

CHARACTERIZATION OF RECEPTORS FOR  
ENCEPHALOMYOCARDITIS VIRUS ON CELLS

CENTRE FOR NEWFOUNDLAND STUDIES

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CHARACTERIZATION OF RECEPTORS FOR  
ENCEPHALOMYOCARDITIS VIRUS ON CELLS

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## ABSTRACT

A great deal more is known about viruses than about the receptor structures that allow entry of viruses into cells. The work reported in this thesis was aimed at finding out more about the structure of a receptor for the mouse encephalomyocarditis (EMC) virus that is found on some mouse cells and on human erythroid cells. Since previous reports claimed that the receptor for EMC virus on the human erythrocyte is glycoprotein A (Allaway et al., 1986), a virus-Sepharose column was used to study the binding of glycoprotein A to the column as well as to study binding between the virus and its surface receptors on human erythrocytes and Krebs II ascites tumour cells. Glycoprotein A was retained on the virus-Sepharose column and was eluted with 0.02 M sodium phosphate buffer pH 8.0 containing 0.02 to 0.05 M NaCl. This result indicates that weak ionic interactions are involved between the virus and glycoprotein A.

Radio-labelled molecules from red cell membranes solubilized in detergent were found to bind to EMC virus-Sepharose and analysis of eluates by electrophoresis showed that the bound material contained both glycoproteins A and B. When glycoproteins A and B isolated by gel filtration on Biogel columns were separately applied to the EMC virus-Sepharose column, both sial-glycoproteins (glycoproteins A and B) were retained on the virus column. Contrary to previous reports this result suggests that both glycoproteins A and B may serve as receptors for EMC virus.

Radio-labelled Krebs cell membranes were solubilized in detergent and applied to the virus-Sepharose column. The column eluate, analysed by SDS-PAGE showed a peak at the origin of the gel and this material was sensitive to trypsin treatment but not treatment with neuraminidase which releases sialic acids. This result suggests that the receptor for EMC virus on the Krebs cell may be a non sialylated glycoprotein.

In a second approach, to characterize the receptor for EMC virus on cells, anti-idiotypic antibodies were used. Antibodies to the EMC virus were raised in New

Zealand White rabbits; these antibodies were purified by affinity chromatography and then injected into the same breed of rabbits to produce anti-idiotypic antibodies. Such antibodies should mimic the binding properties of the virus itself. The anti-idiotypic antibodies were found to:

1. bind to glycoprotein immobilised on nitrocellulose.
2. immunoprecipitate glycoprotein from solubilized radiolabelled red cell membranes.
3. agglutinate both human and sheep erythrocytes but not bovine or rabbit red blood cells.

These results suggest that anti-idiotypic antibodies against EMC virus have been successfully made and these antibodies bind to the receptor for EMC virus on human erythrocytes.



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## LIST OF ABBREVIATIONS

AEMC-----anti-EMC virus antibodies

DOC-----sodium deoxycholate

EDTA-----ethylenediaminetetraacetic acid, (Tetrasodium salt)

ELISA-----enzyme-linked immunosorbent assay

EMC virus-----encephalomyocarditis virus

FMDV-----foot-and-mouth disease virus

HA-----haemagglutination (test)

HA1-----haemagglutination inhibition (test)

HEL cells-----human erythroleukaemic cells

HRV-----human rhinovirus

HTLV-III-----human T cell-lymphotropic virus type III

IgG-----immunoglobulin G

LIS-----lithium diiodosalicylate

NEN-----New England Nuclear (supplier)

PAS-----periodic-acid Schiff's reagent (stain)

PBS-----phosphate buffered saline

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

RPMI-----Roswell Park Memorial Institute (medium)

SDS-PAGE-----sodium dodecyl sulphate-polyacrylamide gel electrophoresis

VAP-----virus attachment protein

V-g-g-----veronal glucose gelatin (diluent for HA test)

VP-----virus protein

WGA-----Wheat germ agglutinin



# Chapter 1

## INTRODUCTION

### 1.1. Virus-Cell Interaction

#### 1.1.1. Historical Background

The study of viruses began with the concept of viruses as agents of disease. In 1892, the Russian scientist Dmitrii Ivanovsky while investigating tobacco diseases, reported that the disease agent which attacked tobacco leaves retained its infectious qualities even after filtration through Chamberland filter-candles which were designed to trap bacteria (Knight, 1974). He assumed that the disease causing agent was a toxin secreted by the bacteria present in the sap of leaves. Beijerinck, working in The Netherlands, repeated Ivanovsky's experiments, in 1898 and demonstrated transmission of mosaic disease of tobacco in bacteria-free filtrates (Knight, 1974). Beijerinck called this pathogen that is smaller than common bacteria, "virus" or "contagious living fluid" (Knight, 1974).

A milestone in studies of the encounter between viruses and cells occurred in 1941 when Hirst discovered that influenza virus agglutinated chick red blood cells. He suggested that influenza virus contained an enzyme since chicken erythrocytes released bound virions when incubated at 37°C and then were unable to adsorb fresh virus. Gottschalk and Lind (1949) and Klenk and his associates (1955) demonstrated that the influenza virus enzyme, since called neuraminidase, released sialic acid which was present on the cell surface and was required for attachment (see Burness, 1980).

The discovery by Kathan, Winzler and Johnson (1961), that this sialoprotein

erythrocyte receptor for myxoviruses also carried MN blood group determinants, stimulated research on receptors. Holland (1961) investigated species and organ specificity of poliovirus and he demonstrated that infectious viral RNA obviated the need for receptors, while initiation of infection with intact poliovirus was receptor mediated. The results of many years of study subsequently revealed the properties and distribution of receptors for a number of viruses. Lonberg-Holm and Whitely (1976) and McClintock and coworkers (1980) estimated the number of receptors per cell for several different virus-cell interactions to be between  $10^4$  and  $5 \times 10^6$ . Co and his collaborators (1985) isolated and characterized the mammalian reovirus receptor as a protein with a molecular mass of 67 kilodaltons. Allaway and associates (1986) reported that the erythrocyte receptor for encephalomyocarditis (EMC) virus is located on a specific site on the sialoglycoprotein, glycophorin A.

Today the concept is generally accepted that receptor specificity is a major determinant of cellular tropism, influencing the course of events leading to disease.

### 1.1.2. General Properties of Viruses

A virus is an infectious entity whose genome is either RNA or DNA, which reproduces inside a living cell using the cellular synthetic machinery to direct synthesis of specialized components that have the ability to transfer the viral genome to other cells (Taylor and Hershey, 1986). Viruses, particularly those with spherical morphology, vary in size from about 22 nm to 300 nm in diameter. In contrast, bacteria (cocci) are approximately 1000 nm and erythrocytes are 7500 nm in diameter. Viruses consist basically of a core of nucleic acid surrounded by a protein coat and because of this simplicity viruses have an obligate requirement for intracellular growth, depending heavily upon host cell metabolic components.

Picornaviruses are small nonenveloped viruses which exhibit icosahedral symmetry. The viral capsid is 22 - 30 nm in diameter (Rueckert, 1985; Mathews, 1979) and is made of sixty copies of each of four polypeptides named VP1, VP2,

VP3 and VP4, in order of decreasing molecular weight (Rueckert, 1976). The genome consists of one molecule of positive sense, single stranded RNA.

The Picornavirus family is divided into four genera: Cardioviruses, Enteroviruses, Rhinoviruses and Aphoviruses. The virus used in this study, encephalomyocarditis (EMC) virus, belongs to the Cardiovirus group of viruses. Cardioviruses can infect man, pigs, elephants, and squirrels, even though they are generally regarded as murine viruses (Rueckert, 1985).

The Enteroviruses inhabit mostly the human alimentary tract and its members include polio and coxsackie viruses. The human rhinoviruses have a special adaptation to the nasopharyngeal region and in this group are the major etiologic agents of the common cold in adults and children. Aphoviruses previously known as Foot-and-mouth disease viruses (FMDV) infect cloven-footed animals, especially cattle, goats, pigs and sheep; rarely, humans can be infected.

### 1.1.3. Virus Attachment To Cells

Virus multiplication in an infected cell can be divided into eight operational stages. Attachment of viruses to specific receptors on the surface of susceptible cells is the first step in viral infection after which penetration and uncoating take place. Other stages, (sometimes regarded as one stage: biosynthesis) include transcription, translation and replication which occur repeatedly as new virus-specific proteins, such as RNA and/or DNA, molecules are synthesized in an infected cell. The last stages involve assembly and release of new virus particles. Viral replication in a susceptible host may be cytocidal resulting in host cell's death or non cytocidal in which case the host cell survives despite infection by virus.

Two elements are involved in the attachment process. The virion component which recognizes a cellular receptor, termed the virus attachment protein (VAP), and the cellular molecule recognized by the VAP, called the cellular receptor. The structures or loci on the surface of the cell which can actually be occupied by

virions are called receptor sites. There may be fewer receptor sites than receptors (Lonberg-Holm and Philipson, 1974).

Different viruses attach to different cell types and sometimes even the same virus may attach to different cells. For instance, reovirus type 3 attaches both to freshly isolated human lymphocytes and to a murine fibroblast cell line called L-cells (Weiner et al., 1980) and coxsackie B viruses attach to mouse and primate cells (Kunin and Halmagyi, 1961). Structures on the plasma membrane which serve as viral receptors may have specificity for a single virus or different viruses may share the same receptor (Tardieu et al., 1982). For example, the various subtypes of coxsackie B viruses may share a receptor on HeLa cells which is distinct from poliovirus, coxsackie A virus and echovirus receptors, while coxsackie B3 and adenovirus type 2 may compete for a common receptor (Lonberg-Holm, Crowell and Philipson, 1976).

#### 1.1.4. Factors Which Influence Attachment

Attachment is influenced by physical conditions such as temperature, pH, ionic strength and cell concentration (Lonberg-Holm and Philipson; 1974). There is a reduction in the rate of attachment by rhinoviruses at temperatures below 37°C probably due to the need for receptors to diffuse laterally through the membrane (Dimmock, 1982). The binding of EMC virus to L929 and Friend cells was reduced as the temperature was raised possibly due to an increase in the rate of dissociation but binding to HeLa cells was unaffected by temperature. Attachment of Mengo virus, another Cardiovirus, to L929 cells was independent of temperature (McClintock, Billups and Notkins, 1980).

Attachment of virions to their receptor is mainly an electrostatic interaction that is strongly influenced by the charge distribution on both the ligand and the receptor (Perutz, 1978; Bailey, Miller and Lenard, 1984). Binding of virus does not occur in the absence of ions (Holland and McLaren, 1959). Some rhinoviruses, coxsackie A9 and FMDV 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 this virus is covered with tightly packed haemagglutinin and neuraminidase glycoproteins. 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. Even though the carbohydrate on the virus is not required for infectivity (Collins and Knight, 1978), the neuraminidase activity destroys sialic acid containing receptors. 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 they inhibit neuraminidase activity (Merz et al., 1981; Huang, 1974).

Changes in cell concentration which affect the number of receptors available also affect attachment in a directly proportional manner. Virus concentration can have an effect upon attachment because saturation occurs as the upper limit is approached and any reduction in the rate of attachment would be as a result of competition for receptor sites. Other factors which influence the rate of attachment include reagents such as sucrose that increase viscosity, decreasing 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 Chopin, 1962).

### 1.1.5. Inhibition Of Attachment

Attachment of viruses to cells can be inhibited by specific antiviral or anticeellular antibodies, or monosaccharides or by dextran sulphate which is a negatively charged polymer (Ozaki and Kumagai, 1972). For instance, N-acetyl glucosamine reduces binding of Echoviruses to cells (Holland and McLaren, 1959; Schmidt et al., 1964).

## 1.2. Virus Attachment Proteins

### 1.2.1. Viral Components Which Recognize Cellular Receptors

While attempting to identify virus attachment proteins (VAPs) it was shown that attachment of virions to cellular receptors did not occur when a specific protein at the surface of the virions was altered by digesting with enzymes (Cavanagh et al., 1977; Moore and Cowan, 1978). However, most of the evidence bearing on the identification of VAPs as that part of a virus particle which recognizes a cellular receptor has been obtained by studies of virus-antibody interactions (Dimmock, 1982). The assumption has been that specific neutralizing antibodies complexed directly with the virus binding component thereby preventing virus attachment to a cell. Even though some investigators (Dimmock, 1982) argue that this reasoning may be fallacious because neutralization may in fact be the result of conformational rearrangements of the virus proteins other than those which directly bind antibody, specific virion structures which may be involved in attachment have been reported (see section 1.2.2).

### 1.2.2. Attachment Protein For Picornaviruses

In the Picornavirus family the viral capsid consists of four polypeptides. VP4 seems to be located on the inside of the capsid near the RNA while the three larger proteins are exposed, with VP1 being the most abundant surface protein (Tardieu et al., 1982; Lonberg-Holm and Crowell, 1986).

Several reports have suggested different functions for picornavirus attachment proteins. Rössmann and associates (1985) have shown by X-ray crystallographic analysis of human rhinovirus type 14, that the virus surface contains a cleft (2.5 nm deep and 1.2 to 3.0 nm wide) which is probably the binding site for host cell receptors. One side of this cleft is formed by VP1; the other side, by VP2 and VP3.

Halperin and coworkers (1964) found that shortly after poliovirus binds to cells,

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a fraction of the attached virus elutes. This eluted virus has lost the polypeptide VP4 and is no longer infectious (Lonberg-Holm and Philipson, 1974). Also antibody to poliovirus VP4 neutralized infectivity (Breindl, 1971). These experiments suggested that VP4 is the virus attachment component (Talbot et al., 1973).

VP1 is the most exposed protein on the surface of the capsid and other reports claim that FMDV infectivity was neutralized by antibody to VP1 (Meloan et al., 1970). When VP1 is cleaved, by trypsin treatment of virions, the virus is incapable of binding to cells (Meloan et al., 1970). Assuming there was no change in the conformation of the virus attachment proteins these results suggest that VP1 alone is the attachment protein.

Like VP1, VP2 on coxsackie virus B3 is present at the capsid surface and Beatrice and coworkers (1980) have suggested that it is the virus attachment protein because Coxsackie virus B3 is neutralized by antibodies to VP2.

Such conflicting results have prompted investigators (Lonberg-Holm, 1974) to suggest that no single protein on picornaviruses functions as the viral attachment protein but that cooperative interactions among the viral proteins result in a unique conformational state which allows binding. This idea would agree with data obtained from recent X-ray crystallographic studies described above (Rossmann et al., 1985).

### 1.2.3. Virus Attachment Proteins Of Other Viruses

Adenoviruses are larger than Picornaviruses and like Picornaviruses are nonenveloped viruses with icosahedral symmetry. Adenoviruses possess apical projections called pentons which have been identified as the site of attachment to cells (Boulanger and Lonberg-Holm, 1981; Morgan et al., 1969). Penton antibody inhibited attachment and binding was competitively blocked by excess of purified penton fibre protein.

Reoviruses have an outer capsid which is composed of three polypeptides:  $\sigma 1$ ,  $\sigma 3$ , and  $\sigma 1$  (Smith et al., 1969; Weiner et al., 1981). The  $\sigma 1$  polypeptide which makes up 1 - 2% of the outer capsid and is located at the vertices of the icosahedral structure (Lee et al., 1981), is the major determinant of reovirus interactions with cells (Weiner et al., 1980).

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 (Kohn, 1985). In herpes simplex virus the gB glycoprotein appears in a dimeric form in the viral envelope and is essential for the penetration and for infectivity of the virus (Person et al., 1982). For instance 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 which belongs to the family of Paramyxoviruses, has two surface glycoproteins, GP90 and GP70. GP90 is reported to serve as the attachment protein (Fennie and Gurin, 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 (Kohn, 1975) but it is not known which glycoprotein is involved in virus attachment to cells.



### 1.3. Virus Receptors

#### 1.3.1. Cellular Receptors

The cell surface membrane consists of a phospholipid bilayer in which cholesterol and various kinds of protein molecules are embedded. Sugars form an integral part of glycolipids and glycoproteins which are confined to the outer monolayer of the cell membrane. Cholesterol and phospholipid are the major membrane lipids and together they make the membrane less permeable to hydrophilic molecules (Breischer, 1965). A significant amount of the glycolipid on the cell membrane is present as cerebroside, a group of glycolipids which contain neutral sugars like glucose and galactose, or as gangliosides which contain sialic acid in addition to sugars (Tooze, 1973). The biological role of these glycolipids is not known even though gangliosides have been demonstrated to serve as receptors for cholera toxins (Cuatrecasas, 1973), interferon (Bessancon and Ankel, 1974), tetanus toxin (Van Heyningen and Allan-Miller, 1961), and Sendai virus (Markwell et al., 1984). However, Allaway and associates (1986) claim that gangliosides found in human erythrocyte surface membranes do not serve as receptors for EMC virus.

Cell surface receptors function as binding structures for a wide variety of ligands including antigens, hormones, toxins and viruses. A virus receptor is the structure on the membrane surface of a cell to which virus binds prior to entering the cell. Such virus-cell interactions may have other important biologic consequences apart from infection, such as affecting cell function by triggering surface structures which affect cellular metabolism or being important for the generation of an immune response against the virus. It is unlikely that the structures on the cell surface which serve as receptors for viruses evolved merely for the purpose of virus binding. For instance, bacteriophage receptors are components of transport systems for low molecular weight sugars (Kohn, 1985). But, the presence or absence of a specific receptor often determines whether or not the cell can be infected by a virus.

### 1.3.2. Virus Receptors On Erythrocytes

Some viruses can attach to receptors located on the surface of certain kinds of erythrocytes. When certain viruses and cells react in this manner, adjacent cells become bridged resulting in the formation of an organized lattice structure in a process termed haemagglutination. The species of animals whose red blood cells agglutinate in this way vary depending on the virus. The biological significance of this interaction between viruses and erythrocytes, which are incapable of being infected, is unknown, but McClintock et al (1980) have suggested that such interactions may decrease the susceptibility of the host to infection by aiding clearance of the virus and by presenting the virus more effectively as antigen to immunocompetent cells.

The receptor for many viruses on human erythrocytes is glycophorin (Business, 1980; Lonberg-Holm and Crowell, 1986), the major red cell surface membrane sialoglycoprotein which is also the MN blood group antigen (Anstee, 1981). It is now known that there are several glycophorins. Some of these glycophorins are designated A2, A, B and AB, and represent the dimer and monomer forms of glycophorin A, the monomer form of glycophorin B, and a hetero-dimer comprising one molecule each of glycophorin A and glycophorin B, respectively (Anstee, 1981). Glycophorin A was reported to serve as a human erythrocyte receptor for Influenza A (Lonberg-Holm and Crowell, 1986) and EMC virus (Allaway et al., 1986):

### 1.3.3. Virus Receptors On Host Cells

Viruses are obligate intracellular parasites. Different cell types act as hosts to various viruses and virus receptors on such host cells act as points of attachment for the viruses. Some viruses recognize a single receptor on different cell types. For example, the receptor for reovirus T3 on neurons, lymphocytes and L-cells has been reported as the beta-adrenergic hormone receptor (Co et al., 1985).

Host cell receptors differ from virus to virus. The receptor for HTLV-III on T

lymphocytes is the CD4 (T4) antigen (Dangleish et al., 1984; Klatzmann et al., 1984). The receptor for Rabies virus on mouse muscle cells may be an acetylcholine receptor (Barrage, Tignor and Smith, 1982; Wunner et al., 1984), while the receptors for Sendai virus on susceptible cells are suggested to be gangliosides (Markwell et al., 1984). Phospholipids or glycolipids can serve as the Vesicular stomatitis virus receptor on fibroblasts (Bailey, Miller and Lenard, 1984; Schlegel and Wade, 1985).

#### 1.3.4. Receptors For Picornaviruses On Host Cells

According to Crowell and Siak (1978), cellular receptors for the human enteroviruses are limited to the external surface of the plasma membrane. These receptors are considered to be components of the membrane since no receptor activity could be demonstrated on any intracellular components. The receptors for Coxsackie virus B3 are located on both the microvilli and the body of HeLa cells (Roising et al., 1975). The cellular location of receptors utilized by cardioviruses has not been determined but some are known to be neuraminidase-sensitive (Lonberg-Holm and Crowell, 1986), suggesting that these receptors contain sialic acid. When Allaway et al (1986) examined the role of sialic acid in virus binding to cells they found that the carboxyl group, unlike the polyhydroxy side chain in sialic acid, is required for EMC virus attachment.

Trypsin inactivated poliovirus receptors on HeLa cells while chymotrypsin inactivated the receptors for group B Coxsackie viruses (Zajac and Crowell, 1965; Kraemer, 1966), suggesting that these receptors are proteins and differ for the different viruses.

## 1.4. Isolation of Virus Receptors

### 1.4.1. Various Ways To Isolate Virus Receptors

A variety of approaches have been used to isolate virus receptors from whole cells or plasma membranes. Receptors containing lipids can be isolated by chloroform-methanol extraction by modifying the technique of Kritchevsky and Shapiro (1967). Procedures used to isolate glycoprotein receptors include the use of hot phenol (Howe et al., 1972), or phenol in combination with either lithium diiodosalicylate (LIS) (Marchesi and Andrews, 1972), or sodium deoxycholate (Segrest et al., 1970), or Triton X-100 (Fukuda and Osawa, 1973). Other methods for isolating glycoprotein receptors have 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 (35mg of receptor material per gram of membrane protein); the major disadvantage is that up to 10 mol LIS per mol of receptor material is retained (Segrest et al., 1970). Further, the LIS method does not completely remove pigments such as haeme from the preparation (Segrest et al., 1970). The sodium deoxycholate procedure while relatively purer, gives a lower yield (Segrest et al., 1970). Hot phenol has been shown to cause chemical modification of the receptor material (Segrest et al., 1970).

Different strategies have been used for isolation of picornavirus host-cell receptors. The receptor for Coxsackievirus B3 on HeLa cells was isolated by extraction of the Coxsackievirus-receptor complex from treated cell membranes (Mapoles et al., 1985), using the sodium deoxycholate and Triton X-100. 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. Tomassini and Colonna (1986) have used antireceptor monoclonal antibodies to isolate and purify the receptor for human rhinovirus type 14.

(HRV-14) on HeLa cells. However, a major disadvantage of this approach could be the apparent low frequency at which monoclonal-antibodies are generated; out of thousands of assays, only one hybridoma cell culture supernatant was identified which could protect cells from HRV-14 infection (Tomassini and Colonna, 1986).

#### 1.4.2. Experimental Strategy,

In the present study, two methods will be used in attempts to isolate the receptor for encephalomyocarditis (EMC) virus on cells that support virus growth: affinity chromatography on virus-Sepharose and the use of anti-idiotypic antibodies raised in rabbits against anti-EMC antibodies.

Purified EMC virus will be covalently linked to cyanogen bromide-activated Sepharose 4B to make a virus column. This column will be studied for stability at different temperatures and pH, and its saturation point in terms of how much receptor material it can accommodate, will be determined. The conditions for elution of receptor material solubilized in sodium deoxycholate and the effect of sodium deoxycholate upon the receptor with regard to its ability to bind virus after solubilization will also be determined. Then radiolabelled membrane preparations of various cells which contain receptor material specific for EMC virus will be solubilized in detergent and applied to the virus column. Specific virus receptor material should get bound to EMC virus attached to the Sepharose and be retained on the column, while material not retained gets washed off the column. Receptor material eluted from the column will then be characterized by gel electrophoresis with autoradiography.

The rationale behind the use of anti-idiotypic antibodies as cell membrane probes is based on Jerne's network theory (Jerne, 1974) of idiotypes. According to this theory, anti-EMC anti-idiotypic antibodies prepared by immunizing rabbits with affinity purified anti-EMC antibodies, would be structurally similar to EMC virus and the anti-idiotypic antibodies should be able to mimic the physiologic activity of the virus such as binding to the receptor for EMC virus. In the present

study the antibody-receptor complex formed after binding will be isolated by immunoprecipitation with protein A Sepharose and characterized by gel electrophoresis. This technique was first used by Sege and Peterson (1978) to isolate the insulin receptor. 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 report that these antibodies effectively bound to a 67 kilodalton cellular receptor protein which also bound virus. This approach has several advantages. Anti-cellular or anti-viral antisera 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 very specific whereas viruses have been known to bind non-specifically to inanimate surfaces such as glass, nitrocellulose and carbon (Valentine and Allison, 1959; Boche and Quilligan, 1966). Also, from a safety point of view, it is easier to work with antibodies than viruses.

#### 1.4.3. Aims And Objectives

This study began with the general objective of developing a specific and sensitive bioassay to identify glycoprotein, which is the receptor for EMC virus on human erythrocytes. A sensitive method of assay would be required to measure the biological activity of the affinity purified anti-EMC antibodies and receptor material specific for EMC virus on host cells. Enzyme-linked immunosorbent assay (ELISA) and haemagglutination inhibition tests were used for these purposes.

The particular aims of the project reported in this thesis are:

- (a) To make a virus affinity column and use this column to isolate the receptor for EMC virus on a host cell.
- (b) To make anti-idiotypic antibodies against anti-EMC antibodies and to use these antibodies to identify the receptor for EMC virus on a cell that supports virus growth.

The objective of this study is to identify and clarify the mode of interaction of EMC virus with its receptor on host cells.

## Chapter 2

# MATERIALS AND METHODS

### 2.1. Cells Used

#### 2.1.1. K562 Cells

These are human erythroleukaemic cells which express the erythroid specific cell surface glycoproteins, glyophorins A and B (Gahnberg et al., 1979; Fukuda, 1980). This cell-line was originally established in 1975, from the pleural effusate of a patient in the terminal stage of chronic myelogenous leukaemia with blast crisis (Lozzio and Lozzio, 1975).

Apart from expressing glyophorins A and B, K562 cells possess other cell surface glycoproteins which are distinctly different from those of mature erythrocytes. Two major glycoproteins, gp105 and gp95 of K562 cells, are similar to those of a myeloblastoid cell line and the same glycoproteins have been found as minor components in erythroblasts cultured *in vitro* (Fukuda, 1980). K562 cells also express both a granulocytic marker identified by the My-1 antibodies and an erythroid marker, spectrin, in the same cells (Marie et al., 1981). Some K562 cells have been found to be bipotent in that they can undergo differentiation into erythrocytes or megakaryocytes depending on the culture conditions (Vainchenko et al., 1981) and K562 cells can be induced with sodium butyrate to produce embryonic haemoglobin (Anderson et al., 1979). The fact that these cells have several characteristics in common with other blood cell lineages has been attributed to their immature stage of development (Stamatoyannopoulos et al., 1981).



Other erythroid surface proteins expressed by K562 cells include the blood group-N antigen and the i antigen whereas band 3 and ABB antigens are absent. (Fukuda, 1980). When these cells are treated with various "inducers" they do not respond by expressing the cell surface markers found on more mature cells and according to Yurchenko and Furthmayr (1980), inducing agents do not increase the amount of glycophorins or spectrin in K562 cells. When K562 cells are treated with tumour promoting phorbol esters, glycophorin A, a cell surface marker specific to later stages of maturation of erythroid cells is lost but gp95 which is specific to early stages is retained (Fukuda, M., 1981).

K562 cells used in this study were grown from a stock kindly provided by Dr. Alan Scheeter at the Laboratory of Chemical Biology, NIH, USA. The cells were grown at 37°C in suspension in Roswell Park Memorial Institute (RPMI) medium 1640 (see Appendix) in the presence of 5% carbon dioxide. Cells were diluted to  $5 \times 10^4$  cells/ml and subcultured when they reached a density of  $0.8 \times 10^6$  cells/ml. These cells were preserved by suspending them at a concentration of  $5 \times 10^6$  cells/ml in RPMI medium 1640 containing 10% (w/v) glycerol and then freezing the cells at -70°C overnight. The frozen cells were stored in liquid nitrogen.

### 2.1.2: HEL Cells

HEL cells are a human erythroleukaemic cell line established in 1982 from a patient with Hodgkin's disease who later developed erythroleukaemia (Tabilio et al., 1984). This cell line has some erythroid features including the production of haemoglobin and the expression of glycophorin A (Tabilio et al., 1984). In addition to these erythroid features, markers specific for other cell lineages such as platelet membrane glycoproteins and platelet  $\alpha$ -granules are also expressed in the HEL cell line (Tabilio et al., 1984).

HEL cells used in this study were grown from a culture kindly given to us by Dr. David Anstee, South Western Regional Blood Transfusion Centre, Bristol, England. These cells were maintained at 37°C in a 5% carbon dioxide atmosphere in suspension in Earle's medium (see Appendix) supplemented with 10% horse

serum, 100 units/ml of penicillin and 10 units/ml of streptomycin (final concentrations).

### 2.1.3. HeLa Cells

HeLa cells were established in 1952 from a human carcinoma of the cervix (Gey et al., 1952). Two strains of HeLa cells were used in this study. The S3 strain of HeLa cells were grown from a culture kindly given to us by Dr. Banfield Younghusband in this faculty. These cells were maintained in suspension at 37°C in a 5% carbon dioxide atmosphere in Eagle's medium, Joklik modified (Gibco laboratories, N.Y., USA), containing 100 units/ml of penicillin and streptomycin, 3.5% calf serum and 3.5% foetal calf serum. The JW strain of HeLa cells, from our own stock stored in liquid nitrogen, were maintained in monolayer cultures in the same Eagle's medium but containing 10% foetal calf serum (final concentrations). JW HeLa cells were harvested from petri dishes using a rubber policeman because proteolytic enzyme digestion would possibly destroy the receptors in which we were interested.

### 2.1.4. Red Blood Cells

Heparinized bovine and sheep bloods were supplied from the Animal Care unit in this faculty. Outdated human type O blood was obtained from the Haematology department in this faculty. Bloods were stored at 4°C and when required cells were collected by centrifugation at 2000 rpm for 5 minutes and washed at least three times in 0.85% NaCl solution until free of haemoglobin released from lysed cells.

### 2.1.5. Krebs II Ascites Tumour Cells

This cell line was established from a carcinoma arising from the inguinal region of a hybrid male mouse (Klein and Klein, 1951). After several passages of the solid tumour by subcutaneous injection into similar mice, haemorrhagic exudates appeared. Repeated injections of this exudate into the peritoneum of mice yielded ascites tumours rather than solid tumours (Klein and Klein, 1951).

Krebs II ascites cells were grown in the peritoneal cavity of heterogeneous albino mice. Krebs cells used to grow virus were maintained at 37°C in swirling (80 rpm) suspension cultures in Earle's medium (see Appendix ) distributed into 100 ml quantities in 1 L Erlenmeyer flasks (see section 2.2.1 for growing virus).

### 2.1.5.1. Harvesting Krebs II Ascites Tumour Cells

#### Materials:

- i. Phosphate-buffered saline (PBS), pH 7.3 (see Appendix ).
- ii. Calcium and Magnesium free PBS (Ca-Mg free PBS), prepared as described in the Appendix.
- iii. Trypan blue, 0.1% in PBS.
- iv. 70% ethanol.

#### Method:

Heterogenous albino mice weighing about 20-30 g were inoculated intraperitoneally with 0.1 ml of a suspension of  $10^8$  cells/ml. Mice bearing seven day tumours were killed by cervical dislocation. The abdomen was washed with 70% ethanol and a small incision made into the sterilized area with scissors. The skin was pulled open to reveal the peritoneum with the mouse held over a funnel in centrifuge bottle containing about 50 ml Ca-Mg free PBS, chilled in an ice bath. The peritoneal wall was punctured to allow cells to drain into the funnel. Two successive washes with Ca-Mg free PBS were sufficient to flush most of the cells from the cavity.

These freshly harvested cells were washed in Ca-Mg free PBS by several rounds of centrifugation at 1500 rpm for 5 minutes to remove red blood cells and ascitic fluid after which cells (0.1 ml) suspended in PBS were diluted 1:10 in 0.1% Trypan blue and counted to adjust to a final concentration of  $10^8$  unstained cells/ml.

## 2.2. Encephalomyocarditis (EMC) Virus

### 2.2.1. Growth Of EMC Virus In Krebs Cells

#### Materials:

- i. Earle's saline growth medium (see Appendix)
- ii. 0.1% Trypan blue in PBS.

#### Method:

i. Krebs cells in suspension ( $10^8$  cells/ml) were infected with the K2 strain of EMC virus (Sanders et al., 1958) at a multiplicity of infection of at least 3, and the suspension was maintained at  $4^{\circ}\text{C}$  for 30 minutes to allow adsorption which encouraged synchronous infection of cells by the virus.

ii. The cell suspension was diluted to  $10^7$  cells/ml with the Earle's medium warmed to  $37^{\circ}\text{C}$  and then 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  $37^{\circ}\text{C}$  overnight. An uninfected control was prepared in a similar way.

iv. About 12 to 18 hours later the cultures were removed from swirlers and cells in 0.1 ml of well mixed culture were stained by diluting 1:10 with 0.9 ml 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 (section 2.2.3.) and crude virus preparations, which comprise suspensions of virus plus cells and cellular debris, were stored at  $-20^{\circ}\text{C}$ .

Radiolabelled virus was prepared by adding  $^3\text{H}$  amino acids or  $^3\text{H}$  adenosine from NEN, to the cell cultures (5  $\mu\text{Ci}/\text{ml}$ , final concentration) 3 hours after incubation had begun.

### 2.2.2. Purification Of EMC Virus

EMC virus was purified according to the method described by Burness (1969).

#### Materials:

- i. Gold ( $4^{\circ}\text{C}$ ) 10% acetic acid.
- ii. Trypsin (8575 BAEE units/mg solid; Sigma) 2.5 mg/ml in deionised water.
- iii. Pancreatic ribonuclease solution (RNase) from Sigma, 100  $\mu\text{g}/\text{ml}$  in deionised water.
- iv. 1.0 M NaCl i.e. 58.44 g/L of distilled water.
- v. Organic solvent mixture: 1 volume butoxyethanol plus 2 volumes ethoxyethanol.
- vi. 2.5 M phosphate: contains 433.45 g/L of  $\text{K}_2\text{HPO}_4$ , adjusted to pH 7.5 with concentrated  $\text{H}_3\text{PO}_4$  and filtered when necessary.
- vii. 0.5 M phosphate buffer, pH 8.0 (see Appendix ).
- viii.  $\text{NaCl-P}_4$  which consists of 10 ml of 1.0 M NaCl and 4 ml of 0.5 M phosphate buffer pH 8.0, made up to 100 ml with distilled water.
- ix. P18-NaCl contains 178.43 g  $\text{Na}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ , 31.83 g  $\text{K}_2\text{HPO}_4$ , and 11.69 g

NaCl adjusted to pH 8.0 with concentrated  $H_3PO_4$  and made up volume to 2 litres with distilled water.

### Method:

#### i. Clarification.

Crude virus preparations were thawed and the suspension clarified in 250 ml bottles by centrifugation at 10,000 rpm for 15 minutes using a Beckman JA-20 rotor. The supernatant fluid containing virus was collected in a large beaker on ice for acid precipitation.

#### ii. Acid Precipitation

Cardioviruses, which includes EMC virus, are unstable at pH 5 to 6 in the presence of 0.1 M halide ions (Spier, 1962; Young, 1986). Therefore to prevent degradation, the virus was cooled to 0°C and cold 10% acetic acid was added rapidly with stirring to bring to pH 4.8. Precipitation was allowed to occur for at least 15 minutes at 0°C after which the precipitate was collected by centrifugation at 2000 rpm for 10 minutes in the cold. The supernatant was discarded and the pellets were combined and resuspended in about 40 ml of P18-NaCl solution. The suspension was homogenized with a Tissumizer (Tekmar company, Ohio, USA).

#### iii. Organic Solvent Extraction

To one volume of virus suspension in a graduated cylinder was added one volume of 2.5 M phosphate buffer and 0.8 volume of organic solvent mixture. After gentle mixing by inverting the cylinder about 20 times, the contents were transferred to 250 ml bottles and centrifuged at 5000 rpm for 5 minutes using a Beckman JA-20 rotor. Upper and lower layers were removed completely by aspiration, leaving behind the precipitated interface. The interfaces from several bottles were combined and resuspended in about 30 ml of P18-NaCl using the Tissumizer.

The suspension was clarified by centrifugation at 10,000 rpm for 15 minutes using a Beckman JA-20 rotor. The supernatant fluid containing virus was transferred to a measuring cylinder and the precipitate was reextracted twice by homogenization with the Tissumizer and centrifugation at 10,000 rpm for 15 minutes. The combined supernatants were centrifuged at 40,000 rpm for 90 minutes using a Beckman SW40Ti rotor. The supernatant was discarded and the virus pellet was covered with 0.5 ml P<sub>1</sub>S-NaCl and left to soften for two hours or overnight.

#### iv. Enzyme Treatment

Each virus pellet was treated with 0.1 ml of RNase solution and incubated at 37°C for 30 minutes. To each pellet was added 0.1 ml of trypsin and incubation continued for a further 30 minutes at 37°C. Added in this order trypsin degrades the ribonuclease as well as protein from cellular material. The pellets were resuspended and combined.

The suspension was clarified by centrifugation at 10,000 rpm for 10 minutes in a Beckman-JA-20 rotor and the supernatant was transferred to clean SW40Ti tubes while the pellet was put aside. The tubes used for centrifugation were washed with 1 ml of P<sub>1</sub>S-NaCl solution which was then added to the precipitates that were put aside and after resuspension and recentrifugation at 10,000 rpm for 10 minutes the supernatant collected. The combined supernatant fluids were centrifuged at 40,000 rpm for 90 minutes in a SW40Ti rotor. The tubes were drained and 1 ml of NaCl-P<sub>1</sub>O<sub>4</sub> was added to the pellet which were left to soften for at least two hours or overnight at 4°C. Virus pellets in the different tubes were resuspended and combined; 10 ul was diluted to 1 ml in NaCl-P<sub>1</sub>O<sub>4</sub> and the concentration of virus estimated. Using a 1 cm (pathlength) cuvette, a suspension containing 100 ug/ml of virus has an extinction (optical density) of 0.77 at 260 nm. The purified virus was stored at -20°C.

### 2.2.3. Haemagglutination (HA) Test

#### Materials:

i. Mayer's veronal buffer was prepared as described by Jungblut (1958).

Mayer's veronal buffer contains equal volumes of solutions A and B and was stored at 4°C.

Solution A: 0.91 g of 5, 5 diethylbarbituric acid was dissolved in 500 ml of hot (75°C) deionised water.

Solution B: 13.892 g KCl, 0.028 g CaCl<sub>2</sub>, 0.079 g MgCl<sub>2</sub>, and 0.102 g KOH were dissolved in 500 ml of deionised water.

ii. Veronal buffer-glucose-gelatin (V-g-g) was made up of 8 volumes of veronal buffer, 1 volume of 4.5% glucose and 1 volume of 1% gelatin.

iii. V-shaped microtitre plates and 50 ul stainless steel microdiluters (Linbro, Flow labs. Inc., USA).

iv. Sheep, human or bovine red blood cells were washed in 0.85% NaCl solution by centrifugation at 2500 rpm for 5 minutes until the supernatant was free of haemoglobin from lysed cells. A 0.2% red cell suspension in V-g-g was made by diluting 10 ul of packed cells to 5 ml.

#### Method:

i. 50 ul of V-g-g was dispensed into each well of a V-shaped microtitre plate.

ii. Virus suspension was taken up in a 50 ul microdiluter and mixed with V-g-g in the first well; this dilutes the virus suspension 1:2.

iii. Using the same microdiluter, 50 ul was removed from the first well and mixed with the V-g-g in the second well. After mixing, 50 ul was removed and



transferred to the third well. This mix and transfer procedure was repeated to make a series of doubling dilutions. A volume of 50 ul was removed from the last well and discarded.

iv. The procedure was repeated in an adjacent row using virus of known IIA titre as a positive control. Another row was prepared containing only V-g-g, representing a negative control.

v. 50 ul of a 0.2% suspension of red blood cells was dispensed into all the wells and the titration plate was covered and incubated for at least 25 hours at 4°C before reading.

vi. The end point was the highest dilution showing a trace of haemagglutination. The number of IIA units per ml is the dilution of the virus at the end point multiplied by 1000 and divided by 50 ul, the volume of the microdiluter.

#### 2.2.4. Haemagglutination Inhibition Test (HAI)

##### Materials:

- i. Immune and preimmune anti-EMC sera inactivated at 56°C for 60 minutes.
- ii. 4-8 IIA units of virus in suspension.
- iii. Veronal buffer, veronal-glucose-gelatin, V-shaped microtitre plates, microdiluters and red blood cells as described above (section 2.2.3.) for the haemagglutination test.

##### Method:

i. 50 ul of V-g-g was dispensed into each well in a V-shaped titration plate and heat inactivated immune serum was taken up in a 50 ul microdiluter and mixed with V-g-g in the first well. The microdiluter was rotated to mix the contents, this diluted the serum 1:2. Using the same microdiluter, 50 ul was removed from this

same well and mixed with the V-g-g in the next well. After mixing, 50  $\mu$ l was removed and transferred to the third well. This mix and transfer procedure was repeated to make a series of doubling dilutions. A volume of 50  $\mu$ l was removed from the last well and discarded.

ii. The procedure was repeated in an adjacent row using preimmune serum as a negative serum control; another row was prepared containing only V-g-g representing a negative diluent control.

iii. 50  $\mu$ l of 4 HA units of virus in V-g-g was added to each well and the mixture was incubated for 60 minutes at room temperature. Then 50  $\mu$ l of a 0.2% suspension of red blood cells was dispensed into all the wells and the titration plate was covered and incubated for at least 5 hours at 4°C before reading. The end point, which was used to express the activity of each serum sample, was the highest dilution of antiserum inhibiting haemagglutination of virus. The volume of the diluter was not taken into account as was done for the haemagglutination test (see section 2.2.3.).

## 2.3. Preparation of Cell Membranes

### 2.3.1. Human Red Blood Cell Membranes

#### Materials:

##### i. Buffer solutions.

Isotonic buffer: 0.15 M NaCl plus 0.005 M  $\text{Na}_2\text{HPO}_4$  adjusted to pH 8.0 with concentrated  $\text{H}_3\text{PO}_4$ .

Hypotonic buffer: 0.005 M  $\text{Na}_2\text{HPO}_4$  adjusted to pH 8.0 with concentrated  $\text{H}_3\text{PO}_4$ .

ii. Outdated human blood was obtained from the Haematology department in this faculty and stored at 4°C in blood donor bags containing citrate phosphate dextrose adenine as anticoagulant.

**Method:**

i. The blood was distributed in 250 ml centrifuge bottles and diluted with an equal volume of cold isotonic buffer. The suspension was centrifuged at 2500 rpm for 10 minutes at 4°C using a swinging bucket rotor. The supernatant and the buffy coat were aspirated and the packed cells were washed at least three times until the supernatant was free of haemoglobin from lysed cells.

ii. The cells were lysed by mixing about 1 ml of packed red blood cells with 40 ml of cold hypotonic buffer and the suspension was centrifuged at 9000 rpm for 25 minutes at 4°C using a Beckman JA-20 rotor. The deep red supernatant containing haemoglobin was aspirated leaving behind the red translucent pellet of packed ghosts. This washing procedure was repeated at least twice until the pellets were white and the supernatant was clear.

iii. Pellets of membranes in different tubes were pooled together and the total volume noted. Protein concentration was measured by the Lowry (1951) method and sialic acid content was determined by the thiobarbituric acid method of Warren (1950).

### 2.3.2. Membranes From K562 Cells, HeLa Cells and Krebs II Ascites Tumour Cells

These membranes were prepared using the procedure described by Atkinson and Summers (1971).

**Materials:**

- i. Cells in suspension.
- ii. 10 mM Tris pH 8.0 containing 15 mM sodium iodoacetate (Tris-iodoacetate).
- iii. 30 mM MgCl<sub>2</sub>.

iv. 100 mM NaCl.

v. 30% sucrose (w/v); 45% sucrose (w/v).

**Method:**

i. The suspension of cells was centrifuged at 1000 rpm for 5 minutes at 4°C to pack the cells. The volume of packed cells was noted and the cells were resuspended in 20 volumes of 10 mM Tris-iodoacetate and left to swell for 5 minutes in an ice bath.

ii. Five volumes of cold distilled water was added to the suspension which was left to stand for another 5 minutes. The swollen cells were ruptured with five strokes of a stainless steel Dounce homogenizer which has a clearance of 0.002 inches.

iii. 0.1 volumes of 30 mM  $MgCl_2$  and 0.1 volumes of 100 mM NaCl were added to the homogenate to stabilize the nuclei.

iv. The homogenate was centrifuged at 1000 g for 1 minute to separate the nuclei from cellular membranes. The supernatant contained the membranes and the pellet contained nuclei. The supernatant was saved.

v. The pellet was resuspended in 4 volumes of Tris-iodoacetate, 3 mM  $MgCl_2$  and 10 mM NaCl and centrifuged at 1000 g for 1 minute to recover more membranes and increase the yield.

vi. The supernatants were combined, aliquoted into 2.5 ml amounts and layered onto a sucrose gradient which was prepared by pipetting 15 ml of 30% sucrose into a 30 ml Corex centrifuge tube. Then 5 ml of the 45% sucrose solution was taken with a syringe and deposited at the bottom of the same Corex tube.

vii. The sucrose pad layered with crude membrane preparations, was centrifuged at 7000 g for 20 minutes in a swingout H1134 rotor. The plasma membrane ghosts

appeared as a white opaque layer at the 30-45% sucrose interface and these were carefully removed with a syringe fitted with a 14 gauge needle.

viii. The plasma membrane ghosts were layered onto a fresh 30-45% sucrose gradient and centrifuged again at 7000 g for 20 minutes in the cold. This procedure was repeated at least twice to further remove any contaminating organelles from the membrane preparations.

ix. After the final centrifugation the purified plasma membranes of each cell line were removed from the 30-45% sucrose interface with a syringe and centrifuged at 1000 g for 5 minutes to get rid of the sucrose. Packed membranes from the same cell line were combined and protein concentration was measured by the method of Lowry et al., (1951). Purified membranes were stored at  $-20^{\circ}\text{C}$ .

### 2.3.3. Labelling Membranes With Periodate-Tritiated Sodium Borohydride.

Purified cell membranes were labelled with tritium according to the method described by Gahmberg and Andersson (1977).

#### Materials:

- i.  $\text{NaCl-PO}_4$  buffer, pH 7.4, which contains 0.15 M NaCl and 0.01 M sodium phosphate.
- ii. 2 mM sodium metaperiodate ( $\text{NaIO}_4$ ) in  $\text{NaCl-PO}_4$ .
- iii. 10% glycerol (w/v).
- iv. Cold 0.01 M NaOH.
- v. Tritiated sodium borohydride ( $\text{NaB}^3\text{H}_4$ , 500 mCi/minol) supplied by Amersham Canada Limited.

vi. Non-tritiated sodium borohydride ( $\text{NaBH}_4$ ).

vii. 24 mM (1%) sodium deoxycholate in 0.02 M sodium phosphate buffer pH 8.0.

viii. Purified plasma cell membranes.

#### Method:

i. 1 ml of packed membranes were washed twice in  $\text{NaCl-PO}_4$  by centrifugation at 2500 rpm for 10 minutes at  $4^\circ\text{C}$  and then the volume was made up to 2 ml with  $\text{NaCl-PO}_4$ . To the membrane suspension was added 200  $\mu\text{l}$  of 2 mM sodium metaperiodate in  $\text{NaCl-PO}_4$  and incubation continued for 10 minutes at  $4^\circ\text{C}$ .

ii. After incubation, 20  $\mu\text{l}$  of 10% glycerol per ml of membrane suspension was added while stirring to react with excess  $\text{NaIO}_4$ . The membrane suspension was washed twice in  $\text{NaCl-PO}_4$  by centrifugation at 2500 rpm for 10 minutes at  $4^\circ\text{C}$  and resuspended in 5 ml of  $\text{NaCl-PO}_4$ .

iii. Membranes were reduced by addition of 5 mCi of carrier free  $\text{NaB}^{3}\text{H}_4$  in 200  $\mu\text{l}$  of ice cold 0.01 M NaOH and kept for 30 minutes at room temperature. After incubation, unlabelled  $\text{NaBH}_4$  was added to a final concentration of 1 mM to reduce any remaining aldehydes on the surface. The membranes were then washed by centrifugation at least three times in  $\text{NaCl-PO}_4$  until radioactivity in the supernatant was close to background. The pellet was suspended in 1 ml of  $\text{NaCl-PO}_4$  and 20  $\mu\text{l}$  of this suspension was solubilized in 12 mM sodium deoxycholate in sodium phosphate buffer, and counted for radioactivity using a Beckman LS 8100 liquid scintillation system. Tritiated membrane preparations were stored at  $-20^\circ\text{C}$ .

## 2.4. Glycophorin

There are several types of human glycophorin. A typical glycophorin isolate consists of glycophorin A (80%), glycophorin B (12%) and glycophorin C (8%) which is a mixture of at least two other components (Anstee, 1981).

### 2.4.1. Isolation Of Glycophorin From Human Erythrocyte Membranes

Glycophorin was prepared from human erythrocytes by the method of Marchesi and Andrews (1971):

#### Materials:

- i. Lithium diiodosalicylate (LIS) 0.3 M; freshly prepared.
- ii. Tris hydrochloride 0.05 M, pH 7.5.
- iii. 50% phenol in water (v/v).
- iv. Cold 100% ethanol.
- v. Dialysis bags with molecular weight cutoffs of 6000-8000 and 12000-14000

#### Method:

- i. Red cell membranes were suspended in 0.3 M LIS in 0.05-M Tris, pH 7.5, at a concentration of approximately 25 mg/ml of protein, and stirred at room temperature for 15 minutes.
- ii. Two volumes of distilled water were added and the turbid suspension was stirred for 15 minutes at 4°C and then centrifuged at 4500 rpm for 90 minutes using a Beckman 50.2T rotor.
- iii. The supernatant fluid which contained most of the membrane proteins was decanted and mixed with an equal volume of freshly prepared 50% phenol in

water. The mixture was stirred at 4°C for 15 minutes and centrifuged at 4000 rpm for 60 minutes using a Beckman JA-20 rotor. The material separated into two phases; the upper phase (aqueous) contained most of the soluble glycoprotein.

iv. The aqueous layer was removed and dialyzed against several changes of distilled water at 4°C for 36 hours. The molecular weight cutoff of the dialysis bag was 12000-14000.

v. The dialyzed material was lyophilized and then resuspended in cold 100% ethanol. This material was stirred for 1-2 hours at 4°C and centrifuged at 5000 rpm for 20 minutes in a JA-20 rotor, to collect the precipitate. The supernatant was discarded and the ethanol washing procedure was repeated three times to further remove any phenol from the sediment.

vi. The washed pellet was suspended in 1 to 2 ml of distilled water and dialyzed against water in the cold overnight using the dialysis bag with molecular weight cutoff 6000-8000. The dialyzed material was then centrifuged at 10,000 rpm for 30 minutes at 4°C in a JA-20 rotor. The clear supernatant which contained the soluble glycoprotein was collected. Protein concentration was measured by the Lowry (1951) method and sialic acid content was determined by the thiobarbituric acid method of Warren (1959). The glycoprotein was stored at -20°C.

#### 2.4.2. Iodination of Glycophorin

Glycophorin was iodinated according to the method described by Markwell and Fox (1978).

##### Materials:

- i. Glycophorin in solution.
- ii.  $^{131}\text{I}$  supplied by NEN products.
- iii. 5% bovine albumin solution in 0.02 M sodium phosphate buffer pH 8.0 (BSA solution).



iv. Disposable Sephadex G-25 columns from Pharmacia (internal diameter 1.5 cm, bed height 5 cm, bed volume 0.1 ml).

v. Glass test tubes coated with iodogen (see appendix for preparation of tubes).

### Methods

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  $\mu$ g iodogen was rinsed with sodium phosphate buffer immediately before use to remove any loose flakes of iodogen. Approximately 100  $\mu$ g of glycothorin in solution was transferred to the coated tube and, working in a fume hood, 200  $\mu$ Ci of  $^{131}$ I was added. 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 applied to the Sephadex column. The column was washed with 5 ml of 0.02 M sodium phosphate buffer and 1 ml fractions were collected and their radioactivity measured in a gamma counter.

#### 2.4.3. Labelling Glycothorin With $^{3}$ H-Acetic Anhydride

Acetylation of glycothorin was performed as described by Pardoe and Burness (1980).

#### Materials:

- i. Glycothorin in solution.
- ii. Saturated sodium acetate in water.
- iii.  $^3$ H-acetic anhydride (activity: 50 mCi/mmol, concentration: 5 mCi/5  $\mu$ l) supplied by NEN and prepared according to supplier's instructions.

#### Method:

- i. To a test tube containing 0.2 ml distilled water and 0.5 ml saturated sodium

acetate was transferred 125  $\mu$ g of glycophorin. 2 mCi of  $^3\text{H}$ -acetic anhydride was added and the tube was kept for 15 minutes at room temperature. Subsequently, 1 mCi of  $^3\text{H}$ -acetic anhydride was added every 15 minutes for 45 minutes at room temperature.

ii. The sample was dialysed against several changes of distilled water for at least 24 hours until the radioactivity of the diffusate was at background level. After dialysis, 10  $\mu$ l of the labelled glycophorin was added to 10 ml of scintillation liquid (Aquasol) and counted for radioactivity in an LS 335 Beckman scintillation counter. The  $^3\text{H}$ -glycophorin was stored at  $-20^\circ\text{C}$ .

## 2.5. Preparation of Virus-Sepharose 4B Affinity Columns

### Materials:

- i. 1 mM HCl.
- ii. Coupling buffer pH 8.3, consisting of 0.1 M  $\text{NaHCO}_3$  containing 0.5 M NaCl.
- iii. Blocking buffer pH 8.0, consisting of 0.2 M glycine.
- iv. Acetate buffer pH 4.0, which consists of 0.1 M sodium acetate containing 0.5 M NaCl, adjusted to pH 4.0 with acetic acid.
- v. Purified EMC virus (0.1-1.0 mg) in coupling buffer.
- vi. Cyanogen bromide (CNBr) activated Cl-Sepharose 4B supplied by Pharmacia.

### Method:

- i. One g of the activated Sepharose was weighed out into a 50 ml beaker and 10 to 20 ml of 1 mM HCl added. The swollen suspension was emptied into a funnel with a sintered glass filter and washed for 15 minutes with 200 ml of 1 mM HCl under vacuum.

ii. The ligand (purified virus) was dissolved in 10 ml coupling buffer in a stoppered 15 ml test tube and the activated Sepharose was then transferred from the sintered glass filter into the tube containing the ligand in solution. The mixture was rotated end-over-end using a multi purpose rotator (Scientific industries Inc.) at 6 rpm for 1 hour at room temperature after which the slurry was emptied into the sintered glass filter again and excess ligand was washed off with 20 ml of coupling buffer.

iii. The virus-Sepharose was transferred to a stoppered 15 ml test 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 washed with three cycles of alternating pH. Each cycle consisted of a wash with 10 ml of acetate buffer, pH 4.0, followed by a 10 ml wash with coupling buffer pH 8.3. The product was stored at 4°C in 0.02 M phosphate buffer pH 8.0, containing 0.1% NaN<sub>3</sub> as a preservative.

iv. To estimate how much virus was attached to the activated Sepharose, a similar preparation was made using tritiated virus and all the product was counted for radioactivity using a Beckman LS 335 liquid scintillation counter.

## 2.6. Antisera Used In This Study

### 2.6.1. Anti-EMC virus and antiglycophoria immune sera.

Anti-EMC and antiglycophoria antisera were prepared in this laboratory by Dr. Graham Allaway. Anti-EMC antibodies (AEMC) were raised by injecting purified EMC virus into New Zealand white rabbits. Antiglycophoria serum was obtained by a similar procedure using glycophoria isolated from human erythrocytes as immunogen. Anti-EMC antibodies was to be used to produce anti-EMC idiotypic antibodies. To determine if these AEMC antibodies were biologically active and specific for EMC virus, gel immunodiffusion, Enzyme-linked immunosorbent assay (ELISA) and haemagglutination inhibition tests were used as described below. The biological activity and specificity of antiglycophoria serum was determined by ELISA and the haemagglutination inhibition test.

### 2.6.1.1. Gel immunodiffusion test.

#### Materials:

- i. 1% agarose in PBS.
- ii. Purified EMC virus diluted to 2 mg/ml with PBS.
- iii. Petri dishes, 1.5 inches in diameter.
- iv. AEMC immune and preimmune sera diluted 1:10 with PBS.

#### Method:

i. Solidified agarose stored at 4°C was liquified by heating in a Microwave oven for 1 to 2 minutes. Approximately 1.5 ml of molten agarose was pipetted into each petri dish and left covered to set at room temperature for 30 to 45 minutes before transferring to 4°C for a further 30 minutes.

ii. Six wells 7 mm in diameter and 2 mm apart were punched into the solid agarose gel using a cork borer and the gel inside the wells was removed by suction. 100 µg of purified virus (antigen) was added to the centre well. Each of the five surrounding wells was loaded with 50 µl of 1:10 dilution of anti-EMC immune or preimmune sera. One of the surrounding wells contained only PBS as a negative diluent control.

iii. A similar preparation was set up in which the centre well contained influenza virus (PR8 strain from our own stock) and the surrounding wells were exactly as described above. All petri dishes were incubated in a moist chamber at 37°C for 72 hours and then examined for precipitin lines.

### 2.6.1.2. Enzyme-linked immunosorbent assay (ELISA) using anti-EMC serum.

#### Materials:

- i. Polyvinyl 96 well flat bottom EIA microtitre plates (Costar, Cambridge MA USA).
- ii. Photometric plate reader (Titertex, Multiskan), micropipettes and tips.
- iii. Serum which consists of Peroxidase-goat anti-rabbit IgG conjugate (Bio-Rad Co.).
- iv. Anti-EMC virus immune and preimmune sera.
- v. Peroxidase substrate kit (Bio-Rad Co.) consists of two solutions. One solution contains 2% Azino-di{3-ethyl benzthiazoline sulphonate (6)} and the other contains  $H_2O_2$ . The substrate solution was prepared according to the supplier's instructions by mixing equal amounts of the two solutions.
- vi. Washing buffer contains 0.05% Tween 20 (v/v) in PBS (Tween-PBS)
- vii. General diluent contains 0.05% Tween 20 (v/v) plus 2% BSA in PBS.
- viii. Diluent for conjugate contains 2% BSA in PBS.
- ix. Blocking buffer consists of 1% BSA plus 10% horse serum in PBS.
- x. 0.05 M  $NaHCO_3$  buffer, pH 9.6.
- xi. 2% oxalic acid in deionised water.

#### Method:

- i. Purified EMC virus was diluted to 200 ug/ml in 0.05 M  $NaHCO_3$  buffer pH

9.6 and 50 ul of the diluted virus were added to each well of polyvinyl microtitre plates. The plates were incubated for 60 minutes at 37°C and then stored overnight at 4°C. Unbound virus was removed by washing the plate three times in Tween-PBS. 250 ul of blocking buffer was added to each well to block any sites without bound virus, and the plates were incubated for 60 minutes at 37°C. The plates were again washed three times with Tween-PBS.

ii. Anti-EMC virus and preimmune sera were diluted 1:10 in 0.05% Tween 20 in PBS containing 2% bovine serum albumin (BSA) and 50 ul of serial two fold dilutions of the 1:10 diluted antisera were added to the wells. The plates were incubated for 60 minutes at 37°C, washed with Tween-PBS at least three times, and 50 ul of the conjugate diluted 1:3000 in 2% BSA in PBS was added to all wells. The plates were again incubated at 37°C for 60 minutes and washed three times with Tween-PBS.

iii. 50 ul of the substrate solution was added to all microtitre wells. The enzyme reaction was allowed to proceed for 60 minutes at 37°C in the dark and then terminated by the addition of 50 ul of 2% oxalic acid to each well. The intense blue-green colour which developed in positives was detected by absorbance at 405 nm using a photometric plate reader. Readings obtained with preimmune serum were considered as background and designated zero value. Using immune sera, any readings above this background level were regarded as positive.

#### 2.6.1.3. ELISA using anti-glycophorin immune serum.

In some experiments anti-glycophorin serum was titrated by the ELISA technique. First, glycophorin or red blood cells which contain glycophorin, were attached to polyvinyl microtitre plates. 50 ul of glycophorin (diluted to 200 ug protein per ml in 0.05 M  $\text{NaHCO}_3$  buffer pH 9.6); was added to each well of the microtitre plates and the plates were incubated for 60 minutes at 37°C and then stored overnight at 4°C.

When red cells were used the wells were precoated by addition of 50 ul of 1

mg/ml poly-L-lysine (Miles laboratories Inc, USA) in PBS to each well and incubation for at least 30 minutes at room temperature (Epstein and Lunney, 1985). Red blood cells washed in PBS were diluted to a concentration of  $5 \times 10^6$  cells/ml and 100  $\mu$ l of this cell suspension was added to each well. The plates were centrifuged for 10 minutes at 1000 rpm, incubated for 15 minutes at room temperature and then washed three times in washing buffer. The unattached sites in the wells were blocked by adding 250  $\mu$ l of blocking buffer.

Then the procedure was continued as described in the preceding section (2.6.1.2.) using anti-glycophorin in place of anti-EMC serum.

#### 2.6.1.4. Indirect Immunofluorescence.

##### Materials:

- i. 2% bovine serum albumin in PBS (BSA-PBS).
- ii. Immune serum (anti-glycophorin or anti-idiotypic serum) or preimmune serum each diluted 1:10 in BSA-PBS.
- iii. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit serum diluted 1:32 in BSA-PBS (Miles laboratories Inc, USA).
- iv. 90% glycerol in PBS (v/v).

##### Method:

Human erythrocytes (50  $\mu$ l of packed cells) and K562 cells ( $1 \times 10^7$  cells/ml) suspended in PBS were incubated with 200  $\mu$ l of immune or preimmune serum diluted 1:10, for 1 hour at room temperature. Following incubation, the cells were washed at least three times by suspending in BSA-PBS followed by centrifugation for about 2 minutes in a Beckman microfuge. Then 50  $\mu$ l of 1:32 fluorescein-conjugated goat anti-rabbit IgG were added and the tubes incubated in the dark for 1 hour at room temperature. The cells were washed again at least three times in BSA-PBS and resuspended in 100 to 200  $\mu$ l of 90% glycerol in PBS. A drop of

each sample was transferred to a glass slide and examined for fluorescence in a Zeiss Universal fluorescence microscope equipped with epilluminateur and a high pressure mercury lamp.

### 2.6.1.5. Purification of EMC virus anti-serum by affinity chromatography

#### Materials:

- i. Virus-Sepharose column.
- ii. Globulin fraction of anti-EMC serum prepared by ammonium sulphate precipitation of whole serum as described by Hudson and Hay (1980).
- iii. 0.02 M sodium phosphate buffer pH 8.0.
- iv. 0.17 M glycine-HCl buffer pH 2.3 containing 0.5 M NaCl.
- v. 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 0.5 mg of purified EMC virus to 1 g of CNBr-activated Sepharose 4B as described previously (see section 2.5.). The globulin fraction (1 ml) of anti-EMC virus immune serum (HA1 titre  $2 \times 10^3$ ), was added to the virus-Sepharose, shaken for 1 hour at room temperature and rotated overnight at 4°C. The virus-Sepharose with adsorbed antibodies was packed into a column and washed successively with phosphate buffer; with glycine-HCl containing NaCl; and with Triton X-100 in phosphate buffer. The volume of each wash was 20 ml collected in 1 ml fractions. ELISA tests using EMC virus attached to polyvinyl microtitre plates were performed on each wash 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.



## 2.6.2. Anti-idiotypic Antiserum

### 2.6.2.1. Production of anti-EMC anti-idiotypic antibodies.

#### Materials:

- i. Affinity purified anti-EMC IgG antibodies (purified AEMC).
- ii. Freund's adjuvant; complete and incomplete.
- iii. Two New Zealand white rabbits supplied by the Animal Care unit, Faculty of Medicine, MUN. These rabbits were from the same breed of rabbits as those used to produce anti-EMC antibodies (see 2.6.1.).

#### Method:

i. Affinity purified anti-EMC 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 ug 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 presence of anti-EMC anti-idiotypic antibodies by the haemagglutination test. Then an intramuscular booster of 30 ug of purified AEMC IgG in Freund's incomplete adjuvant was given to the rabbits and they were allowed to rest for another 3 weeks while 2 ml blood samples were taken every week and screened for anti-idiotypic antibodies. Two intramuscular boosters of 30 ug of IgG each in Freund's incomplete adjuvant were given on weeks 6 and 9. Ten days after the ninth week the rabbits were anaesthetized by intramuscular injection of 100 mg of Ketamine and bled to death by cardiac puncture. Sera were stored at -20°C.

### 2.6.2.2. Inhibition of virus infection by anti-idiotypic antibody through receptor blockade

#### Method:

HEL cells grown in suspension were washed once with PBS (Ca-Mg free) and resuspended in fresh RPMI medium (see appendix) at a concentration of  $2 \times 10^6$  cells per ml. These cells were incubated with 200  $\mu$ l of anti-idiotypic or preimmune antiserum for 30 minutes at room temperature. Following incubation the cells were spun down at 2000 rpm for 5 minutes to remove excess serum. The cells were resuspended in 200  $\mu$ l of RPMI medium and 100  $\mu$ l of crude virus preparation was added to the cells and incubated for 30 minutes at 4°C. Diluent (3 ml RPMI) was added to dilute to  $5 \times 10^6$  cells per ml and the cells were incubated for 24 hours at 37°C. Infected cells in 0.1 ml of well mixed culture were stained by diluting 1:10 with 0.9 ml of 0.1% trypan blue in a test tube and counted to check for cell death. A positive control (cells infected with virus, without antibodies) and negative controls (cells plus antibody without virus, cells alone, without virus or antibodies) were included and these cells were also stained with trypan blue and examined for cell death.

### 2.7. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was performed according to Fairbanks et al. (1971) or Laemmli (1970) using either cylindrical or slab gels.

#### 2.7.1. Fairbanks' system

##### Materials:

##### i. Stock solutions.

(a) Con. AcBis contained 40 g acrylamide, 1.5 g N, N'-methylene-bis-acrylamide (Bis), in 100 ml deionised water.

(b) 10 X buffer (pH 7.4) contained 1 M Tris (40 ml), 2 M sodium acetate (10 ml), 0.2 M EDTA (10 ml), adjusted to pH 7.4 with acetic acid and made up volume to 100 ml with deionised water.

(c) 10% (w/w) SDS.

(d) 1.5% (w/v) ammonium persulphate.

(e) 0.5% (v/v) tetramethylethylenediamine (TEMED).

ii. Electrophoresis buffer: 10 X buffer (100 ml), 10% SDS (100 ml), deionised water (800 ml).

iii. Gel overlay contains 0.1% SDS, 0.15% ammonium persulphate and 0.05% TEMED (final concentrations).

iv. 2X sample buffer consists of 1% SDS, 10% sucrose, 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8) and 40 mM dithiothreitol (DTT), to final concentrations.

v. Tracking dye: 10 ug/ml Pyrouin Y in deionised water.

#### Method:

i. The gel was prepared by mixing the concentrated stock solutions in the following order and proportions: Con AcBis (1.4 ml), 10X buffer (1.0 ml), 10% SDS (1.0 ml), deionised water (5.1 ml), 1.5% ammonium persulphate (1.0 ml), 0.5% TEMED (0.5 ml). The air was evacuated from the mixture for 2 to 5 minutes at room temperature and about 8.5 ml of the mixture was transferred to glass tubes (10 cm in length, 0.5 cm internal diameter). Each column was overlaid gently with gel overlay and left to stand at room temperature. When polymerization was complete (about 45 minutes), the tops of the gels were rinsed with about 0.5 ml of electrophoresis buffer. The gels were then left to stand for at least 12 hours at room temperature.

ii. Samples were prepared for electrophoresis by adding to them an equal volume of 2 X sample buffer and about 5  $\mu$ l of the tracking dye, and incubating at 37°C for 15-30 minutes to promote reduction of disulphide bonds by DTT and to dissociate membranes. Samples containing salts were dialyzed at 4°C overnight. The samples (50  $\mu$ l/tube gel) were loaded onto the gels and electrophoresis was carried out at constant voltage (75 volts) using electrophoresis equipment supplied by Buchler Instruments (Searle), USA.

### 2.7.2. Laemmli system.

#### Materials:

##### i. Stock solutions.

(a) 4 X upper Tris (0.5 M) buffer, pH 6.8 contains 0.4 g SDS and 3 g Tris in 50 ml deionised water. The solution was adjusted to pH 6.8 with 1 M HCl and the volume was made up to 100 ml with deionised water.

(b) 4 X 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 and the volume was made up to 150 ml with deionised water.

(c) Sample buffer consists of 4 ml deionised water, 1 ml 0.5 M Tris pH 6.8, 0.8 ml glycerol, 1.0 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) 30% acrylamide.

ii. Ammonium persulphate 100  $\mu$ g/ml in deionised water, freshly prepared.

iii. Tetramethylethylenediamine (TEMED).

iv. Electrophoresis buffer contains 3 g Tris, 114 g glycine and 10 g SDS, dissolved in 1 litre of deionised water.

#### Method:

The separating (lower) gel was prepared by mixing acrylamide-Bis (3.3 ml), lower Tris buffer (2.5 ml), deionised water (4.2 ml), ammonium persulphate (33 ul) and TEMED (8.0 ul). The gel mixture was degassed for 5 minutes; about 8.5 ml of the gel mixture was then transferred to glass tubes (10 cm in length, 0.5 cm internal diameter) and layered with butanol. When polymerization was complete (about 45 minutes), the top of the gels were rinsed three times with deionised water and overlaid with 0.5 ml of the upper gel mixture which contains 30% acrylamide (0.65 ml), upper tris buffer (1.3 ml), deionised water (3.05 ml), ammonium persulphate (25 ul) and TEMED (5 ul). The upper gel was layered with butanol and allowed to set at room temperature (about 30 minutes) after which the top of the gels were rinsed several times with deionised water. The gels were used immediately to prevent alteration in buffer pH by diffusion.

Samples were prepared for electrophoresis by adding an equal volume of the sample buffer followed by boiling for 5 minutes. Electrophoresis was carried out at constant voltage (75 volts) or at 3 mAmps/tube gel.

#### 2.7.3. Gel staining

After electrophoresis, the gels were stained for carbohydrate using the periodic acid-Schiff (PAS) procedure (Fairbanks et al., 1971) and for protein with Coomassie blue (Fairbanks et al., 1971).

##### 2.7.3.1. PAS staining

###### Materials:

- i. Fixative: 25% isopropyl alcohol (2-propanol), 10% acetic acid.
- ii. 0.5% periodic acid.

iii. 0.5% sodium arsenate in 5% acetic acid.

iv. 0.1% sodium arsenate in 5% acetic acid.

v. 5% acetic acid.

vi. 0.1% sodium metabisulphite in 0.01 M HCl ( $\text{Na}_2\text{S}_2\text{O}_5/\text{HCl}$ ).

vii. Schiff's reagent was prepared by dissolving 2.5 g of Basic fuchsin in 500 ml of distilled water and then adding 5 g of sodium metabisulphite and 50 ml of 1 M HCl. The solution was stirred for several hours and then decolourized by adding about 2 g of activated charcoal (Fisher brand, also called NORIT-A) while stirring and filtered.

#### Method:

Gels were removed from the glass tubes and soaked in fixative for 2 hours at room temperature. Following fixation the gels were stained in test tubes containing 0.5% periodic acid for another 2 hours. Subsequently, the gels were washed in 0.5% sodium arsenate-acetic acid for 30-60 minutes; in 0.1% sodium arsenate-acetic acid for 20 minutes (twice); and in 5% acetic acid for 10-20 minutes. The gels were then transferred to tubes containing 10 ml of Schiff's reagent and left to stain overnight at 4°C. After staining the gels were put in a flask containing  $\text{Na}_2\text{S}_2\text{O}_5/\text{HCl}$  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 staining tubes containing  $\text{Na}_2\text{S}_2\text{O}_5/\text{HCl}$ .

#### 2.7.3.2. Coomassie blue staining

##### Materials:

i. Fixative: 25% isopropylalcohol in 10% acetic acid.

ii. Staining solution: 0.05% Coomassie brilliant blue.

iii. Destaining solution: 10% methanol in 10% acetic acid.

**Method:**

Gels were soaked in fixative for 2 hours 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 staining tubes containing destaining solution.

**2.7.4. Immunoprecipitation of receptor material from detergent solubilized membranes**

**Method:**

Tritiated membrane preparations ( $2 \times 10^5$  to  $1.5 \times 10^6$  cpm), labelled by the periodate- $\text{NaB}^{3}\text{H}_4$  technique were solubilized in 12 mM sodium deoxycholate (final concentration) and then incubated with 90  $\mu\text{g}$  of antibody (polyclonal rabbit anti-idiotypic, antiglycophorin, or preimmune serum) for one hour at room temperature. 50  $\mu\text{l}$  of Sepharose protein A (supplied by Pharmacia as 1 mg protein A/ml in 0.9% NaCl) was next added and incubated for 2 hours at room temperature. Beads were collected by centrifugation in a Beckman microfuge for 2 minutes, washed three times in solubilization buffer (0.25% Nonidet, 0.5% Triton X-100 in 0.02 M phosphate buffer pH 8.0) and then twice in phosphate buffered saline. The pellets (about 30  $\mu\text{l}$ ) were mixed with an equal volume of Laemmli sample buffer and boiled for 5 minutes. After boiling, the mixture was centrifuged again in a Beckman microfuge for 2 minutes. The supernatant was collected with a pipette and 1  $\mu\text{l}$  of this sample was counted using a liquid scintillation system; the rest of the sample was analysed by electrophoresis on a 10% acrylamide slab gel using the Laemmli system (see section 2.7.2). Following fluorography using Enhance (supplied by NEN) according to the supplier's instructions, dried gels were exposed to Kodak X-Omat RP film at -70°C for 5 weeks. After exposure the film was processed according to the procedure supplied by Kodak (given on each

box containing Kodak films) and then examined visually for the presence or absence of bands.

### 2.7.5. Enzyme treatment of membrane preparations

#### Method:

Membrane preparations (100  $\mu$ l) of Krebs, HeLa, K562 and red blood cells labelled by the periodate-NaB<sup>112</sup>I<sub>4</sub> technique, were incubated with 5  $\mu$ l of neuraminidase (activity 1 RU/ml; supplied by Calbiochem-Bering Corp., USA) or 20  $\mu$ l of 2% trypsin (8575 BAEE units/mg solid; Sigma) for 1 hour at 37°C. After incubation, samples were added to an equal volume of Laemmli sample buffer and run on a SDS/PAGE gel as described above (see 2.7.2.). Following electrophoresis, the tube gels were cut into 1 mm fractions (using a Gilson gel cutter) and the radioactivity in each fraction was measured in a scintillation counter.



## Chapter 3

# RESULTS

### 3.1. Receptor characterization by affinity chromatography

#### 3.1.1. Properties of EMC virus-Sepharose column

To determine how efficiently EMC virus can be covalently linked to Sepharose,  $^3\text{H}$ -amino acid labelled EMC virus of known radioactivity was coupled to cyanogen bromide-activated CH-Sepharose 4B as described in section 2.5. Then the total labelled virus-Sepharose conjugate was transferred to a vial containing scintillation fluid and counted for radioactivity. About 80% of the radioactivity remained covalently linked to the Sepharose even after washing with  $\text{NaHCO}_3$  buffer pH 8.3 and sodium acetate buffer pH 4.0 containing 0.5 M NaCl.

A non-labelled virus-Sepharose conjugate was prepared and transferred in 0.02 M sodium phosphate buffer pH 8.0, to a 0.7 cm internal diameter glass column. The void volume of this column was 3 ml as determined by applying 1 ml of 0.2 mg/ml DNA or 1 ml of a solution containing 0.1 mg DNA and 0.1 mg adenosine (final concentrations) to the column, and measuring the absorbance (at 260 nm) of the eluate fractions collected without allowing any time for adsorption to take place (figure 3-1).

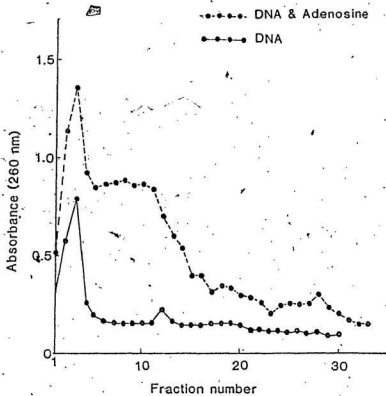
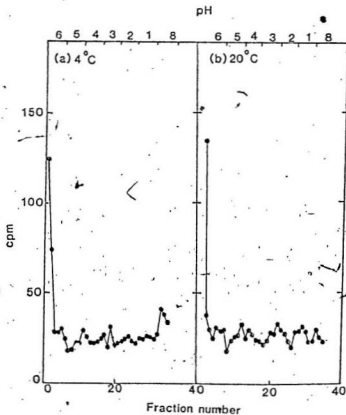


Figure 3-1: Void volume of an EMC virus-Sepharose column

EMC virus is known to be unstable and loses its infectivity at 37°C in the presence of halide ions between pH 5 and 7 (Speir, 1962; Young, 1986). Because the procedure used to couple EMC virus to Sepharose involved several alternate washes in NaHCO<sub>3</sub> buffer pH 8.3 and sodium acetate buffer pH 4.0 at room temperature (20-25°C), it was considered necessary at the beginning to show that virus linked to Sepharose was not inactivated nor was its ability to bind receptor material adversely affected by the pH or temperature at which the column was prepared or stored. Stability of the virus-Sepharose column between pH 1 and 8 was examined by first washing the column prepared with <sup>3</sup>H-labelled virus at room temperature with 0.02 M phosphate buffer pH 6.0 at a flow rate of 0.3 ml/min 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 using the same virus column the experiment was repeated at 4°C. Almost all the virus remained covalently-linked to the Sepharose (figure 3-2) and when the column was tested for its ability to bind radiolabelled glyco-phorin (tested by the procedure described below), compared to a similar virus-Sepharose column which was not subjected to variable pH washes, no significant difference was observed in percent binding.



**Figure 3-2:** Effect of various pH buffers at 4°C and 20°C on EMC virus coupled to Sepharose. A  $^3\text{H}$ -labelled virus-Sepharose column was washed with phosphate at various pHs and any virus released was detected by measuring radioactivity in fractions collected.

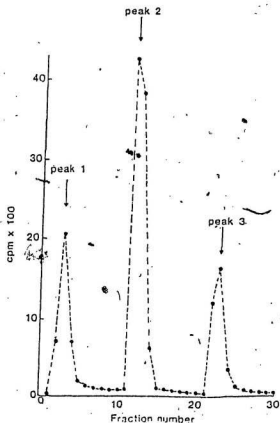
The receptor for EMC virus on human erythrocytes was reported to be glycophorin A (Allaway et al., 1986). To determine whether a virus-Sepharose column can bind the red cell receptor, glycophorin was extracted from human erythrocyte membranes (see section 2.4), labelled by  $^3\text{H}$ -acetylation and 2  $\mu\text{g}$  (equivalent to about 20000 cpm) of radiolabelled glycophorin was added to a 0.5 mg EMC virus column in 0.02 M phosphate buffer pH 8.0. After allowing 30 minutes for adsorption to take place, the column was washed with phosphate buffer until unbound material was fully removed. Then the column was washed with phosphate buffer containing 0.2 M NaCl followed by the same buffer containing 0.1% (1.6 mM) Triton X-100. Chromatographic runs were performed at flow rates of 0.3 ml/min at room temperature and fractions of 1 ml were collected for radioactivity measurements.

On average, the total recovery of radioactivity was about 78% of that added to the column of which about 25% of the recovered radioactivity was not retained on the virus-Sepharose column (peak 1, figure 3-3)

while 54% was retained and eluted from the column with phosphate buffer containing 0.2 M NaCl (peak 2) and 21% came off only when the column was washed with phosphate buffer containing 0.1% Triton X-100 (peak 3).

The presence of three peaks suggested several possibilities. The first possibility was that the retained material (peaks 2 and 3) was held back non specifically probably because separation occurred on the column according to size. If so, peaks 2 and 3 would be released by further washing with 0.02 M phosphate buffer pH 8.0 without the addition of 0.2 M NaCl or 0.1% Triton X-100. Another possibility was that the unbound material (peak 1) was not held back because the column was saturated. A third possible reason for obtaining three peaks was attributed simply to the fact that the peaks might be different because they contained different components of the original glycophorin applied to the column.

To test the first possibility, tritiated glycophorin was applied to the column



**Figure 3-3:** Chromatography of glycohorin on a 0.5 mg virus-Sepharose column. Two ug of  $^3\text{H}$  acetylated glycohorin in 0.02 M phosphate buffer pH 8.0 was added to the column and, following adsorption the column was washed with 0.02 M buffer, buffer containing 0.2 M NaCl, and then finally buffer containing 0.1% Triton X-100. Radioactivity, in fractions collected was measured.

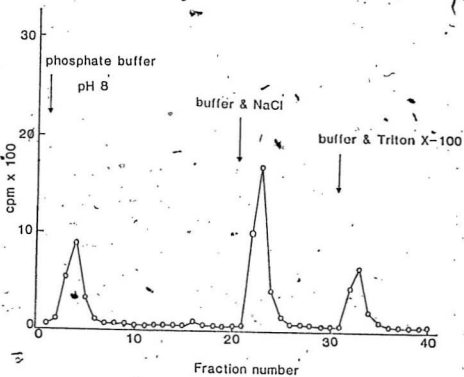


Figure 3-4: Chromatography of glycoprotein on a 0.5 mg virus-Sepharose column: effect of prolonged washing with 0.02 M phosphate buffer on the elution profile.

followed by extensive washing with 0.02 M phosphate buffer pH 8.0 (figure 3-4). As before (see figure 3-3), approximately 28% of the glycoprotein applied to the column was not held back; the rest was retained on the column and was eluted only when the column was washed with phosphate buffer containing 0.2 M NaCl or 0.1% Triton X-100. Prolonged washing with phosphate buffer alone did not effect elution of the retained material. This result showed that the three peaks obtained were not due to separation of glycoprotein on the virus-Sepharose column based on size and suggested that some other mechanism might be responsible.

The possibility that peak 1 was generated through column saturation was discounted by several findings. Firstly, increasing the amount of tritiated glycoprotein added to the 0.5 mg virus-Sepharose from 2 to 8  $\mu$ g resulted in relatively more glycoprotein binding to the column but the pattern in terms of percent glycoprotein coming off or retained on the column, remained essentially unaltered (figure 3-5). This result indicated that the column was not saturated since it could accommodate more than 2  $\mu$ g of glycoprotein.

Further evidence that peak 1 did not arise through column saturation was obtained when attempts were made to determine the maximum capacity of the column. Various amounts (50 to 200  $\mu$ g) of non radioactive ("cold") glycoprotein were mixed with 2  $\mu$ g of  $^3$ H-labelled glycoprotein. Each mixture (comprising a specific amount of "cold" glycoprotein plus 2  $\mu$ g of radiolabelled glycoprotein) was applied to the virus column and allowed 30 minutes for adsorption to occur. The column was washed with phosphate buffer, phosphate buffer containing NaCl and phosphate buffer containing Triton X-100. The column was regarded as saturated with glycoprotein when it failed to retain most of the radioactive glycoprotein. The 0.5 mg virus-Sepharose column accommodated up to 200  $\mu$ g of glycoprotein (figure 3-6), confirming previous observations that the column was not saturated by 2  $\mu$ g of glycoprotein (see figure 3-3) while also suggesting that peaks 1, 2 and 3 probably consist of different components of the receptor material.

Another method to determine whether peak 1 arose through column saturation



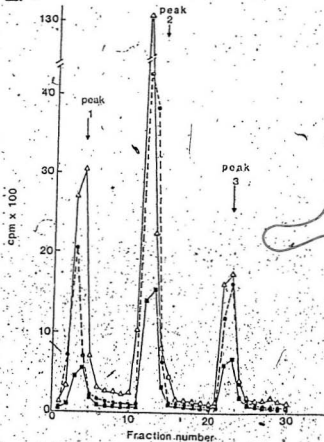
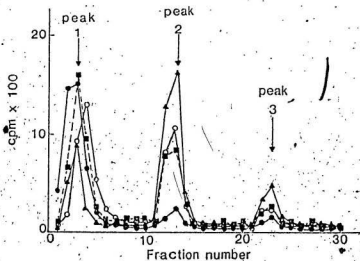


Figure 3-5: Chromatography of glycoflavin on a 0.5 mg virus-Sepharose column: effect of varying the concentration of glycoflavin on the elution profile. ■—■ 2 ug; ●—● 5 ug; △—△ 8 ug of glycoflavin.



**Figure 3-6:** Determination of the maximum capacity of a 0.5 mg virus column. The column was saturated with non-radioactive glycyphorin (\*cold\*), mixed with radioactive glycyphorin and regarded as saturated when no more radiolabelled glycyphorin bound to the column. ▲—▲ 2 ug <sup>3</sup>H glycyphorin. ○—○ 50 ug, ■—■ 100 ug, and ●—● 200 ug of \*cold\* glycyphorin.

was to test the effect of keeping the amount of glycophorin constant (2  $\mu$ g) but to increase the amount of virus covalently bound to the Sepharose. Therefore, virus-Sepharose columns containing 0.1 mg, 0.5 mg and 1.0 mg of purified EMC virus were prepared and 2  $\mu$ g of radioactive glycophorin was applied to each column. Following adsorption for 30 minutes each column was washed with phosphate buffer, buffer containing NaCl and the same buffer containing 0.1% Triton X-100. Then the binding profiles of the three columns were compared.

The results (figure 3-7) showed that regardless of the amount of virus coupled to the Sepharose (0.1 to 1.0 mg) the percent binding of glycophorin to the various virus columns did not vary significantly. Although increasing the amount of virus (for example, 0.1 to 0.5 mg) resulted in relatively more glycophorin (i.e.  $\mu$ g of protein) binding to the column, in each case 20-30% of the tritiated glycophorin was not retained on any of the columns and was washed off with 0.02 M phosphate buffer pH 8.0. Approximately 45-60% was retained and eluted with phosphate buffer containing 0.2 M NaCl while 15-25% was retained but eluted only with phosphate buffer containing 0.1% Triton X-100. These results suggest that peak 1 was not generated through saturation of the column.

To check whether peak 1 arose because it contained material different from that in peaks 2 and 3, peak 1 was collected and reappplied to the virus-Sepharose column. It was found that over 90% of this material was also not retained in the second run but eluted in the void volume which implied that peak 1 contained material that did not bind to the virus. In contrast, when material in peak 2 was reappplied to the column, about 80% was held back after the column was washed with phosphate buffer although the material differed from the first run in that 80% was released from the column only when washed with 0.02 M phosphate buffer containing 0.1% Triton X-100 and phosphate buffer containing 0.2 M NaCl eluted only 0% of this material. This implied that peak 2 contained material which bound to the virus but when eluted from the column this material became aggregated causing it to come off only in the presence of phosphate buffer containing detergent. About 60% of the material in peak 3 was not held back

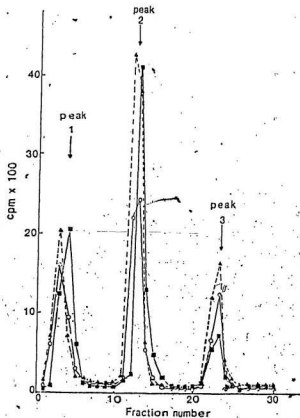


Figure 3-7: Chromatography of glycoprotein on EMC virus-Sepharose column: effect of varying the concentration of virus on the elution profile.

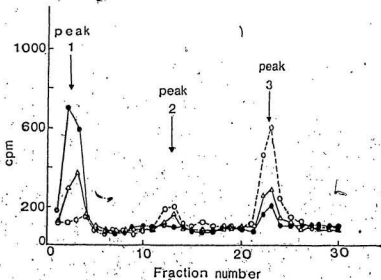
○—○ 0.1 mg; ▲—▲ 0.5 mg;  
 ■—■ 1.0 mg of virus.

when reappplied to the column and over 30% of this material could be eluted only with phosphate buffer containing Triton X-100 (figure 3-8).

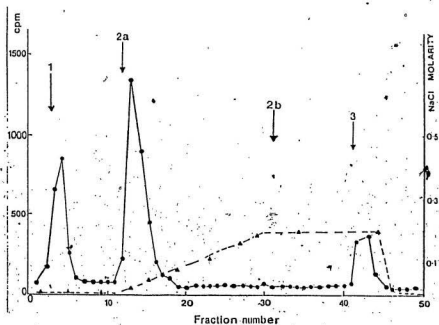
In order to determine the least concentration of NaCl required to break the glycophorin-virus bond, glycophorin bound to virus on the Sepharose column was eluted using a salt gradient of 0-0.2 M NaCl in 0.02 M phosphate buffer pH 8.0. The concentration of NaCl was determined from a standard curve of known NaCl molarity measured on a conductivity bridge (Model 31, Yellow Springs Instruments Co., USA). Conductivity readings obtained with phosphate buffer without any NaCl were subtracted from the results of phosphate buffer containing NaCl. The result shows that glycophorin attached to EMC virus-Sepharose was released by 0.02 to 0.05 M NaCl (figure 3-9). When this experiment was repeated in phosphate buffer containing 0.02 M NaCl and the same buffer containing 0.05 M NaCl, over 30% of the glycophorin attached to virus linked to Sepharose was eluted with phosphate buffer containing 0.05 M NaCl (peak 2b). A small fraction, less than 20% was eluted when the column was washed with phosphate buffer containing 0.02 M NaCl (peak 2a) and about 25% (peak 3) was eluted with phosphate buffer containing 0.1% Triton X-100 (figure 3-10).

### 3.1.2. Analysis of glycophorin and erythrocyte membrane preparations

Since we were interested in characterizing the receptor for EMC virus on a nucleated cell by affinity chromatography, it was considered important to show first that the virus-Sepharose column could be used to isolate the known receptor for EMC virus on the human red cell membrane, glycophorin A (Allaway et al., 1986). In these investigations, it was essential to be able to identify the various components in the red cell surface membrane. Therefore glycophorins were extracted from human erythrocytes, labelled by <sup>3</sup>H acetylation and 10-20 µg of the radiolabelled glycophorin analysed by electrophoresis on SDS gels. When the gels were cut into 1 mm fractions and counted for radioactivity, the pattern obtained revealed the presence of at least four glycoproteins which have been identified by periodic acid-Schiff (PAS) reagent staining which detects

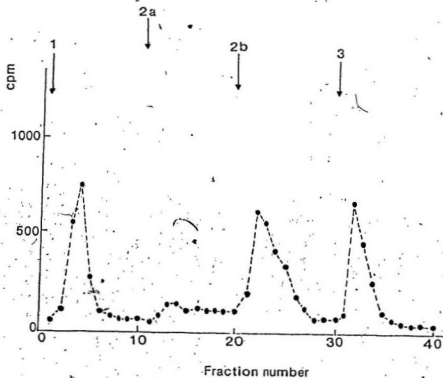


**Figure 3-8:** Chromatography of glycoprotein on a 0.5 mg. virus-Sepharose column: reapplication of peaks 1, 2, and 3 from previous runs. ●—● peak 1; ○--○ peak 2; ▲—▲ peak 3 from previous run.



**Figure 3-9:** Chromatography of glycephorin on a 0.5 mg virus-Sepharose column washed with 0 to 0.2 M NaCl gradient. Arrows indicate: 0.02 M phosphate buffer wash (1); 0 to 0.2 M NaCl gradient (2a); 0.2 M NaCl wash (2b); phosphate buffer containing  $0.1\% \text{ Triton X-100}$  (3).

●—●  $^3\text{H}$  glycephorin;  
 ▲---▲ NaCl gradient.

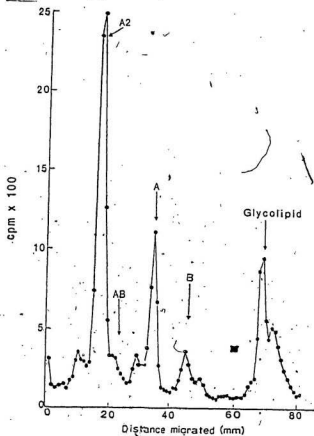


**Figure 3-10:** Chromatography of glycoprotein on a 0.5 mg virus-Sepharose column washed with 0.05 M NaCl. Arrows indicate: phosphate buffer wash (1); 0.02 M NaCl wash (2a); 0.05 M NaCl wash (2b); phosphate buffer containing 0.1% Triton X-100 (3).

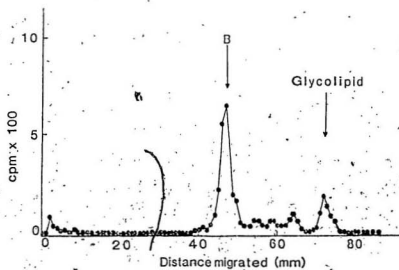


glycoproteins (Fairbanks et al., 1971). The peaks designated A2, A, B and A<sub>1</sub> (figure 3-11) represent the dimer and monomer forms of glycoprotein A, the monomer form of glycoprotein B, and a hetero-dimer comprising one molecule each of glycoprotein A and glycoprotein B, respectively (Anstee, 1981). The glycolipids ran as a major peak at the electrophoretic front where all molecules of 10 Kd or less, including breakdown products are likely to be found (Dunker and Rueckert, 1971). It is noteworthy that even in the presence of sodium dodecyl sulphate, glycoproteins tended to aggregate and were present as homo- and hetero-dimers. In order to identify the components of the various peaks obtained when glycoprotein was applied to the virus-Sepharose column, material in peaks 1 and 2 (column eluates) were each combined and following dialysis and lyophilization, analysed by electrophoresis on SDS gels. The results show that peak 1 contained material which ran in the region of glycoprotein B (figure 3-12) whereas peak 2 (which is material retained on the column and eluted with buffer and NaCl) showed the presence of both glycoproteins A and B (figure 3-13). This result implied that both glycoproteins A and B can bind to EMC virus.

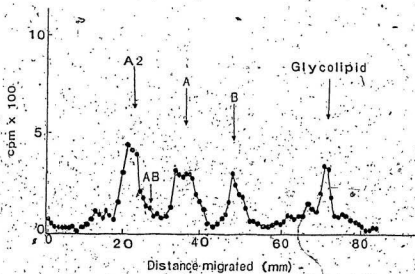
Glycoprotein is present as dimers or trimers even in the presence of SDS (see figure 3-11; Pardoe and Burness 1981). However, in the presence of sodium deoxycholate (DOC) some of the dimer glycoprotein A2 is converted to the monomer glycoprotein A as shown in figure 3-14. To discount the possibility that glycoprotein B was retained on the column simply by forming an aggregate with the receptor glycoprotein A, it was necessary to ensure that the glycoproteins were in monomeric form by adding DOC in order to disrupt aggregates. However the DOC had to be used at a concentration which was sufficient to effect solubilization of the receptor without causing elution of glycoprotein from the virus column. To determine this concentration, a <sup>3</sup>H-labelled glycoprotein preparation was added to the column which was then washed with a linear 0 to 24 mM (1%) DOC gradient in phosphate buffer pH 8.0. The results show that at DOC concentrations above 6 mM over 90% of the glycoprotein previously retained on the column was eluted (figure 3-15) and at 6 mM or less most of this glycoprotein remained bound to the virus on the column.



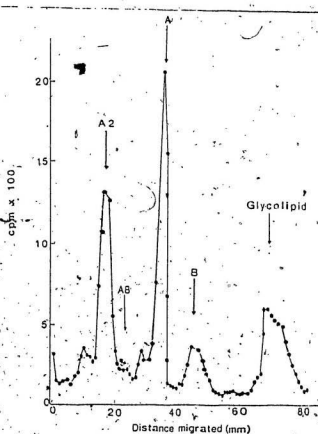
**Figure 3-11:** SDS-PAGE of a  $^3\text{Hl}$  acetylated glycophorin preparation. The gel was cut into 1 mm fractions and counted for radioactivity. Arrows indicate: dimer (A2) and monomer (A) forms of glycophorin A; monomer (B) of glycophorin B; hetero-dimer (AB) of glycophorins A and B; and the region containing glycolipids.



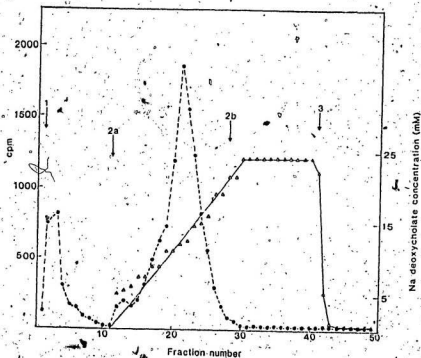
**Figure 3-12:** SDS-PAGE of peak I (eluate of glycoprotein on virus-Sepharose column). The gel was cut into 1 mm fractions and counted for radioactivity. Arrow (B) indicates region where glycoprotein B is normally present, based on fraction number.



**Figure 3-13:** SDS-PAGE of peak 2 (eluate of glycoprotein on virus-Sepharose column). The gel was cut into 1 mm fractions and counted for radioactivity. Arrows indicate the regions where various components of glycoprotein ran on parallel gels.



**Figure 3-14:** SDS-PAGE of glycophorin solubilized in 12 mM Na deoxycholate. The gel was cut into 1 mm fractions and counted for radioactivity. Arrows indicate the regions where various components of glycophorin ran on parallel gels stained with PAS and identified by comparison with Fairbanks et al., (1971).

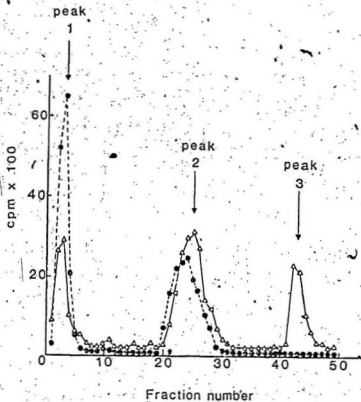


**Figure 3-15:** Chromatography of  $^3\text{H}$  acetylated glycephorin on a 0.5 mg virus-Sepharose column washed with 0 to 24 mM Na deoxycholate gradient. Arrows indicate: 0.02 M phosphate buffer wash (1); 0 to 24 mM deoxycholate gradient (2a); 24 mM deoxycholate wash (2b); 0.1% Triton X-100 in phosphate buffer (3).  
 ●---●  $^3\text{H}$  glycephorin;  
 ▲---▲ deoxycholate concentration.

Although the DOC disrupted the glycoprotein-virus bond at 12 mM concentration, it was found that diluting the detergent to 6 mM or less following solubilization in 12 mM DOC allowed most of the glycoprotein to bind to virus-Sepharose giving profiles similar to those obtained in the absence of DOC (figure 3-16).

To determine if DOC affected the distribution of components,  $^{131}\text{I}$  labelled glycoprotein was solubilized in 12 mM DOC, diluted 1:2 in 0.02 M sodium phosphate buffer pH 8.0 and then applied to the virus column. The column was washed with 0.02 M sodium phosphate buffer pH 8.0 containing 6 mM DOC to elute peak 1 followed by a wash in the same buffer containing 0.05 M NaCl in the presence of 6 mM DOC which eluted peak 2. The two peaks were collected separately and after dialysis and lyophilization, material in each peak was mixed with 10  $\mu\text{g}$  of  $^{125}\text{I}$  labelled glycoprotein and then analysed by electrophoresis on SDS gels. Using the  $^{125}\text{I}$  glycoprotein as a marker, it was found that material in peak 1 ran in the region corresponding to glycoprotein B (figure 3-17) while that in peak 2 ran in the positions expected for monomeric forms of both glycoproteins A and B (figure 3-18).

This result was similar to that shown in figure 3-13 and suggested again that not only glycoprotein A bound to EMC virus-Sepharose which was expected, but that glycoprotein B also bound even under conditions where the possibility of aggregation had been eliminated by including DOC throughout.



**Figure 3-16:** Chromatography of glycoprotein solubilized in 12 mM Na deoxycholate on a 0.5 mg virus-Sepharose column. ●-● glycoprotein with deoxycholate; △-△ glycoprotein without deoxycholate.



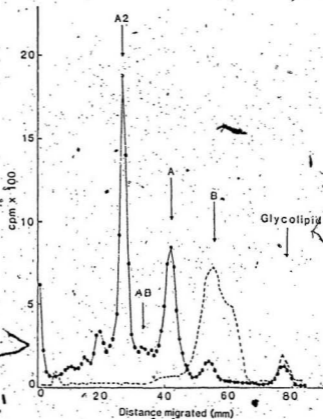
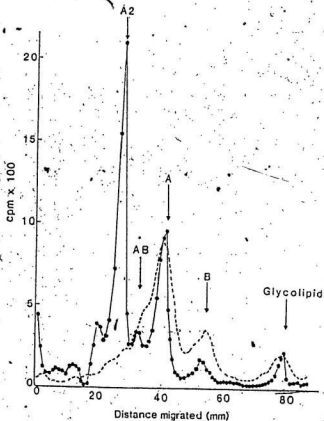


Figure 3-17: SDS-PAGE of glycoforin combined with peak 1 (eluate of glycoforin on virus-Sepharose column). Gels were cut into 1 mm fractions and counted for radioactivity. Arrows indicate regions where various components of glycoforin ran on parallel gels. ●—●  $^{125}\text{I}$  glycoforin; ---  $^{131}\text{I}$  labelled peak 1.



**Figure 3-18:** SDS-PAGE of glycohorin combined with peak 2 (eluate of glycohorin on virus-Sepharose column). Gels were cut in 1 mm fractions and counted for radioactivity. Arrows indicate regions where various components of glycohorin ran on parallel gels. ●—●  $^{125}\text{I}$  glycohorin; ---○---  $^{131}\text{I}$  labelled peak 2.

A glycophorin preparation which had been separated in this laboratory by gel filtration on a Bio-Gel A-1.5m column into three peaks reported to contain predominantly glycophorin A (peak A), glycophorin B (peak B) and other components (peak C), respectively (Furthmayr, 1978) was kindly given to me by Ms. Ingrid Pardoe. Small samples of peaks A and B were analysed by SDS gel electrophoresis and it was confirmed that the two contained predominantly glycophorins A and B, respectively (L. Pardoe, personal communication). The remaining material in each peak was labelled separately by  $^3\text{H}$ -acetylation along with some of the original glycophorin that was used for separation on the Bio-Gel column. About 2  $\mu\text{g}$  of  $^3\text{H}$ -labelled peak A was solubilized in 12 mM DOC for 30 minutes at room temperature, diluted to 6 mM and applied to the virus-Sepharose column which was then washed with phosphate buffer containing 6 mM DOC followed by a wash in sodium phosphate buffer containing 6 mM DOC and 0.05 M NaCl. A similar experiment was performed using about 2  $\mu\text{g}$  of  $^3\text{H}$ -labelled peak B. Peak A contained material which was not retained on the virus-Sepharose column (peak 1, figure 3-10) and material which bound to the column (peak 2). Similarly, peak B contained both peaks 1 and 2 (see figure 3-10). These results implied that both glycophorins A and B bound to EMC virus coupled to Sepharose.

To isolate the receptor for EMC virus from the red cell membrane, first, erythrocyte membrane preparations were labelled by the periodate- $^3\text{H}$ -borohydride method which labels glycosylated material (Gahnberg and Andersson, 1977). Some of the  $^3\text{H}$ -membranes was analysed by SDS-PAGE to make sure the glycophorins were labelled. Then the  $^3\text{H}$ -labelled membranes were solubilized in 12 mM DOC and diluted 1:2 after which they were applied to the virus-Sepharose column and the column was washed as described for peak A immediately above. Peak 1 (material from red cell membranes not retained on the column) and peak 2 (material from red cell membranes retained on the column) were dialysed and lyophilised separately and then analysed by SDS-PAGE. The results showed that peak 1 contained material which corresponds to glycophorin B on a parallel gel containing  $^3\text{H}$ -labelled human erythrocyte membranes while peak

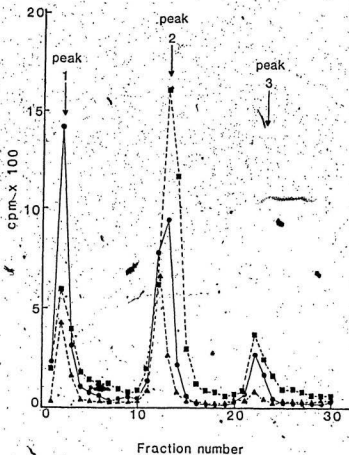
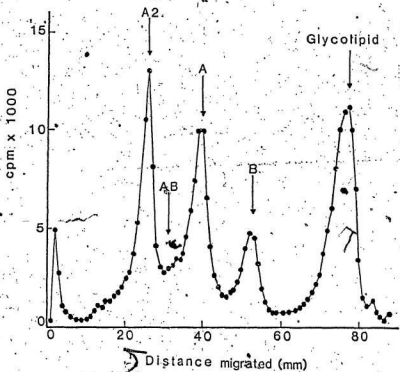


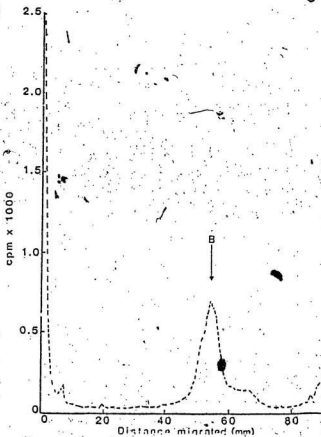
Figure 3-10: Chromatography of  $^3\text{H}$ -acetylated glycoprotein A and glycoprotein B preparations solubilized in 12 mM Na deoxycholate on virus-Sepharose column.

- original glycoprotein;
- glycoprotein A;
- △--△ glycoprotein B.

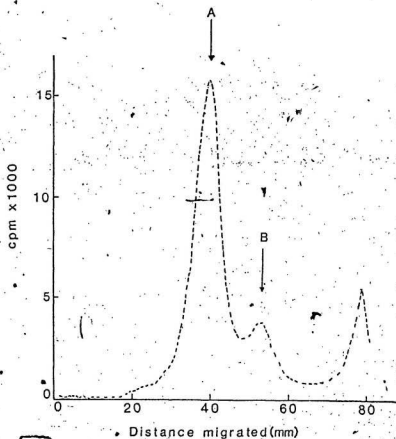
2 contained material which corresponds with glycoprotein A and glycoprotein B (see figures 3-20, 3-21, 3-22). As before (see figure 3-10) these results indicated that both glycoproteins A and B bound to EMC virus coupled to Sepharose.



**Figure 3-20:** SDS-PAGE of  $^3\text{H}$ -borohydride labelled human erythrocyte membranes solubilized in 12 mM Na deoxycholate; The gel was cut into 1 mm fractions and counted for radioactivity. Arrows indicate regions where various components of glycophorin are normally present.



**Figure 3-21:** SDS-PAGE of peak 1 (eluate of detergent solubilized erythrocyte membranes on virus column). The gel was cut into 1 mm fractions and counted for radioactivity. Arrow (B) indicates the region where glycoprotein B is normally present.



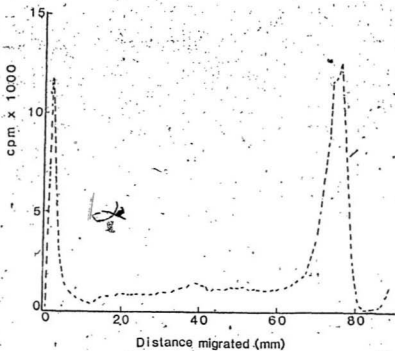
**Figure 3-22:** SDS-PAGE of peak 2 (eluate of detergent solubilized erythrocyte membranes on virus column). The gel was cut into 1 mm fractions and counted for radioactivity. Arrows (A) and (B) indicate the regions where glycoporphins A and B are normally present.



### 3.1.3. Analysis of Krebs cell membrane preparation

To isolate the receptor for EMC virus from the Krebs cell, first, membrane preparations were labelled by the periodate- $^{32}\text{P}$ -borohydride method (Gähmberg and Andersson, 1977) and then analysed by SDS-PAGE on 10% gels. Two major peaks were detected, one of low electrophoretic mobility at the top of the gel and the other at the bottom of the gel (figure 3-23).

Next, the  $^{32}\text{P}$ -labelled Krebs membranes were solubilized in 12 mM-DOC and diluted 1:2 after which they were applied to the virus-Sepharose column and the column was washed as described for peak A above (section 3.1.2). Peak 1 (material from Krebs membrane not retained on the column) and peak 2 (material from Krebs membrane retained on the column) were analysed by SDS-PAGE. The results showed that the peak at the top of the gel was missing following SDS-PAGE analysis of peak 1 (figure 3-24), whereas the material that was retained showed a peak at the origin of the 10% gel indicating the presence of a high molecular weight material which binds to virus (figure 3-25).



**Figure 3-23:** SDS-PAGE of  $^3\text{H}$ -borohydride labelled Krebs cell membranes solubilized in 12 mM Na deoxycholate. The gel was cut into 1 mm fractions and counted for radioactivity.

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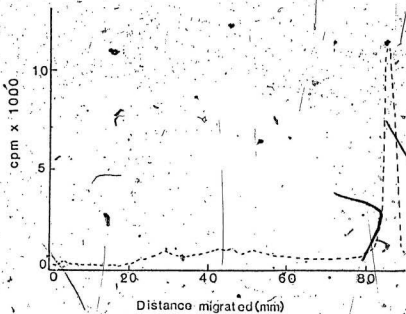
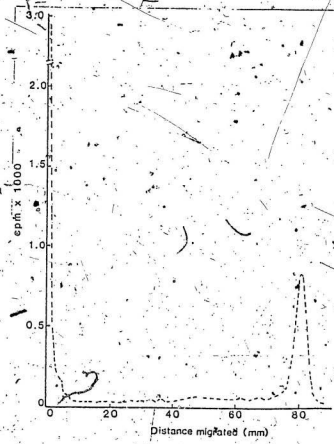


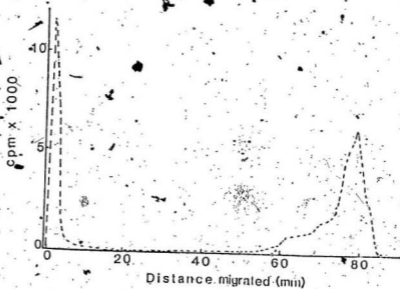
Figure 3-24: SDS-PAGE of peak 1 (eluate of solubilized  $^3\text{H}$ -borohydride labelled Krebs membranes on virus column). The gel was cut into 1 mm fractions and counted for radioactivity.



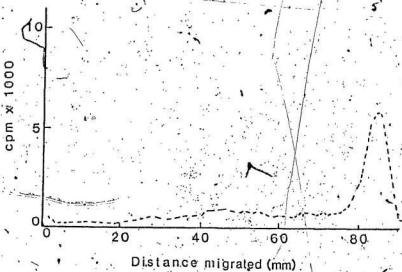
**Figure 3-25:** SDS-PAGE of peak 2 (eluate of solubilized <sup>3</sup>H-borohydride-labelled Krebs membranes on virus column). The gel was cut into 1 mm fractions and counted for radioactivity.

Neuraminidase treatment of human erythrocytes releases sialic acid and prevents EMC virus from attaching to treated red cells (Berness and Pardoe, 1983). To determine whether the high molecular weight material present in peak 2 above (material from Krebs membrane retained on the column) contained sialic acid, periodate-<sup>3</sup>H-borohydride labelled Krebs cell membrane preparations were treated with *Vibrio cholerae* neuraminidase as described in section 2.7.5. and then analysed by SDS-PAGE. The results (compared to untreated <sup>3</sup>H-labelled Krebs membranes analysed on SDS gels) showed that neuraminidase treatment had no effect on either of the two major peaks (Figure 3-26). This result suggested that the high molecular weight material present in peak 2, which appeared at the top of the gel, may be non sialylated.

To analyse the nature of the peaks obtained further, a similar experiment was performed using periodate-<sup>3</sup>H-borohydride labelled Krebs membrane preparations treated with trypsin. After SDS-PAGE analysis, the results showed that treatment of the Krebs membranes with trypsin completely eliminated the peak at the top of the gel (Figure 3-27) indicating that peak 2 was protein.



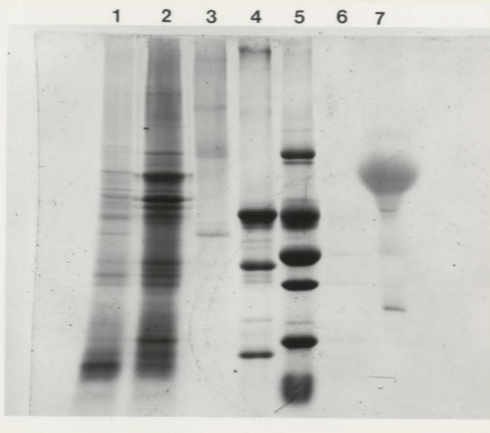
**Figure 3-20:** Effect of neuraminidase on <sup>3</sup>H-borohydride labelled Krebs membranes. The membranes were incubated with neuraminidase, collected by centrifugation and analysed by SDS-PAGE. The gel was cut into 1 mm fractions and counted for radioactivity.



**Figure 3-27:** Effect of trypsin on <sup>3</sup>H-prohydroxide labelled Krebs membranes: The membranes were incubated with trypsin, collected by centrifugation and analysed by SDS-PAGE. The gel was cut into 1 mm fractions and counted for radioactivity.

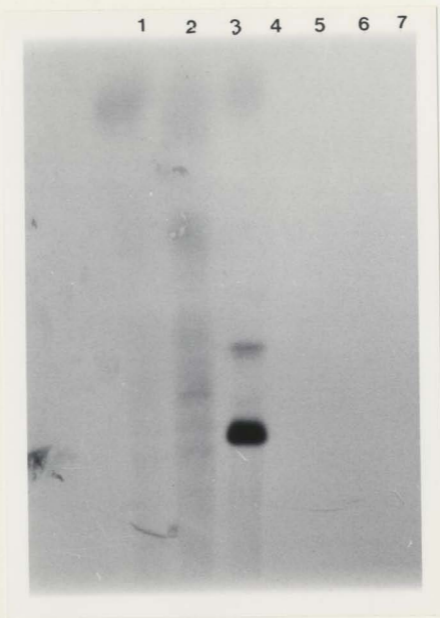
In order to determine the size of the high molecular weight material in peak 2 (material from Krebs membrane retained on the column), SDS-PAGE analysis of the retained material was repeated using this time a 5-20% SDS polyacrylamide gel and stained for protein with Coomassie blue followed by autoradiography. Three protein bands were revealed by Coomassie staining (lane 6, figure 3-28). However, no band was seen when the gel was exposed to Kodak X-Omat RI film at 70°C. for three weeks whereas the  $^3\text{H}$ -borohydride labelled membranes prepared from Krebs, 1562 and red cells showed several bands (figure 3-29).





**Figure 3-28:** SDS-PAGE of membranes on 5-20% gradient gels stained with Coomassie blue. Lanes 1 to 3 contain  $^3\text{H}$ -borohydride labelled K562, Krebs and RBC membranes respectively; lanes 4 and 5 non radiolabelled protein markers; lane 6, peak 2 (eluate of solubilized  $^3\text{H}$ -Krebs membrane on virus-Sepharose column); lane 7 contains non radiolabelled glycophorin.

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**Figure 3-29:** Autoradiogram of the 5-20% gradient gel shown in figure 3-28.

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## Chapter 4

# RESULTS

### 4.1. Receptor characterization using antiidiotypic antibodies

#### 4.1.1. Activity and specificity of anti-EMC serum

Anti-EMC virus antiserum was tested for its ability to inhibit virus haemagglutination. Biological activity (or specificity) was also checked using ELISA (section 2.0.1.2.) and the Ouchterlony (gel diffusion) test (section 2.6.1.1.). Haemagglutination inhibition (HAI) and ELISA tests showed the anti-EMC immune serum had HAI titre of  $4 \times 10^3$  and ELISA activity of  $5 \times 10^4$  ELISA Units (EU) per ml (EU/ml was calculated by dividing the endpoint, i.e. a dilution of 1:2560, by the volume of the diluter 50  $\mu$ l and multiplying by 1000). The Ouchterlony test result showed a precipitin line between anti-EMC and EMC virus but not between anti-EMC and influenza virus, PBS or preimmune rabbit serum (figure 4-1), indicating that the anti-EMC immune serum was specific for EMC virus.

#### 4.1.2. Affinity purification of anti-EMC virus antibodies

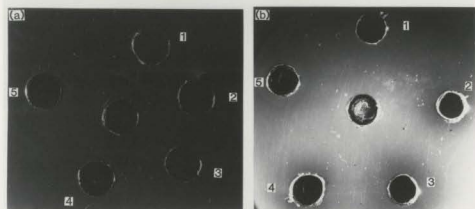
The purpose of this experiment was to get enough pure anti-EMC antibodies to make anti-idiotypic antibodies and purity rather than percent recovery was important. Therefore, anti-EMC antibodies were adsorbed on a virus-Sepharose column which was then washed successively with 0.02 M sodium phosphate buffer pH 8.0, 0.17 M glycine-HCl buffer pH 2.3, and 0.1% Triton X-100 in the same phosphate buffer. The fractions collected were each tested for anti-virus activity using ELISA and for protein concentration by measuring absorbance at 280 nm.

The results showed that material not retained on the column had a high protein concentration with high ELISA activity whereas anti-EMC antibodies specifically adsorbed on the column but released with glycine-HCl had high ELISA activity with low protein concentration, indicating that the virus-Sepharose column was able to purify the immune antibodies (figure 4-2).

#### 4.1.3. Biological activity and properties of anti-EMC antiidiotypic antibodies

Anti-idiotypic antibodies were produced by injecting affinity purified anti-EMC antibodies into two rabbits (see section 2.6.2.1). Because these anti-idiotypic antibodies should physiologically mimic the activity of EMC virus, they were subjected to tests to compare their biological activity with that of EMC virus.

Serum from samples of blood taken from both rabbits every week, beginning three weeks after immunisation with anti-EMC antibodies, were screened for biological activity by the haemagglutination test and the results obtained (not shown) showed no haemagglutination activity. However, when serum taken from one of the rabbits ten days after the ninth week (post immunisation) was tested for ability to agglutinate red cells, it was found that this serum contained anti-idiotypic antibodies which like the virus (Angel and Burness, 1977), agglutinated human (IIA titre 1:512) and sheep erythrocytes (IIA titre 1:256) but not bovine nor rabbit red cells. Anti-EMC and preimmune sera which were also tested as controls failed to agglutinate human, sheep, bovine or rabbit red cells.



**Figure 4-1:** Gel immunodiffusion test:  
 biological activity of anti-EMC  
 antibodies. The centre well in (a) contains  
 EMC virus while the centre well in (b)  
 contains influenza virus. Wells 1 to 3 in both  
 (a) and (b) contain anti-EMC, antiglycophorin  
 antiserum and preimmune serum, respectively.  
 Well no. 4 is empty and well no. 5  
 contains PBS.

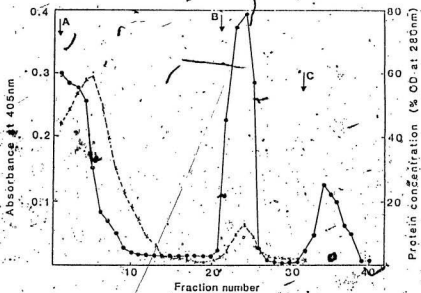


Figure 4-2. Chromatography of anti-EMC antibodies on an EMC virus-Sepharose column. Fractions collected were tested for ELISA activity and protein concentration. Arrows indicate start of wash with: 0.02 M phosphate buffer pH 8 (A); 0.17 M glycine-HCl pH 2.3 (B); phosphate buffer containing 0.1% Triton X-100 (C). ●-● ELISA activity; x--x protein concentration.

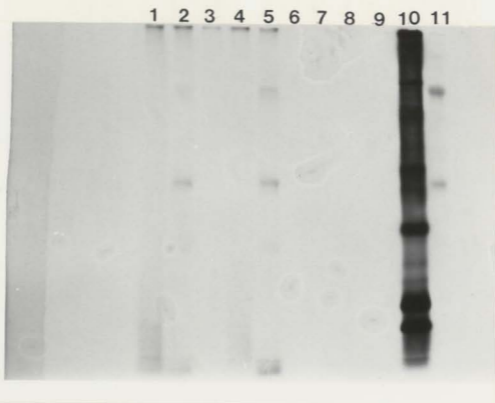
To determine whether these anti-idiotypic antibodies would, like the virus, recognize the reported receptor for EMC virus on the red cell, glycoporphin was immobilized by adsorption to nitrocellulose and tested with the anti-idiotypic antibodies in an immunobinding assay. This assay was qualitative (colour development) and was a modification of the ELISA test described in section 2.6.1.2, replacing micrititre plates with nitrocellulose as the support system. The results showed the anti-idiotypic antibodies and antiglycophorin antibodies recognized glycophorin whereas anti-EMC antibodies and preimmune serum failed to react with glycophorin.

To further establish that these anti-idiotypic antibodies react with the receptor for EMC virus on cell membranes, the immunoprecipitation test was performed as described in section 2.7.4. Periodate-<sup>3</sup>H-borohydride labelled Krebs and human erythrocyte membranes were solubilized in 12 mM DOC, diluted 1:2 and then incubated with the anti-idiotypic antibodies. The receptor-antibody complex was precipitated with Sepharose protein A and analysed by SDS-PAGE followed by autoradiography. The anti-idiotypic antibodies immunoprecipitated receptor material from human erythrocyte membranes solubilized in 12 mM DOC but no receptor material from solubilized Krebs cell membranes was detected (figure 4-3).

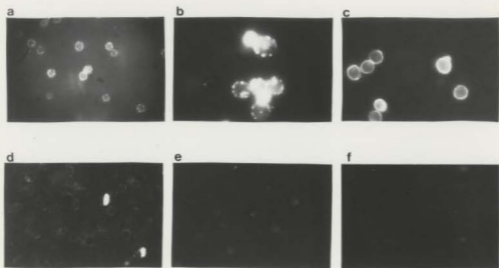
Since the anti-idiotypic antibodies like the virus recognized the red cell surface receptor for EMC virus, attempts were made to block virus infection of HEL cells by treating these cells with the antibodies prior to infecting the cells with EMC virus as described in section 2.6.2.2. After incubating the cells with virus overnight at 37°C, the cells were stained with trypan blue to check for cell death. The result (not shown) was that the anti-idiotypic antibodies failed to protect the HEL cells (over 80% cell death) from infection with EMC virus. Negative controls comprising (a) HEL cells plus antibody but without virus and (b) HEL cells alone, each showed less than 10% cell death while a positive control (HEL cells plus virus without antibody) showed over 80% cell death. Assuming enough antibody was used, this result suggested that the anti-idiotypic antibodies do not recognize the receptor for EMC virus on the HEL cell membrane. However, another explanation is the possibility that not enough antibody was used in the test.

Another method used to determine whether these anti-idiotypic antibodies recognize the receptor for EMC virus on cells that support virus growth was the fluorescent antibody test (see section 2.6.1.4). Immune and preimmune sera raised in rabbits were added to cells and after incubation followed by several washes, FITC-conjugated goat anti-rabbit antibodies were added. Following another incubation and several washes the cells were suspended in glycerol-PBS, mounted on a glass slide and examined with a fluorescent microscope. Fluorescent antibody test results showed that antiglycophorin antibodies reacted positively (i.e. gave fluorescence) with human erythrocytes, K562 and HEL cells while preimmune serum did not give any fluorescence. Compared to the antiglycophorin antibodies which were used as a control; anti-idiotypic antibodies reacted positively with human red cells but showed no fluorescence on K562 cells (figure 4-4). This result suggested that the anti-idiotypic antibodies did not recognize glycophorin on K562 cells.





**Figure 4-3:** Immunoprecipitation of receptors for EMC virus in solubilized membranes using anti-idiotypic antibodies. Lanes 1 to 4 contain  $^3\text{H}$ -borohydride labelled Krebs, RBC, HeLa and K562 membranes respectively with antiidiotypic antibodies; lane 5  $^3\text{H}$ -RBC with antiglycophorin serum; lane 10  $^{14}\text{C}$  labelled protein marker; lane 11  $^3\text{H}$ -acetylated glycophorin. Lanes 6 to 9 served as controls with preimmune serum.



**Figure 4-4:** Analysis of receptors for EMC virus on cells by fluorescence microscopy using goat anti-rabbit antibodies conjugated with FITC. (a) RBC; (b) K562 and (c) HEL with antiglycophorin. (d) RBC and (e) K562 with antiidiotypic antibodies. (f) RBC with preimmune serum.

## Chapter 5

# DISCUSSION

### 5.1. Affinity chromatography

#### 5.1.1. Stability of virus column

EMC virus-Sepharose columns used in this study were subjected to several washes in high and low pH buffers during preparation but were shown to be stable in the presence of low pH buffer. The virus-Sepharose column was used for several months at room temperature for repeated binding and elution of glycoprotein without any significant reduction in percent binding.

Previous reports have shown that poliovirus is relatively stable at 37°C in isotonic NaCl but rapidly loses both infectivity and antigenicity above 50°C in isotonic solutions (Speir, 1961a; Speir, 1961b). At low temperatures Mengo virus retains a high level of infectivity over a wide range of pH when diluted in 0.1 M phosphate buffer but loses its infectivity at 37°C in the presence of halide ions between pH 5 and pH 7 (Speir, 1962; Young, 1986). In phosphate buffered hypertonic salt, Mengo virus is inactivated at 0°C at low pH, but it is relatively stable at 56°C between pH 5 and 7 (Speir, 1962). The stability of EMC virus, which belongs to the same serotype as Mengo virus (Rueckerl, 1985), under conditions which inactivate or cause Mengo and Polio viruses to lose infectivity, is probably due to the rapidity of the procedure used when the virus-Sepharose was washed with buffers of pH 4 and 8.3. It is possible that because the virus-Sepharose was subjected to quick alternate washes with buffers of pH 4.0 and 8.3, the pH change occurred almost immediately so that the virus was not exposed at pH 5-7 for too long.

### 5.1.2. Analysis of glycophorin

In the present study, chromatography of glycophorin on virus-Sepharose columns revealed three peaks, designated peaks 1, 2 and 3. Three possibilities were considered for the origin of the peaks.

1. The retained material (peaks 2 and 3) was held-back non specifically probably because separation occurred on the column according to size.
2. The unbound material (peak 1) was not held back because the column was saturated.
3. The three peaks were different because they contained different components of the original glycophorin applied to the column.

Experiments showed that the appearance of several peaks was not a result of separation on the column according to size nor was it due to the column being saturated with glycophorin. Material in the various peaks were different based on their ability to bind EMC virus coupled to Sepharose. When the various peaks were reappplied to the virus column over 90% of the material in peak 1 was not held-back whereas material in peak 2, which was previously retained on the column and eluted with phosphate buffer containing NaCl, was retained the second time but could be eluted from the column only with phosphate buffer containing 0.1% Triton X-100. The behaviour of material in these peaks suggested that peak 2 bound specifically to virus-Sepharose but when eluted from the column following dialysis, lyophilisation and rechromatography, this material became aggregated causing it to come off only in the presence of phosphate buffer containing Triton X-100. In contrast to peak 2, peak 1 contained material which did not bind to EMC virus. Peak 3 probably also contained aggregated material which was trapped on the column and became solubilized in the presence of detergent. This argument is supported by evidence that glycophorin is present as monomers in 0.5% sodium deoxycholate or 0.1% Triton X-100 but in the absence of detergent as heterogeneous, large-sized aggregates (Parlor and Burmess, 1981). Experiments carried out in this laboratory using stepwise elution rather than gradient elution used here have shown that about 10% of glycophorin applied to a similar glycine-Sepharose column remained on the column and came off only when

the column was washed with phosphate buffer containing detergent (Allaway and Burness, in press).

It has been reported previously that only glycophorin A is involved in the attachment of EMC virus to red cells (Allaway and Burness, 1986; Allaway et al., 1986) based on the inability of EMC virus to attach to En(a-) erythrocytes, which although haematologically normal, lack glycophorin A but contain the other glycoproteins and gangliosides (Taliano et al., 1980). To test this, the components of the various peaks obtained when glycoprotein preparations were subjected to affinity chromatography on virus-Sepharose were analysed by SDS-PAGE. Peak 1 revealed material which ran in the region of glycoprotein B whereas peak 2 showed the presence of both glycoproteins A and B suggesting that glycoprotein A and glycoprotein B can serve as receptors for EMC virus. An alternative explanation was that glycoprotein B was probably aggregated on the receptor, glycoprotein A. This possibility was discounted by the results obtained following solubilization of glycoprotein and purified membranes preparations in 12 mM sodium deoxycholate, to prevent aggregation, before application on the virus column. SDS-PAGE analysis of peak 2 using solubilized membranes and glycoprotein preparations again showed the presence of both glycoproteins A and B. Furthermore, when glycoproteins A and B separated by gel filtration on a Biogel column were each separately applied to the virus column, both sin glycoproteins were retained. It was concluded that both glycoproteins A and B bind to EMC virus coupled to Sepharose and therefore probably serve as receptors for EMC virus.

The conclusion that only glycoprotein A can serve as the receptor for EMC virus based on the En(a-) results obtained can be misleading. First, the report showed that EMC virus attachment to En(a-) erythrocytes is not zero; 3% of the virus used at a virus/cell ratio of  $1.5 \times 10^7$  did attach to En(a-) cells (Allaway and Burness, 1986). The fact that some attachment occurred at all is significant and indicates that EMC virus can bind to red cells which lack glycoprotein A and possibly binds to glycoprotein B which is known to be present on En(a-)

erythrocytes. Besides, Mengo virus which is a *Cardiovirus* belonging to the same serotype as EMC virus and considered to be a strain of EMC (Rueckert, 1985), binds to En(a-) cells (Pardoe and Burness, personal communication) which shows that glycoprotein B can serve as a virus receptor, and particularly for a *Cardiovirus*.

Glycoprotein A isolated from human red cell membranes contains 131 amino acids and approximately 70 of the amino acids in the N-terminal of the molecule are external to the erythrocyte surface membrane (Burness, 1980). The  $\text{NH}_2$  termini of both glycoprotein A and glycoprotein B are identical in amino acid sequence and sites of glycosylation up to residue 26, (numbered from the  $\text{NH}_2$  terminus) while glycoprotein B lacks the segment containing amino acids 27 to 58 on glycoprotein A (Burness, 1980). Previous reports claim that EMC virus attached only to glycoprotein A and such attachment involves the region containing amino acids 35 to about 50 (Allaway and Burness, 1986). The same report argues that the lack of receptor activity in glycoprotein B suggested that the  $\text{NH}_2$ -terminal section of glycoprotein, including amino acids 1 to 25, is not involved in EMC virus attachment. However, both EMC and Mengo viruses bind to Dantu red cells (Pardoe and Burness, personal communication) which have fused glycoprotein B-A and do not contain the segment from amino acids 35 to 50 which were reported to be part of the site of attachment for EMC virus on glycoprotein A. This result indicates that EMC virus does not bind to glycoprotein A alone.

Further evidence to support the conclusion that glycoprotein B can serve as a virus receptor is the experiments reported in this thesis which have shown that when glycoprotein is applied to an EMC virus-Sepharose column, having eliminated the possibility of aggregation by detergent solubilization, the material retained on the virus column comprises glycoprotein A and glycoprotein B.

Material present in peak 1 (column eluate; analysed by SDS-PAGE) which acts like glycoprotein B has probably lost its ability to bind to virus because the sialic

acids are not in optimum position or this material is not in the ideal conformation for virus binding.

### 5.1.3. Analysis of Krebs cell membrane

Previous reports from this laboratory showed that SDS-PAGE of a purified Krebs membrane-derived glycoprotein, capable of inhibiting agglutination of human red cells by EMC virus, revealed a single PAS staining band near the origin of the gel with a molecular weight of 225000 (Sheppard, MSc Thesis 1980). This is consistent with findings reported in this thesis which showed that when Krebs membranes are solubilized in sodium deoxycholate and applied to the virus-Sepharose column, the material retained contains one or more components which is (are) found near the origin on 10% polyacrylamide during analysis by SDS-PAGE suggesting the presence of high molecular weight material which binds to virus.

The nature of this putative receptor was examined using enzymes. Because this peak was not sensitive to neuraminidase treatment which releases sialic acid but was sensitive to treatment with trypsin and since the Krebs membrane preparations were labelled by the periodate-<sup>3</sup>H-borohydride method which labels glycosylated material (see figure 3-20.), it was concluded that the receptor for EMC virus on Krebs cells may be a non sialylated glycoprotein. This result is in agreement with complementary experiments performed in this laboratory which showed that neuraminidase treatment of Krebs cells reduced attachment of <sup>3</sup>H EMC virus and infectivity only by 30% whereas similar treatment of red cells caused 90% reduction of attachment, indicating that sialic acid may not be as important in binding Krebs cells as to red cells. However, an alternative explanation of this result is that Krebs cells probably contain sialic acid but because these cells are of mouse origin their sialic acid might be resistant to *Vibrio cholerae* neuraminidase (Burness, 1980).

#### 5.1.4. Analysis of virus-receptor bond

Elution of material specifically retained on the virus-Sepharose column which presumably is the receptor was achieved using a linear 0.02 to 0.05 M NaCl in phosphate buffer rather than 0.2 M NaCl, suggesting involvement of weak ionic interactions between the virus and its cell surface receptor. In contrast EMC virus binds to glycophorin which itself is already attached to a wheatgerm agglutinin-Sepharose column in the presence of 0.1 M NaCl (Pardoe and Burness, 1980). A possible explanation for this contradiction in stability of the virus-red cell receptor bond to salt is that because of the covalent attachment of the virus to Sepharose the affinity of the virus for its receptor is reduced and this may account for the requirement of a lower concentration of NaCl to break the bond between glycophorin virus. A reduction in the affinity of virus for its receptor could also occur by steric hindrance or if coupling to Sepharose caused disruption of the virus.

It is significant to note that while elution of glycophorin from the column with detergent occurred at concentrations above 6 mM the same effect could not be achieved with NaCl at concentrations below 20 mM. This shows that the presence of Na ions at 6 mM is not, in itself, a sufficient condition for breaking the glycophorin-virus bond. It has been suggested that while elution of glycophorin by NaCl probably results from breakage of weak ionic bonds between virus and receptor, the much lower concentration of detergent required indicates involvement of hydrophobic bonding since the detergent concentration required for elution is similar to that which disrupts hydrophobic bonds between membrane components (Allaway and Burness, in press).

#### 5.2. Antiidiotypic antibody studies

As a first step in the preparation of anti-idiotypic antibodies, the biological activity and specificity of polyclonal anti-EMC serum was investigated. The HA1 titre of the anti-EMC immune serum was  $4 \times 10^3$  and the ELISA activity was  $5 \times 10^4$  ELISA Units/ml. The anti-EMC antibodies reacted positively (precipitin line)



with EMC virus but not with influenza virus as shown by gel immunodiffusion test, nor did the preimmune serum react with EMC virus. It was concluded that the anti-EMC serum contained antibodies specific for EMC virus.

Anti-EMC antibodies purified on the virus-Sepharose column showed peak (antibodies retained on the column and eluted with glycine-HCl pH 2.3) had high ELISA activity with low protein concentration indicating that purification did take place. The high level of ELISA activity detected in the peak not retained on the column could be due to saturation of the virus column with antibodies resulting in premature emergence of the antibodies.

The antiidiotypic antibodies immunoprecipitated glycoprotein from solubilized red cell membranes but no receptor material was detected from Krebs cells by the method used. This is probably because the number of EMC virus receptors on the Krebs cell are far less than those on the red cell. It is possible that the antiidiotypic antibodies recognized the radiolabelled receptor on both Krebs and red cells. But because the number of receptors on the Krebs cell are few, the amount of radiolabelled receptors immunoprecipitated from the Krebs cell were insufficient for detection compared to those on the red cell.

Another possible explanation is simply that although the antiidiotypic antibodies recognize the receptor for EMC virus on red cells they do not recognize the Krebs cell receptor. This implies that the attachment site for EMC virus on a host cell is different from that on a red cell.

K562 (Fukuda, 1980) and human red blood cells (Anstee, 1981) are reported to contain glycoprotein and EMC virus attaches to both cell types. Using the fluorescent antibody technique it was shown that the anti-idiotypic antibodies recognized the receptor for EMC virus on the human red cell but failed to bind to glycoprotein on K562 cells. This result might be explained by variation in the number of receptors for EMC virus between K562 cells and red cells. Apart from the quantity of glycoprotein present on the cell surface as receptors, the

carbohydrate structure and the configuration of the glycoprotein molecule may be an important factor in EMC virus attachment to cells containing glycoprotein. For instance glycoprotein A isolated from mature erythrocytes contains 15 O-linked glycosidic oligosaccharides whereas glycoprotein A isolated from K562 cells contain a relatively small amount of O-linked oligosaccharides.

Further experiments need to be carried out in order to answer several questions raised in the present study. For example,

1. do Krebs cells contain sialic acid which is resistant to *Vibrio cholerae* neuraminidase.
2. what is the number of receptors for EMC virus on host-cells compared to cells that do not support virus growth.
3. do K562 cells support EMC virus growth.
4. would antiglycoprotein block infection of K562 cells with EMC virus.

## Appendix A

### A.1. Media

#### A.1.1. Earle's saline growth medium

Earle's medium used to grow Krebs cells consists of 100 ml of 10X Earle's balanced salt solution (GIBCO, USA) supplemented with 10 ml inactivated horse serum, 100 units/ml penicillin and 10 units/ml streptomycin (final concentrations), 50 ml of 4.4%  $\text{NaHCO}_3$  saturated with  $\text{CO}_2$  and the volume was made up to 1 litre with sterile deionised water.

#### A.1.2. RPMI Medium 1640

Roswell Park Memorial Institute medium 1640 was prepared by adding 10.38 g of RPMI 1640 powder, (Flow laboratories Inc.) to about 800 ml of deionised water. HCl (1 M) was added gradually while stirring until all the powder was completely dissolved and by then the colour had turned from pink to yellowish. Two grams of sodium bicarbonate was added to the solution which was adjusted to pH 7.4 with 1 M NaOH. The volume was made up to 1 litre with deionised water and the medium filtered and stored at 4°C.

Foetal calf serum (10%), Heps (10mM), penicillin (100 units/ml) and streptomycin (10 units/ml), each at final concentration, were added just before use.

## A.2. Buffers

### A.2.1. PBS pH 7.3

Solution A: NaCl 40.0 g, KCl 1.0 g,  $\text{Na}_2\text{HPO}_4$  5.75 g and  $\text{KH}_2\text{PO}_4$  1.0 g were dissolved in 4 litres of deionised water. Solution B:  $\text{CaCl}_2$  0.5 g and  $\text{MgCl}_2$  0.5 g were dissolved in 1 litre of deionised water.

Solutions A and B were sterilized separately at  $121^\circ\text{C}$  for 20 minutes. When cooled, solution B was added to solution A and stirred vigorously. 500,000 units of penicillin and 50,000 units (1 mega unit) of streptomycin were added to the solution mixture which was then aliquoted into 100 ml amounts in screw capped bottles and stored at  $4^\circ\text{C}$ .

### A.2.2. Ca-Mg free PBS

Calcium and Magnesium free PBS was made up as for PBS and contains 10 g NaCl, 1.0 g KCl, 5.75g  $\text{Na}_2\text{HPO}_4$  and 1.0 g  $\text{KH}_2\text{PO}_4$  dissolved in 5 litres of deionised water. When used in the harvesting of Krebs ascitis tumour cells, EDTA (0.001 M, final concentration) was added to the Ca-Mg free PBS to prevent agglutination of ascitic fluid.

### A.2.3. 0.5 M Sodium phosphate buffer, pH 8.0

solution A: 70.98 g/L of  $\text{Na}_2\text{HPO}_4$  in deionised water.

solution B: 69.0 g/L of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  in deionised water.

Solution A was mixed with an equal volume of solution B to give a buffer solution of pH 8.0.

### A.3. Serum Treatment With Neuraminidase

Serum samples were treated with neuraminidase to remove any non-specific inhibitors present in the serum. For this purpose, 20 ul of anti-EMC serum was mixed with 100 ul of neuraminidase (activity 1 IU/ml) supplied by Calbiochem-Behring Corp., USA. The mixture was incubated for 18 hours at 37°C. After the incubation, 80 ul of veronal-glycose-gelatin was added to the mixture to bring the volume up to 200 ul representing a 1:10 dilution of the serum. This treated serum was used in the haemagglutination inhibition test.

### A.4. Preparation of reaction vessel for Iodination using Iodogen (Markwell and Fox, 1978)

200 ug of chloroglycoluril (available as Iodogen from Pierce Chemical Co.) was dissolved in 400 ul of chloroform. To each of a number of glass tubes was transferred 20 ul (i.e. 10 ug) of the Iodogen solution which was plated on the surface of the test tube by drying under a stream of N<sub>2</sub> gas at room temperature. Test tubes coated with Iodogen were stored in a dessicator at room temperature and used within six months.

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