1987; Varki *et al.*, 1991). Although the HPLC system used in this study prevents de-O-acetylation by rapidly eluting substituted sialic acids (Manzi *et al.*, 1990), attempts to determine whether BMP cells contain O-acetylated sialic acids were unsuccessful, perhaps due to the close retention times of O-acetylated sialic acids (Manzi *et al.*, 1990). However, it was found that total cellular sialic acid released by acid hydrolysis contained N-glycolyl neuraminic acid (Fig. 3.12 c). It could not be determined if the acid-releasable sialic acid was located intracellularly or cell surface bound. Assuming that the residual N-glycolyl neuraminic acid released after acid hydrolysis of neuraminidase treated cells was present on the cell surface, the result in Fig. 3.12 f suggests that N-glycolyl neuraminic acid is resistant to *V. cholerae* neuraminidase. If

this is correct, the residual virus binding of about 55% obtained after neuraminidase treatment of cells may be due to either the presence of N-glycolyl neuraminic acid or to O-acetylated sialic acids or both. Further, since the cell surface component that the virus binds to is protein in nature and involves sialic acid residues which are linked to proteins via carbohydrate molecules, and since N-linked sugars are not required for virus to bind to BMP cells, any sugars involved in EMC-D virus binding to BMP cells must be O-linked. Other virus receptors have been shown to require O-linked sugars for proper ligand binding. For example, the receptor for SV40 virus requires O-linked sugars in the form Gal- β (1,3)GalNAc (Clayson & Compans, 1989).

The fact that EMC-D virus binds to sialic acid-containing receptors on BMP cells is not unique. Influenza A and B viruses use Neu5Ac for binding to cells. Studies from our laboratory have shown that Neu5Ac plays a direct role in the attachment of EMC virus to human erythrocytes (Tavakkol & Burness, 1990). Sialic acids are said to perform a direct role when they function as an integral part of the virus binding site on the receptor. In the indirect role, the receptor is held in the required configuration for virus recognition by negatively charged sialic acids which interact with positively charged amino acid residues found in the polypeptide chain of receptors. So far, two viruses, bovine coronavirus (BVC) and influenza C virus have been

reported to use 9-O-acetylated sialic acid for the initiation of infection (Schultze & Herrler, 1992). Although these two very different viruses (BVC is a positive-stranded RNA virus and influenza C virus is a negative-stranded RNA virus) recognize similar sialic acids, it is not yet known whether the presence of 9-O-acetylated sialic acid can be a determinant of tissue tropism for BCV, influenza C virus or any other virus.

4.4. What is the relationship between the putative receptor(s) on BMP cells and glycophorin A?

Glycophorin A has a molecular weight of about 36,000 daltons and is expressed only on erythrocytes and on cells of erythroid lineage such as the human erythroleukaemic cell line, K562 (Anstee, 1990; Cartron *et al.*, 1990). EMC virus binds to human erythrocytes via glycophorin A which is the major cell surface sialoglycoprotein on these cells (Allaway & Burness, 1986). The results of figure 3.14 (peak 2) and figure 3.17 (*e* & *f*) provide evidence that glycophorin binds to EMC-D virus. In figure 3.14, peak 1 is eluted in the void column of the EMC virus column and probably contains aggregated glycophorin, since glycophorin is present as monomers in sodium deoxycholate or Triton X-100, but as heterogeneous, large-sized aggregates in the absence of detergents (Allaway & Burness, 1986).

The possibility that EMC-D virus binds to glyophorin A or a glyophorin A-like molecule on pancreatic β cells is discounted by the huge difference in the molecular weights of glyophorin A and the putative receptor proteins on BMP cells, and by the fact that glyophorin A is normally expressed only on cells of erythroid origin. However, I cannot rule out the possibility that both EMC virus and its diabetogenic variant, EMC-D, may bind to a common structural component present on both glyophorin A and the putative receptor.

4.5. Production of anti-virus anti-idiotypic antibodies

According to the canyon hypothesis (reviewed in section 1.3.5), the receptor binding sites on picornaviruses are in canyons or pits in the capsid wall (Rossmann *et al.*, 1985). Since antibodies are supposedly too large to fit into these deep, narrow depressions, it is postulated that antibodies are not formed against the receptor binding sites. If this is so, it follows that anti-idiotypic antibodies against the receptor cannot be obtained. These predictions were supported by reports that anti-idiotypic antibodies against anti-virus monoclonal antibodies for Cocksackie virus (McClintock *et al.*, 1986) and foot-and-mouth disease virus (Baxt *et al.*, 1986) apparently failed to recognise virus receptors, although more recent reports have shown that the attachment site on foot-and-mouth disease virus is exposed, not located in a

pit (Acharya *et al.*, 1989; Fox *et al.*, 1989).

The anti-idiotypic antibodies used in this report were raised by immunizing New Zealand white rabbits with affinity purified antibodies against EMC virus, using siblings of the rabbits which were used to produce the anti-EMC antibodies. These antibodies are polyclonal in nature, which means that they were made against a group of idiotypes rather than against a single idioype. Idiotypes are serologically defined determinants located within the combining region of immunoglobulins. The determinants may be amino acid sequences on either the light or heavy chain of the immunoglobulin molecule or may involve both chains. Antibodies against idiotypes may be restricted, when they react only with the immunizing immunoglobulin molecule, or cross-reactive, in which case they react with the same or different immunoglobulins. Cross-reactivity among anti-idiotypes may be due to common amino acid sequences or to the presence of an "internal image" of the antigen which occurs when the tertiary structure of the anti-idiotpe resembles that of the antigen.

The anti-EMC virus anti-idiotypic antibodies were characterized in a previous study by their ability to behave like EMC virus (Baldeh & Burness 1987). The evidence that these antibodies behave like EMC virus was that the

antibodies were capable of binding to purified glycoporphin preparations immobilised on nitrocellulose and they immunoprecipitated glycoporphin A from solubilized radiolabelled red cell membranes. Also, like EMC virus, the antibodies agglutinated both human and sheep erythrocytes but again like the virus, they did not agglutinate bovine or rabbit red blood cells (Baldeh & Burness 1987). In support of my results, other investigations have since produced anti-EMC anti-idiotypic antibodies (Vlasploder *et al.*, 1990).

Our success in producing anti-EMC virus anti-idiotypic antibodies, compared to earlier investigators (Rossmann *et al.*, 1985; Baxt *et al.*, 1986; McClintock *et al.*, 1986), may be due to the following reasons. The binding site on EMC virus for human erythrocyte glycoporphin probably differs from that on nucleated cells and is located in a site accessible to antibodies. Alternatively, perhaps the canyon hypothesis needs modification and the reason for the apparent failure to generate anti-idiotypic antibodies against Cocksackie and foot-and-mouth disease receptors lies elsewhere. For instance, I used polyclonal antibodies throughout, thus covering the widest range of epitopes possible. In addition, it is easier to detect antibodies binding to red cell receptors, which occur in such abundance, than to the low numbers of receptors on nucleated cells. Also, it is possible that the canyon hypothesis does not apply to EMC virus; after all, foot-and-mouth disease virus does not

contain a cleft (Acharya *et al.*, 1989, Fox *et al.*, 1989).

4.6. EMC-D virus receptors and virus-induced IDDM in mice

Type 1 diabetes (IDDM) results from destruction of the insulin-producing pancreatic β -cells. A number of reports have shown that the diabetogenic strain of EMC virus (EMC-D) induces a type 1 diabetes-like syndrome in SJL/J mice but not in C57BL/6J mice (Yoon *et al.*, 1980; Yoon & Notkins, 1983). The histopathological observations reported in this thesis are consistent with such reports that EMC-D virus destroys pancreatic β -cells in susceptible strains of mice.

It is reported in this thesis that both EMC-B and EMC-D viruses are able to replicate in BMP cells although the peak titre for EMC-B virus was lower than that for EMC-D virus. Similar results have been observed in tissues of SJL/J mice infected with these two viruses (Yoon & Notkins, 1983; Jordan *et al.*, 1987). In spite of previous observations that EMC-B virus is a better inducer of interferon than EMC-D virus (Yoon *et al.*, 1980; Cohen *et al.*, 1983), my results show that both viruses productively infect BMP cells. Apart from indicating that BMP cells possess surface receptors for both virus strains, this finding also shows that both viruses have the ability to enter, uncoat, and replicate in mouse pancreatic β -cells. According to Cohen *et al.*,

1983, the greater interferon sensitivity of EMC-B virus is not observed in all cells *in vitro* and perhaps a similar situation exists with BMP cells. Whatever the case may be, Bae and associates (1990) have reported that a single base insertion at the 5' noncoding region of the EMC-B virus genome is probably responsible for this virus' notable interferon-producing ability and that its ability to induce interferon is distinct from its non-diabetogenic activity.

In contrast to EMC-B virus, reports indicate that the diabetogenic activity of EMC-D virus may be due to point mutations within the polyprotein coding region of the virus genome, resulting in changes in the amino acid residues of the leader peptide and in VP1 (Bae *et al.*, 1990). It is suggested that modification of amino acid residues within VP1 of EMC-D virus probably increases the efficiency with which the virus binds to β -cells (Bae *et al.*, 1990). A report by Kaptur and associates (1989) that up to six times more EMC-D than EMC-B virus attached to primary pancreatic β -cells seems to support this suggestion. However, because Kaptur and coworkers also reported that they could not achieve saturation with either virus, one finds it difficult to accept that their results reflect the true situation. While all of these reports demonstrate attempts to understand why one variant of EMC-virus is diabetogenic and another is not, they do not explain why EMC-D virus destroys β -cells from SJL/J mice but not β -cells from C57BL/6J mice.

Although previous reports claim that β -cells from SJL/J mice bound more EMC-D virus than β -cells from C57BL/6J mice (Chairez *et al.*, 1978), in my hands, EMC-D virus bound equally well to β -cells from both mouse strains. A possible explanation for the different results may be contamination with other cell types. The β -cells used by Chairez and associates were isolated by a commonly used procedure which is nonetheless subjective. With the procedure used by Chairez *et al.*, islets which contain β -cells and other cell types are selected using a microscope, disrupted and then allowed to settle so that the β -cells can be isolated. It is possible that the cells used by Chairez and coworkers comprised β -cells, and other contaminating cell types, which may express increased number of receptors for EMC-D virus. In contrast, the β -cells used in my study were stained for insulin using anti-insulin antibodies and then isolated on a fluorescent activated cell sorter (FACS). As such, the possibility that I too used contaminated cells is practically nil. First, pancreatic cells from SJL/J and C57BL/6J mice were stained with FITC-conjugated anti-insulin antibodies and sorted on the FACS. Then the insulin-containing β -cells were selected and used to study EMC-D virus attachment, utilizing rabbit anti-EMC virus antibodies and anti-rabbit phycoerythrin-conjugated antibodies. Alternatively, the increased number of receptors for EMC-D virus observed on β -cells from SJL/J (Chairez *et al.*, 1978) may be due to the effect of cytokines, particularly TNF- α , which is produced by macrophages following

exposure to infectious agents. Receptors for TNF- α have been found on virtually all cells examined and TNF- α is inducible in a wide variety of cell types including pancreatic β -cells (Vilcek & Lee, 1991). Induction of TNF- α induces expression of many proteins, including the cell adhesion molecule ICAM-1, which is also the cell surface receptor for most rhinovirus serotypes. According to a recent report by Topham *et al.* (1992) treatment of pancreatic β -cells with TNF- α increased the number of cell surface receptors for EMC-D virus on these cells. It is therefore conceivable that β -cells obtained from inadvertently infected (bacterially) SJL/J mice would produce TNF- α and bind more EMC-D virus than β -cells from non-infected C57BL/6J mice. Also, the fact that EMC-D virus binds equally well to β -cells from both strains of mice suggests that the cause(s) for susceptibility or resistance to EMC-D virus-induced IDDM in SJL/J and C57BL/6J mice lies elsewhere, not in the presence/absence, or reduction in the number of β -cell receptors for EMC-D virus. However, it is arguable that differences exist between receptors for EMC-D virus on β -cells from SJL and C57BL mice but are not detectable by the methods used.

4.7. Possible mechanism for β -cell destruction

Various studies on whether or not immune mechanisms are involved in the destruction of β -cells following EMC-D viral infection arrived at different

conclusions. Yoon *et al.* (1985) could not find any evidence that immune mechanisms account for the destruction of β -cells in EMC-D virus-infected SJL mice although a previous study had suggested that T lymphocytes were involved (Buschard *et al.*, 1976; Buschard *et al.*, 1983). According to the study by Yoon and associates (1985), EMC-D virus caused diabetes in athymic nude CD-1 mice, in DBA/2J mice immunosuppressed by thymectomy, and in SJL mice immunosuppressed with antilymphocyte serum, suggesting that depletion of lymphocytes does not alter the incidence of the disease. This finding also suggested that IDDM in virus-infected SJL mice results from a direct cytolytic effect of EMC-D virus on pancreatic β -cells. More recent studies showed that Mac-2⁺ macrophages in EMC-D virus-infected SJL/J mice participate in the early phase of β -cell destruction in IDDM whereas other macrophages, CD4⁺ and CD8⁺ T lymphocytes appear in pancreatic islets at the intermediate and late stages of the disease. The study revealed that mice treated with silica, which activates macrophages, showed enhanced destruction of β -cells while removal of macrophages by treating with monoclonal antibody against macrophages resulted in reduced β -cell destruction (Baek & Yoon, 1990). Baek and Yoon (1990) have suggested that β -cells are destroyed via the synergistic actions of activated macrophages and virus infection in which case the β -cells may actually be destroyed by direct cytolytic effect of the virus while the macrophages are present only as scavengers. Another possibility is

that the macrophages actually contribute to β -cell destruction by releasing cytokines such as TNF, interleukin-1 and γ -interferon. Tumour necrosis factor induces cell surface expression of MHC molecules (Vilcek & Lee, 1991). The possibility that TNF or γ -interferon produced by virus-specific response in EMC-D virus-infected SJL/J mice causes aberrant expression of class II MHC molecules on pancreatic β -cells, thus facilitating presentation of normal tissue antigens to the immune system, does not occur (Sarvetnick *et al.*, 1988; Lo *et al.*, 1988; Bohme *et al.*, 1989). Simple expression of class II MHC molecules on the surface of β -cells is not sufficient to initiate an autoimmune response although it may result in IDDM (Sarvetnick *et al.*, 1988; Lo *et al.*, 1988; Bohme *et al.*, 1989). These reports show that the immune processes by which EMC-D virus causes IDDM in SJL/J mice are far from clear.

This thesis reports that the anti-virus anti-idiotypic antibodies, like EMC-D virus, bound to β -cells from SJL/J mice. This finding may indicate a possible role for anti-idiotypic antibodies in the initiation of pancreatic β -cell destruction in EMC-D virus-infected SJL/J mice. Autoimmunity may be initiated via cross-reaction between determinants on β -cells and antibodies produced against idiotypes on the antiviral antibody or for that matter between β -cell determinants and the antiviral antibodies. Similar cases of molecular mimicry have been reported in demyelinating autoimmune disease

in mice where antibodies against Theiler's virus cross-react with host mouse tissues (Oldstone and Notkins, 1986). Further, antiviral antibodies produced by cytomegalovirus-infected IDDM patients have been shown to recognize a 36 kd protein on human pancreatic islet cells (Baekkeskov *et al.*, 1982; Pak *et al.*, 1988), and anti-reovirus anti-idiotypic antibodies bind to reovirus receptors on the surfaces of lymphocytes and nerve cells (Co *et al.*, 1985). Current opinion on the association of idiotypes with autoimmune disease pathogenesis suggests a link between the type of idiootype present (restricted or cross-reactive) and severity of the disease (Mendlovic *et al.*, 1989). The anti-idiotypic antibodies used in this study were made against neutralizing anti-EMC virus antibodies. Since neutralizing antibodies often bind to virus epitopes that are either directly or closely associated with the virus receptor attachment site, the 3-dimensional antigen-binding domain of the antibody might resemble the receptor. As such, the anti-idiotypic antibody's antigen binding domain might resemble the virus attachment site and therefore be capable of interacting with the receptor and thus initiate tissue damage. There is no evidence to suggest that a similar situation does not exist in C57BL/6J mice especially since the anti-idiotypic antibodies also bound to β -cells from C57BL/6J mice. However, since C57BL/6J mice infected with EMC-D virus failed to develop IDDM, perhaps idiotypes produced in virus-infected SJL/J mice are more pathogenic than those in C57BL/6J mice. Pathogenic and non-

pathogenic idiotypes have been observed in patients with systemic lupus erythematosus, in a phenomenon referred to as idiotypic shift (Suenaga *et al.*, 1989). A particular idiotypic was detected when nephritis occurred but when the clinical manifestation changed, a different idiotypic emerged. Similarly, pathogenic idiotypes have been detected in mice (Hahn *et al.*, 1988) and perhaps a similar phenomenon exists between SJL/J and C57BL/6J mice infected with EMC-D virus.

4.8. Summary

In summary, the results described in this thesis are consistent with the receptor for EMC-D virus on BMP cells being a sialylated glycoprotein, possibly involving both Neu5Ac and Neu5Gc but not N-linked sugars. EMC-D virus attachment to BMP cells was saturable and specific and virus binding to these cells was identified as receptor mediated. While Scatchard analysis of the saturation binding data suggests the presence of a single binding site, further analysis of the same data by the Hill method indicates that EMC-D virus has more than one binding site on BMP cells. The binding affinity between EMC-D virus and a homogenous group of binding sites was estimated to be high ($K_d = 1.2 \text{ nM}$) and the number of binding sites for this homogenous group was estimated at 4×10^5 per cell.

Two putative receptor proteins (M.W. 97,000 & 70,000 daltons) were isolated from BMP cells by affinity chromatography. This finding supports the previous conclusion that EMC-D virus has more than one binding site on BMP cells. These proteins bound to EMC-D virus attached to Sepharose. Likewise, EMC-D virus bound to the isolated putative receptor on nitrocellulose in a concentration-dependent manner, and labelled EMC-D virus binding to these proteins was competed for by unlabelled virus. Antibodies raised against the putative EMC-D virus receptor blocked EMC-D virus infection of BMP cells as well as virus binding to β -cells from SJL/J mice. This may suggest that (i) the EMC-D virus receptors on BMP cells are authentic and (ii) that the virus possibly binds to the same receptor on various β -cells. Alternatively, this result may suggest that EMC-D virus recognizes a common molecular structure present within different receptors, on different cells. Data from our laboratory (Ingrid Pardoe, personal communication) indicate that EMC-D virus binds to a similar receptor on a different mouse cell.

It is also concluded that the inability of EMC-D virus to cause IDDM in C57BL/6J may not be due to lack of, or a reduction, in the number of cell surface receptors for EMC-D virus on β -cells from C57BL/6J mice. Pancreatic β -cells from SJL/J and C57BL/6J mice contain about the same number of

receptors for EMC-D virus, so that EMC-D virus binds equally well to β -cells from both mouse strains. Anti-idiotypic antibodies produced against idiotypes on anti-EMC virus antibodies behaved like the virus and bound BMP cells, and β -cells from SJL/J and C57BL/6J mice. It is suggested that anti-EMC virus anti-idiotypic antibodies may play a role in the initiation of β -cell destruction in virus-infected SJL/J mice, possibly via a molecular mimicry mechanism. Although there is no evidence to show that a similar situation does not exist in C57BL/6J mice, it is postulated that anti-EMC virus anti-idiotypic antibodies produced in SJL/J mice may be pathogenic while those in C57BL/6J mice may be non-pathogenic or less pathogenic; such an argument could account for the non diabetogenicity of EMC-D virus in C57BL/6J mice.

4.9. Future studies

Future studies on this project should be aimed at:

i. Further characterization of the putative receptor proteins by mass spectrometry to determine more precisely the molecular weights of the proteins, and amino acid sequencing of the receptor protein. Based on my experience, during failed attempts to sequence the putative receptor protein, I suspect that the N-terminus of the receptor is blocked. If this is the case, it will be necessary to produce peptides, using cyanogen bromide or tryptic cleavage, for sequencing, after separating the peptides by high performance

liquid chromatography. With the amino acid sequence available, a search through data bases will determine if the receptor has been identified as was found for the rhinovirus receptor. If the protein has not been identified, it will be necessary to synthesize oligonucleotide mixtures complementary to the receptor mRNA sequence as deduced from the amino acid sequence, taking into account the degeneracy of the genetic code. These oligonucleotide mixtures can be used for cloning of the EMC-D virus receptor gene. Ultimately, it should be shown that following transfection with cDNA encoding the virus receptor gene cells which lacked the virus receptor and were not infectable by EMC-D virus, acquire the ability to bind the virus and become infectable.

ii. A study to determine whether anti-virus anti-idiotypic antibodies are present in the sera of virus-induced diabetic mice is worthwhile. Such a study could shed some light on my speculation that anti-virus anti-idiotypic antibodies are possibly involved in the initiation of β -cell destruction in virus-infected SJL/J, possibly via a molecular mimicry mechanism.

APPENDIX

1. Formula used to calculate number of binding sites

Binding sites per cell = B_{max} {expressed in moles per litre} X Avogadro's number divided by cell concentration {expressed per litre} (Kappy *et al.*, 1979).

2. Relationship between number of virus particles and moles

Avogadro's Number = 6.023×10^{23}

M.W. of EMC virus = 8.5×10^6 daltons

Specific activity of the radiolabelled virus = 5976 dpm/picomole

Therefore $1 \text{ pM} : 10^{-12} \times 6.023 \times 10^{23} = 6.023 \times 10^{11}$ virus particles

Note: (a). 1 M contains 6.023×10^{23} particles

(b). 1 dalton = 1.64×10^{-24} g

(c). cpm = counting efficiency X dpm (the efficiency, determined from a quench curve, was 50.75%).

3. Strain origin of NOD mice

The non-obese diabetic (NOD) strain was established during the course of an inbreeding programme aimed at developing a cataract-prone mouse

strain, later called CTS mouse (Wicker *et al.*, 1987; Slattery *et al.*, 1990; Miyazaki *et al.*, 1990). CTS mice were developed from an original pair of non-inbred ICR and Clea (sometimes called JCL) mice. At the sixth generation of sib matings of ICR and Clea mice, two strains could be distinguished from each other. One exhibited fasting hyperglycaemia and the other did not. By the 20th generation of sib matings, it was observed that the strain which did not exhibit fasting glycaemia was predisposed to IDDM. This line of mice, which exhibits normal fasting blood glucose levels and is predisposed to IDDM, is called the NOD strain. The other line, which exhibits fasting hyperglycaemia but do not develop IDDM, are called non-obese normal (NON) mice (Wicker *et al.*, 1987; Slattery *et al.*, 1990; Miyazaki *et al.*, 1990).

NOD mice develop diabetes abruptly between 100 and 200 days of age, as well as rapid weight loss and severe glucosuria. Without insulin treatment, they do not survive for more than a month after developing IDDM (Wicker *et al.*, 1987; Slattery *et al.*, 1990).

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