IDENTIFICATION AND CHARACTERIZATION OF A NOVEL CLASS OF RECEPTOR FOR ENCEPHALOMYOCARDITIS VIRUS ON HUMAN CELLS

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YI-MING JIN

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL CLASS OF RECEPTOR FOR ENCEPHALOMYOCARDITIS VIRUS ON HUMAN CELLS

by

© Yi-Ming Jin, M.B.

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Abstract

Encephalomyocarditis (EMC) virus belongs to the genus cardiovirus of the picornavirus family. The virus is important in the etiological study of several virus-induced human diseases. However, little is known about EMC virus attachment to nucleated cells. In this thesis, the results on the characterization of the biochemical nature of EMC virus receptors on two human nucleated cell lines, K562 and HeLa cells, are presented. The study showed that EMC virus bound specifically to both cell types. The number of receptor sites on HeLa cells was in the range of 1.6×10^5 per cell and the dissociation constant for virus binding was 1.1 nM. These results are consistent with previously determined values for the binding of EMC virus to K562 cells. After trypsin digestion of K562 and HeLa cells, regeneration of virus binding activity was inhibited by cycloheximide treatment, suggesting that recovery of the EMC virus-specific receptor is dependent upon intracellular protein synthesis. Further, digestion with proteases and neuraminidase, as well as lectin treatment of intact cells, cell membrane preparations and detergent-solubilized cell membranes, demonstrated that receptors for the virus on both K562 and HeLa cells are sialoglycoproteins. Affinity chromatography employing EMC virus columns isolated 70-kD receptor proteins from K562 and HeLa cells. Virus overlay protein assay revealed that

EMC virus specifically recognized only the 70-kD proteins in both the purified receptor preparations and in detergent solubilized cell membranes, suggesting that virus attachment to K562 and HeLa cells could be exclusively mediated by the identified receptor molecules. Using the chromatofocusing technique, it was found that the receptor on K562 cells is likely more sialylated than that on HeLa cells. Finally, Western blot analysis using anti-glycophorin A antibody revealed that the antibody does not recognize the EMC virus 70-kD receptor on K562 or HeLa cells (the latter does not express glycophorin A). This indicates that the identified receptor proteins may not be glycophorin A, but they represent novel, not yet described EMC virus receptor molecules.

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List of Abbreviation and Symbols Used

BSA	Bovine serum albumin
CRS	Cellular receptor site
CRU	Cellular receptor unit
DMEM	Dulbecco's modified eagle medium
DOC	Sodium deoxycholate
EMC virus	Encephalomyocarditis virus
EDTA	Ethylenediaminetetraacetic acid
HBSS	Hanks' balanced salt solution
ICAM-1	Intercellular adhesion molecule-1
Iodogen	1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril
kD	Kilodalton
Kd	Equilibrium dissociation constant
LFA-1	Lymphocyte function-associated antigen-1
LPA	*Limulus polyphemus* agglutinin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
RPMI medium	Roswell Park Memorial Institute medium

SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
VAP	Viral attachment protein
VOPBA	Virus overlay protein blot assay
WGA	Wheat germ agglutinin

Chapter 1

Introduction

1.1. General Background

Perhaps, Beijerinck (1898) was the first person to use the term "virus" to define a kind of infectious agent which can pass through a bacteria-proof filter and retain infectivity. However, several years before Beijerinck's definition of a "virus", a similar phenomenon had already been observed by the Russian biologist, Iwanowski (Dimmock & Primrose, 1987).

Viruses have been found in every living creature, from single-celled bacteria to mammals. Viral nucleic acids are either DNA or RNA but not both. Viral nucleic acids contain all the necessary genetic information for viral replication. The viral capsid is made up of mutiple identical viral prot eins called capsomeres. The viral capsid encloses either an extended or a condensed nucleic acid core to form a basic viral structure called a nucleocapsid. Except for a few virus families, the complete infectious virus particle, or virion, has a lipid-containing membrane called an "envelope" enclosing its nucleocapsid. The virus acquires its envelope by budding through the host's cellular membrane. The envelope is modified by the insertion of viral glycoproteins into the cell membrane during virion maturation.

The oldest classification scheme for viruses is based on the clinical symptoms and histopathological characteristics of the diseases. Although this old scheme aids the clinicians in dealing with the symptoms and epidemiology of viral diseases, it is no longer satisfactory because many biologically unrelated viruses can cause indistinguishable symptoms in infected hosts. Currently, viruses are classified according to the sheme proposed by the "International Committee on Taxonomy of Viruses". According to this taxnomy, animal viruses are divided into 17 families on the basis of the nature of the genomic nucleic acid (RNA or DNA), mode of viral replication and structure of the virion (which refers to size, shape, symmetry of the capsid, and presence or absence of an envelope, etc.). Classifications of viruses within the family into genera and then species, types and strains are usually based on physico-chemical or serological differences.

By definition, viruses cannot be regarded as microorganisms, because they multiply only within living cells, which supply all the energy, reproductive machinery and molecular precursors. In general, the virus replication cycle includes attachment, penetration, uncoating, transcription, replication of viral nucleic acids, translation, assembly and release of new virions.

Many viruses, but not all, can be grown in cultured cells or fertile eggs. The growth of viruses in cultured cells or fertile eggs can be easily detected

by a variety of methods. Observation of cytopathic effects in cultured cells and death of the embryo, addition of an indicator virus to detect some non-cytopathogenic viruses, and hemagglutination assays will detect most viruses. Viruses can be quantitated by direct counting of the viral particles by electron microscopy or more often by biological titrations such as plaque and pock assays, and by immunoassays employing antibodies specific to viral antigens.

1.2. Picornaviruses and Encephalomyocarditis (EMC) Virus

Picornaviruses are the smallest RNA viruses, 20-30 nm in diameter. Nonenveloped and icosahedral, the virion contains a positive single-stranded RNA genome (Jackson, 1986; Luo, 1987; Chen *et al.*, 1989). The viral capsid is composed of 60 capsomeres, each capsomere containing 4 polypeptides VP1, VP2, VP3 and VP4, which are derived from a single precursor VP0 (Palmenberg & Rueckert, 1982). The linear viral RNA with a molecular weight of about $2x10^6$ daltons, is polyadenylated at the 3'-terminus and contains a small protein (Vpg) at the 5'- terminus (Armstrong *et al.*, 1972; Yogo & Wimmer, 1972; Lee *et al.*, 1977; Flanegan *et al.*, 1977). The infectious viral RNA can act as an mRNA and bind directly to ribosomes for protein translation without a prior transcriptional step. There are four genera in the

picornavirus family: enterovirus, cardiovirus, rhinovirus and aphthovirus (Rueckert, 1990).

Genus enterovirus includes polioviruses, coxsackieviruses, echoviruses, human enteroviruses and hepatitis A virus. The viruses in this genus are important human pathogens (Rueckert, 1990).

Cardioviruses include Columbia SK virus, encephalomyocarditis (EMC) virus, Maus Elberfeld virus, MM virus, mengo virus and Theiler's encephalomyelitis virus (Rueckert, 1990). However, since the viruses within the genus are serologically indistinguishable, they are very often considered to be strains of EMC virus (Rueckert, 1990). Rhinoviruses include human rhinoviruses with over 113 serotypes, and bovine rhinoviruses. Human rhinoviruses are one of the major etiologic agents of the common cold (Rueckert, 1990).

Aphthoviruses include several serotypes of the foot-and-mouth disease virus. These viruses are important in the agricultural economy since they cause a highly infectious disease in cloven-footed animals (Rueckert, 1990).

In this project, EMC virus has been used which was initially found in mice during the investigation of poliomyelitis in 1943 (Jungeblut & Dalldorf, 1943). Although the virus is generally regarded as a murine virus, it has been detected in many other species including swine, rhesus monkeys, wild

mongoose, mosquitoes, and humans (Acland & Littlejohns, 1978; Dea *et al.*, 1991; Dick *et al.*, 1948; Smithburn, 1948; Gajdusek, 1955). In mice, the virus causes severe encephalitis and myocarditis (Dick, 1949; Warren, 1965), whereas in humans, the infection appears as a mild febrile illness with central nervous system involvement (Warren, 1965). The virus is an important pathogen, since it causes various diseases in laboratory animals, that mimic several virus-induced human diseases, including insulin-dependent diabetes mellitus (Craighead & McLane, 1968), vasculitis (Burch & Rayburn, 1977), myocarditis (Gainer, 1974) and polymyositis (Miller *et al.*, 1987).

In most cases, as for other picornaviruses, replication of EMC virus in cultured cells will eventually lead to the release of new virions from lysed cells. There are several EMC variants, among them, the D variant of the virus causes an insulin-dependent diabetes mellitus (IDDM)-like syndrome in susceptible mice. This variant was isolated together with a non-diabetogenic B variant (Yoon *et al.*, 1977, Yoon & Notkins, 1983) from a pool of a myocardiotropic M variant (Craighead, 1966). For this project, an original virus strain (K_2) isolated from infected mice was used (Hoskins & Sanders, 1957).

1.3. Virus-Cell Interaction

The consequences of viral infection of a cell are various and they depend on the character of the invading virus and the type of targeted cell. A lytic infection results in lysis and death of the infected cell. In a nonlytic infection, a persistent infection may occur in which the infected cells may survive and produce progeny virus at low levels or a cell may become malignant. However, to initiate infection of a cell, the virus must attach to its receptor on the cell (Dales, 1973; Lonberg-Holm & Philipson, 1974; Lonberg-Holm, 1981; Tardieu & Weiner, 1982; Marsh & Helenius, 1989; Lentz 1990). There are two essential components involved in this initial interaction, the viral attachment protein (VAP) and the host cellular receptor. The VAP is located on the viral envelope for enveloped viruses or on the capsid for non-enveloped viruses, which mediates the attachment of the virus to the cell surface. The cellular receptor is a complementary structure to VAP, to which the virus binds, and is called the cellular receptor site (CRS) (Lonberg-Holm, 1981). However, because the CRS is often composed of multiple units, the term cellular receptor unit (CRU) is used to refer to these units (Lonberg-Holm, 1981). Viruses can bind to a cellular receptor directly or indirectly through an intermediate molecule which may be associated with the cell surface. Antibodies have been indentified as intermediate molecules for

attachment of several viruses to cells (Webster & Askonas, 1980; Peiris *et al.*, 1981; McGuire *et al.*, 1986). Polyalbumin and the receptor for polymerized human albumin apparently facilitates the binding of hepatitis B virus to hepatocytes (Imai *et al.*, 1979; Thung & Gerber, 1984). Virus cellular receptors can be any host cell surface molecule or even a viral protein produced by a previous virus infection. It was reported that infection of hepatitis delta virus requires the presence of an ongoing infection with hepatitis B virus (Rizzetto *et al.*, 1990), and cell surface influenza hemagglutinin mediates infection of other viruses to cells (Fuller *et al.*, 1985).

Following the interaction between VAP and its receptor, virus entry into the cytoplasm is usually by direct fusion with the plasma membrane or by receptor-mediated endocytosis (Marsh & Helenius, 1989). In the case of picornaviruses, the penetration into host cells is by receptor-mediated endocytosis (Crowell & Landau, 1983).

1.3.1. Viral Attachment Proteins

As mentioned before, viral attachment proteins are located either on the viral envelope or on the capsid. Recently, several such viral attachment

proteins have been described. For example, in reovirus, the VAP has been identified as the σ1 protein, the viral hemagglutinin (Bassel-Duby *et al.*, 1986). The specificity of this protein for recognition of cellular receptors was demonstrated by changing its receptor specificity by the replacement of glutamic acid with lysine at amino acid 419 on the VAP polypeptide (Bassel-Buby *et al.*, 1986). Similarly, a single amino acid change in the influenza virus hemagglutinin led to a change in receptor specificity (Paulson *et al.*, 1986). These findings not only reveal the high specificity of VAP but also suggest the involvement of secondary and tertiary structures in the determination of VAP specificity. In many cases, VAP possesses other functions besides binding to host cells. For example, influenza virus hemagglutinin participates in fusion and virus penetration into the cell (White *et al.*, 1986); the VAP of vesicular stomatitis virus, a sole glycosylated spike protein on the virus surface, can function as a hemolysin, hemagglutinin, and cytotoxin (Schlegel, 1986). Although some viruses such as influenza virus and HIV-1 have very high mutation rates, the binding domains of VAP appear to be conserved as long as the viruses maintain the same cell tropism (Lasky *et al.*, 1987; Weis *et al.*, 1988). The conserved nature of the binding domains of VAP provides a good target for intervention in viral infection.

Studies of picornavirus VAPs resulted in an important hypothesis. The researchers in this field were perplexed by the fact that several VAPs had been identified in other viruses, but not for picornaviruses. However, by using X-ray crystallography and nucleic acid sequence analysis, a deep "canyon" or "pit" was found in the viral capsid (Rossmann *et al.*, 1985; Hogle *et al.*, 1985; Luo *et al.*, 1987; Jenkins *et al.*, 1987). The deepness and narrowness of the "canyon" or "pit" makes access of antibodies to the floor of the "canyon" impossible. The amino acids lining the surface of the "canyon" show greater conservation than those at the surface, and allow viruses to retain their receptor specificity, while at the same time permit viruses to avoid the host's immune system by mutation of residues about the "canyon" rim (Rossmann & Palmenberg, 1988). The "canyon" hypothesis explains several failed attempts to isolate picornavirus receptors by using anti-idiotypic antibodies. However, unlike other picornaviruses, the foot-and-mouth disease virus receptor attachment site appears to be a projecting "loop" on its capsid instead of a "canyon" or "pit" (Acharya *et al.*, 1989). Therefore, the failure to isolate the receptor for the foot-and-mouth disease virus appears not to be explained by this mechanism (see section 1.5).

1.3.2. Plasma Membranes and Cellular Receptors for Viruses

1.3.2.1. Structure and Components of the Plasma Membrane

In most cases, virus receptors are cellular molecules located on the surface of the plasma membrane. The plasma membrane consists of three components, lipids, proteins and carbohydrates that form a fluid structure.

There are three major classes of lipids in the plasma membrane: phospholipids, cholesterols and glycolipids. The amphipathic properties of these lipids enable them to form a continuous bilayered structure, the basic structure of the membrane.

Plasma membrane proteins are embedded in the lipid bilayer or bound to each other. Membrane proteins play major roles in active transport, cellular signalling, membrane-associated enzymatic catalysis, etc. Many membrane proteins are termed transmembrane proteins because their polypeptide chains pass through the lipid bilayer. Membrane proteins can also bind to the lipid bilayer by covalent bonds with one side of the lipid bilayer or by non-covalent bonds with the transmembrane proteins. The transmembrane proteins together with other covalently bound membrane proteins can be released from membranes by disrupting the lipid bilayer with detergents. These membrane proteins are often referred to as integral membrane proteins. The non-covalently bound plasma membrane proteins can be easily released under mild

conditions, such as changes in ionic strength or pH, without disrupting the lipid bilayer. These plasma membrane proteins are termed peripheral membrane proteins (Karp, 1984)

Carbohydrates are present on eukaryotic cell surfaces and are covalently bound to membrane proteins or lipids. Increasing evidence shows that the carbohydrate groups on glycoproteins play important roles in various biological activities of the plasma membrane, such as the interaction of ligand-cellular receptors, protein targeting, cell-cell interactions (Paulson, 1989). In addition, most eukaryotic cells have a carbohydrate-rich zone at the cell surface, called the glycocalyx (Karp, 1984). The biological function of the carbohydrates in the glycocalyx has not yet been elucidated. However, their complexity and location on the cell surface suggest that they may play some role in cell-cell and cell-matrix interactions (Karp, 1984).

1.3.2.2. Cellular Receptors for the Virus

Obviously, cellular receptors are not synthesized for the purpose of providing receptors for virus binding. All three major classes of plasma membrane components, proteins, lipids and carbohydrates have been found to act as virus receptors. Many of these cellular receptors have also other biological functions. Viruses may use hormone or neurotransmitter receptors

as their cellular receptors. For example, reovirus utilizes the β-adrenergic receptor (Co *et al.*, 1985*a*), and vaccinia virus uses the epidermal growth factor receptor for binding to host cells (Eppstein *et al.*, 1985). More recently, CD4, ICAM-1 and class I MHC molecules, all of which belong to the immunoglobulin superfamily, were defined as cellular receptors for human immunodeficiency virus (HIV) (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984), major serotypes of human rhinovirus (Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*, 1989*a*) and simian virus 40 (Breau *et al.*, 1992), respectively. The members of the immunoglobulin family actively engage in immune responses. For example, endogenously synthesized foreign antigens such as viral peptides can be recognized by $CD8^+$ cytotoxic T lymphocytes, only when they are associated with class I MHC molecules, whereas $CD4^+$ helper T cells recognize the exogenous antigens only when the antigens are associated with class II MHC molecules. Although, proteins are most often found to be virus receptors, lipids and carbohydrates also serve as virus receptors, such as phosphatidylinositol for vesicular stomatitis virus (Mastromarino *et al.*, 1987); sialyloligosaccharides for sendai virus (Paulson *et al.*, 1979), Newcastle disease virus (Paulson *et al.*, 1979), polyomavirus (Fried *et al.*, 1981) and influenza virus (Rogers, 1986).

1.4. Obstacles in Virus Receptor Isolation

Isolation of virus receptors has always posed considerable difficulties. The major obstacles in virus receptor isolation are:

(1) Viruses may attach to cells non-specifically, sometimes such non-specific attachment may even lead to infection of cells, as observed in the binding of vesicular stomatitis virus to baby hamster kidney cells (Bailey *et al.*, 1984), making it extremely difficult to distinguish specific binding from non-specific binding.

(2) Viruses may utilize different cellular receptors in different cell lines. This is the case in the attachment of coxsackievirus B3 (RD variant) to HeLa cells and rhabdomyosarcoma cells (Reagan, 1984). A virion may have more than one receptor binding determinant, and each determinant could bind to different cellular receptors, although these determinants may be located on the same molecule of the virion (Reagan *et al.*, 1984; Tignor *et al.*, 1984).

(3) Weak affinity between viruses and their cellular receptors. With a few exceptions, virus-receptor bonds are unstable in the presence of relatively low concentrations of detergent required by most biochemical purification methods to solubilize cell membranes (Dales, 1973; Knipe, 1990). This feature together with the small quantity of virus receptors makes it difficult to apply

most biochemical methods for the purpose of virus receptor purification.

(4) Virus may bind to intermediate molecules, which in turn bind to the cell as mentioned (section 1.3.2.2). This makes it even more difficult to identify and purify virus receptors.

1.5. Methods of Isolation and Characterization of Virus Receptors

Despite the difficulties mentioned above, various methods have been successfully used to isolate and characterize virus receptors. Some successful cases of isolating virus receptors took advantage of a particular property of cellular receptors or the virus-receptor interactions. For example, the receptor for coxsackievirus on HeLa cells has been successfully isolated by taking advantage of the stability of receptor-virus complexes in the presence of non-ionic detergent. Using differential sucrose gradient centrifugation, a conventional virus purification method, Mapoles and his co-workers (1986) achieved greater than 10^4-fold purification of the receptor.

Examining the history of virus receptor studies, one sees that numerous methods have been used for isolation and characterization of the receptors. The methods described below have been most commonly used both in the past and presently.

(1) Physico-chemical methods in virus receptor identification

Saturation and competition binding assays are two of the most commonly used methods in the study of the physical parameters of virus attachment. These methods were originally used in neuropharmacology and endocrinology. They are not necessarily suitable for the study of virus-receptor interactions, because viruses are large particles containing multiple copies of VAP, and in many cases the binding of viruses to cells is virtually irreversible (Lonberg-Holm, 1981; Tardieu *et al.*, 1982). Nevertheless, these methods have been and still are being used in almost every virus receptor study, and are regarded as a framework for the definition of a virus receptor (Tardieu *et al.*, 1982).

(2) Chemical and enzymatic modification of virus receptors.

Many chemical and biochemical agents have been used to alter the virus receptor on plasma membranes in attempts to characterize the biochemical nature of virus receptors. In this category, enzymes have perhaps contributed the most to our early knowledge about the nature of molecules involved in virus binding. As early as 1949, Verlinde and de Baan found that treatment of erythrocytes with neuraminidase, purified from *Vibrio cholerae*, prevented subsequent EMC virus induced hemagglutination (Verlinde & de Baan, 1949).

For this reason, neuraminidase was called a receptor-destroying enzyme (RDE). The sensitivity of the receptor to neuraminidase treatment suggested that sialic acid residues, which were cleaved from the sialylated receptor molecules, were involved in virus attachment. Similarly, the elimination of receptors for group B coxsackieviruses and rhinoviruses by chymotrypsin treatment revealed that those receptors are proteins, and that the receptor polypeptides contain one or more residues of tyrosine, phenylalanine, tryptophan, leucine, methionine, asparagine and glutamine (Crowell & Landau, 1983). However, besides cleaving polypeptide chains, treatment of cells with proteases causes other indirect effects on cells, such as changing the cell membrane conformation and increasing protease secretion from cells (Spelk *et al.*, 1972; Werb & Aggeler, 1978), therefore, extra caution is needed when interpreting the data (Colonno, 1987).

(3) Characterization and isolation of virus receptors using specific biological ligands.

In this category, lectins have been widely used for virus receptor studies. Lectins were originally isolated from plant seeds. They recognize and bind to specific sugar residues on polypeptide chains. For example, wheat germ

agglutinin (WGA) recognizes and binds to N-acetylglucosamine or sialic acid residues, whereas Concanavalin A binds to α-mannosyl. Taking advantage of the specificity of lectin binding to sugar residues, a considerable amount of information about the role of carbohydrates on receptors for viruses has been obtained (section 1.6). Attempts have also been made to isolate virus receptors, including EMC virus receptors, by affinity chromatography on lectin-columns (Pardoe & Burness, 1981; Mischak *et al.*, 1988).

(4) Isolation and characterization of virus receptors using specific anti-receptor antibodies.

The receptor for the major group of rhinoviruses was successfully isolated by affinity chromatography employing monoclonal anti-receptor antibody columns (Tomassini & Colonno, 1986).

(5) Isolation of virus receptors using anti-idiotypic antibodies.

This method is based on Jerne's internal image theory (Jerne, 1974). According to this theory, anti-idiotypic antibodies raised against an antibody to the viral attachment protein may mimic the biological properties of the viral attachment protein and bind to the cellular receptor. An anti-idiotypic

antibody raised against antibodies to the reovirus attachment protein was successfully used to isolate the receptor for reovirus (Co *et al.*, 1985*a*). Unfortunately, this method is not considered suitable for isolation of the receptor for most picornaviruses, including EMC virus, as their receptor binding sites are likely to be embedded in the "canyon" and are physically inaccessible to antibodies (see section 1.3.1).

(6) Transfection of virus receptor gene from receptor-positive to receptor-negative cells.

Using this technique, the gene for poliovirus receptor has been successfully identified (Mendelsohn *et al.*, 1986; Mendelsohn *et al.*, 1989). However, since EMC virus infects the cell lines from many different species, the technique probably cannot be applied for cloning the EMC virus receptor gene.

1.6. Cellular Receptors for Picornaviruses

1.6.1. General Properties of Receptors for Picornaviruses

Early physico-chemical studies on the attachment of picornaviruses to cells revealed that each host cell binds about 10^4 to 10^5 virions (Lonberg-Holm *et al.*, 1976*a*), and that several physical and chemical factors such as temperature,

pH, ionic strength and cell concentration affect virus attachment (Lonberg-Holm, 1981). For example, the attachment of EMC virus to HeLa-S_3 cells was reported to be temperature independent over the range 0^{o} to $40^{o}C$, whereas the attachment of the same virus to L-929 murine cells was progressively reduced with increasing temperature over the same temperature range (McClintock *et al.*, 1980). The attachment of picornaviruses to cells does not occur in the absence of cations (Holland & McLaren, 1959), and divalent cations, Ca^{2+} or Mg^{2+}, are required for attachment by several picornaviruses, including rhinoviruses (Fiala & Kenny, 1967; Lonberg-Holm & Whiteley, 1976), two serotypes of coxsackievirus A (McLaren *et al.*, 1960; Cords *et al.*, 1975) and foot-and-mouth disease virus (Brown *et al.*, 1962). Based on virus competition studies, it was discovered that although there are more than 120 serotypes of human rhinovirus, 90% of them (the major group) share a single cellular receptor (Abraham & Colonno, 1984), while the remaining serotypes (the minor group) with one exception, compete for a second receptor (Colonno *et al.*, 1986; Uncapher *et al.*, 1991). Similar studies on foot-and-mouth disease virus showed that six serotypes of the virus share a common receptor, which is distinct from the receptor for poliovirus and the receptor for EMC virus (Sekiguchi *et al.*, 1982). The discovery that

picornavirus receptors are divided into groups suggests that the binding domains in the VAP among many different serotypes of viruses are highly conserved (Colonno, 1987). These findings raise a new therapeutic possibility to block virus infection by blocking virus attachment to its cell surface receptors.

Early information about the biochemical nature of a receptor for picornaviruses was obtained mainly by treatment of cells or cellular plasma membranes with various enzymes and chemical reagents, and then examining the effects of such treatment on virus attachment. In most cases, it appeared that the receptors for picornaviruses are glycoproteins and they are located on the plasma membranes (Lonberg-Holm & Crowell, 1986; Colonno, 1987). Further biochemical characterization of the virus receptors revealed that some carbohydrate groups attached to the protein polypeptide chains are integral components of the virus receptor. Concanvalin A, a lectin, which binds to components on a cell surface containing α-D-mannopyranosyl-like residues, inhibits attachment and infection of both rhinovirus type-2 and poliovirus type 2 to HeLa cells (Lonberg-Holm, 1975). Similarly, wheat germ agglutinin, binding to components containing N-actylglucosamine or sialic acid residues, prevents the binding of rhinovirus type 15 to host cells (Colonno, 1987). These results suggests that the suguar residues, which the lectins recognize

and bind to, are involved in the attachment of viruses to the receptors.

Knowledge about receptors for picornaviruses has rapidly expanded since the development of monoclonal antibody and recombinant DNA technologies. These techniques led to the isolation and identification of two receptors for picornavirus family, the receptor for poliovirus and the receptor for the rhinovirus.

1.6.2. Receptor for Poliovirus

Poliovirus is one of the most extensively studied picornaviruses. As early as 1959, it was already found that poliovirus replicates only in primate cell lines (McLaren *et al.*, 1959). Later studies demonstrated that only cell debris derived from poliovirus-susceptible primate cells can inhibit infectivity of poliovirus (Holland & McLaren, 1959), and inoculation of purified poliovirus RNA to nature resistant cell lines from species other than primates can produce only one cycle of replication in those cells (DeSomer *et al.*, 1959; Holland *et al.*, 1959). These findings revealed that resistance to poliovirus infection in non-primate cells happens at the level of virus entry rather than at the intracellular level.

The first attempt to search for the receptor for poliovirus by a genetic

approach was accomplished by Miller and his co-workers (1974). Analyzing the chromosome complements of human/mouse hybrid cells lines by chromosome banding methods, they concluded that the poliovirus receptor gene is carried by human chromosome 19. Interestingly, although several monoclonal anti-receptor antibodies were produced by different groups (e.g., Minor *et al.*, 1984; Nobis *et al.*, 1985; Shepley *et al.*, 1988), it was the genetic approach, that finally led to the isolation of the receptor for poliovirus. By using DNA transfection, the gene for the poliovirus receptor was successfully transferred from the virus-susceptible human HeLa cell to the nonsusceptible mouse L cell, and subsequently the receptor gene was cloned (Mendelsohn *et al.*, 1986; Mendelsohn *et al.*, 1989). The polypeptide encoded by the receptor gene is a transmembrane protein with three homologous immunoglobulin-like domains, which groups the molecule into the immunoglobulin superfamily (Mendelsohn *et al.*, 1989). The normal biological function of this unidentified member of the immunoglobulin superfamily has not yet been determined.

1.6.3. Receptor for Rhinoviruses

It is well known that rhinoviruses initiate infection of cells by attaching to a specific cellular receptor (Haff *et al.*, 1966; Stott & Heath, 1970; Thomas *et*

al., 1970). However, for many years, our knowledge about the nature of receptors for rhinoviruses were very limited. We knew that receptors appear to be glycoproteins since pretreatment of cells with trypsin or Concanavalin A inhibits subsequent viral attachment to cells (Stott & Heath, 1970; Lonberg-Holm, 1975). Further studies on the nature of the receptor for rhinovirus, which required the purified receptor, were hampered by the scarcity of receptors on cells. However, success in generating monoclonal anti-receptor antibodies paved the way for subsequent studies of this virus receptor, such as receptor isolation, biochemical characterization, amino acid sequencing, and cloning the receptor gene.

The first successful production of monoclonal antibodies to cellular receptors for the major group rhinoviruses were achieved by immunizing mice with whole human HeLa cells (Colonno *et al.*, 1986). The specificity of the anti-receptor antibodies was proved by: (1) Ability of the anti-receptor antibodies to block attachment and infection of HeLa cells by major group rhinoviruses and group A coxsackieviruses, but not by other viruses, such as minor group rhinoviruses, poliovirus, group B coxsackieviruses or hepatitis A virus (Colonno *et al.*, 1986). These results confirmed results from previous studies indicating that the major group rhinoviruses share the same receptor

with group A coxsackieviruses, but not other picornaviruses (Lonberg-Holm *et al.*, 1976*a*; Abraham & Colonno, 1984). (2) The anti-receptor antibodies attach to a wide variety of human and chimpanzee cells, but not to cells of other species (Colonno *et al.*, 1986). It is known that the major group rhinoviruses only infect humans and chimpanzees (Dick, 1968; Pinto & Huff, 1969). Thus, anti-receptor antibodies showed identical host specificity as that displayed by the major group rhinoviruses (Colonno *et al.*, 1986).

Subsequently, the monoclonal anti-receptor antibodies were used to isolate the rhinovirus receptor. A cellular receptor protein with molecular weight of 90 kD was isolated from detergent solubilized HeLa cell membranes by gel filtration, followed by monoclonal anti-receptor antibody affinity chromatography. The polyclonal antiserum, prepared in rabbits immunized with purified putative receptor protein, inhibited infection of susceptible cells by the major group rhinoviruses and group A coxsackieviruses, but not other viruses (Tomassini & Colonno, 1986). This result confirmed that the 90-kD protein was the major group rhinovirus receptor, or at least the functional component of the receptor. Further biochemical characterization of this 90-kD polypeptide revealed that oligosaccharides constitute 30% of its molecular mass. Seven N-linked glycosylation sites were detected by partial removal of

oligosaccharides from the polypeptide with N-glycanase (Tomassini *et al.*, 1989*a*).

Later, studies from three independent groups revealed that the receptor for major group rhinoviruses is the intercellular adhesion molecule-1 (ICAM-1). ICAM-1 is a member of the immunoglobulin superfamily with five immunoglobulin-like domains (Dustin *et al.*, 1988; Staunton *et al.*, 1988; Simmons *et al.*, 1988) and serves as a cell adhesion molecule for the lymphocyte function-associated antigen-1 (LFA-1) (Makgoba *et al.*, 1988). One group of investigators showed that the amino acid sequences of receptor polypeptides and, the ICAM-1 molecule, and the nucleotide sequences of a cDNA clone of the receptor and the cDNA clone of ICAM-1 were identical (Greve *et al.*, 1989). At the same time, another group showed that the major group rhinoviruses bind specifically to both purified ICAM-1 molecules and to ICAM-1 molecules expressed on cells transfected with the ICAM-1 gene (Staunton *et al.*, 1989). The same conclusion was reached by another group which first isolated the receptor for the major group rhinovirus by monoclonal anti-receptor antibodies (Tomassini & Colonno, 1986). Their studies also showed that the cloned major group rhinovirus receptor cDNA encodes a protein with a sequence nearly identical to that of ICAM-1 (Tomassini *et al.*,

1989*a*). Thus, it is well established that the receptor for the major group rhinoviruses is ICAM-1, a member of the immunoglobulin superfamily.

Recently, the binding domain of the ICAM-1 molecule for the major group rhinovirus has been extensively studied. It was reported that the binding of these viruses to ICAM-1 was blocked only by ICAM-1 monoclonal antibodies, which can block ICAM-1-LFA-1 interaction, but not by antibodies to the other epitopes on the ICAM-1 molecule (Staunton *et al.*, 1989). This finding suggested that binding sites on ICAM-1 for the major group rhinovirus are proximal or identical to that of LFA-1 (Staunton *et al.*, 1989). Furthermore, by employing human-murine ICAM-1 chimeras with single and multiple amino acid mutagenesis, as well as monoclonal antibody epitope mapping, the primary binding site for the major group rhinoviruses was localized to the domain 1 of ICAM-1. Certain amino acids located on the N-terminal domain appear critical for virus binding (Staunton *et al.*, 1990; Lineberger *et al.*, 1990; Register *et al.*, 1991). However, expression of domain 1 and domain 2 appear to be dependent on each other, and the presence of domains 3, 4 and 5 significantly affects the accessibility of the binding site on the ICAM-1 molecule for the major group rhinoviruses (Staunton *et al.*, 1990). Analysis of the domain 1 of ICAM-1 by computer modelling suggested that half of the N-

terminal of domain 1 can fit into the "canyon" on the virion surface and interact with amino acids located at the wall or floor of the "canyon" (Giranda *et al.*, 1990). Again, this finding supports the involvement of the "canyon" in virus-receptor interaction. It is expected that success in determining the co-crystal structure of the domain 1 of ICAM-1 and the major group rhinovirus will provide more information about the mechanism of virus attachment.

It is interesting that several other viruses also use the immunoglobulin superfamily proteins as their receptor or as a necessary component of their receptors. These include CD4 for the human immunodeficiency virus (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984), an unidentified immunoglobulin superfamily protein for poliovirus (Mendelsohn *et al.*, 1989), and MHC class I protein is an integral component of the receptor for simian virus 40 (Breau *et al.*, 1992). The biological significance of the receptors for these viruses being members of the immunoglobulin superfamily remains unclear. Is this phenomenon a coincidence, or a reflection of a unique role of the receptors in virus-cell interaction? One hypothesis suggests that the use of ICAM-1 as the rhinovirus receptor aids the spread of virus within the host (Staunton *et al.*, 1989). This suggestion was based on the fact that ICAM-1 surface expression is highly induced by lymphokines and monokines, which can

be stimulated by viral infection (Staunton *et al.*, 1989).

1.6.4. Receptors for Other Picornaviruses

In contrast to the relatively well characterized receptors for poliovirus and the major group rhinovirus, only limited information about receptors for other picornaviruses is available. A putative receptor for group B coxsackievirus was purified by taking advantage of the fact that the virus-receptor complex is stable after detergent extraction (Krah & Crowell, 1985; Mapoles *et al.*, 1985). The purified receptor polypeptide with molecular weight about 50 kD was used as an immunogen to produce polyclonal and monoclonal antibodies (Hsu *et al.*, 1988). Both types of antibodies prevented infection of HeLa cells by all six serotypes of group B coxsackieviruses (Hsu *et al.*, 1988). Amino acid sequencing of the receptor polypeptide and isolation of the cDNA encoding the polypeptide have not yet been reported.

The status of studies on the receptor for minor group rhinoviruses is at a very similar stage as for the group B coxsackieviruses. A putative virus receptor with molecular weight about 450 kD was detected after the receptor's activity was enriched by lectin affinity chromatography followed by gel permeation and anion exchange chromatography (Mischak *et al.*, 1988).

However, neither the amino acid sequence of the receptor polypeptide nor its cDNA sequence has been reported. The search for the foot-and-mouth disease virus receptor is just beginning to yield results. It has been established that many cells contain various adhesion proteins, integrins, which recognize and interact with many extracellular ligands containing Arg-Gly-Asp sequences (Ruoslahti & Pierschbacher, 1987). The inhibition of attachment of foot-and-mouth disease virus to its target cells by peptides containing Arg-Gly-Asp sequences suggests that one or more integrins are components of the receptor for this virus (Fox *et al.*, 1989).

1.6.5. Receptor for EMC Virus

Studies on the receptor for EMC virus can be traced back four decades. However, very little information is available in terms of the virus receptor on nucleated cells. Most of our knowledge about the receptor for EMC virus has been achieved by studying EMC-erythrocyte interactions.

1.6.5.1. Glycophorin A - the Receptor for EMC Virus on Erythrocytes

As many other viruses, EMC virus attaches to erythrocytes of many species (Angel & Burness, 1977), however, the biological significance of such an

interaction remains unclear. One reasonable explanation is that EMC-erythrocyte binding may aid the host to get rid of the invading virus more effectively, by presenting the virus to immunocompetent cells (McClintock *et al.*, 1980). Early observations showed that hemagglutination caused by EMC virus was inhibited by pre-treatment of erythrocytes with neuraminidase (Angel & Burness, 1977; Enegren & Burness, 1977; Burness & Pardoe, 1981). This finding revealed the involvement of sialic acids in virus attachment.

Glycophorin is a family of glycoproteins. Currently, they are named glycophorin A, glycophorin B, glycophorin C and glycophorin D in human erythrocytes (Dahr, 1986). The first three members of the family normally comprise about 86%, 7.5% and 1.5% of total sialoglycoprotein, respectively, whereas, the glycophorin D can only be detected in purified glycophorin preparations (Dahr, 1986). The studies which led to the conclusion that glycophorin A is the receptor for EMC virus took advantage of the existence of several erythrocyte variants lacking one or more species of glycophorin (Dahr, 1986). Thus, En(a-) cells completely lack glycophorin A, but contain normal amounts of other glycophorins and glycolipids (Taliano,1980; Anstee, 1981); whereas, S-s-U+ erythrocytes lack glycophorin B, but are normal in other aspects (Dahr *et al.*, 1978). The finding that EMC virus was unable to

attach to En(a-) erythrocytes, but attached to S-s-U+ erythrocytes at normal levels (Allaway & Burness, 1986; Allaway *et al.*, 1986), together with the previous conclusion that the EMC virus receptor is a sialoglycoprotein (Verlinde & DeBaan, 1949; Angel & Burness, 1977), revealed that glycophorin A was the receptor for EMC virus on human erythrocytes (Allaway & Burness, 1986).

1.6.5.2. Site of Attachment of EMC Virus on Glycophorin A

A detailed examination of the structure of glycophorins and the segments cleaved by proteases allowed investigators to determine the binding site for EMC virus on glycophorin A (Allaway & Burness, 1986; Allaway *et al.*, 1986). Glycophorin A contains 131 amino acids of which 70 are exposed on the cell surface with one N-linked and 15 identical O-linked sialo-oligosaccharide side chains (Tomita *et al.*, 1978; Dahr, 1986). Glycophorin B is extremely similar to glycophorin A in structure. The structures of glycophorin A and glycophorin B are identical in the region of amino acids 1 to 26 and very similar in the region 56 to 72, but the segment 27 to 55 is absent in glycophorin B. Therefore, the fact that EMC virus does not bind to En(a-) erythrocytes, but binds to S-s-U+ erythrocytes at normal levels strongly

suggests that the binding site for EMC virus on glycophorin A must be located between amino acids 27 to 55, the region that distinguishes glycophorin A from glycophorin B (Allaway & Burness, 1986; Allaway *et al.*, 1986).

This suggestion was also supported by three other experiments. The first experiment showed that treatment of erythrocytes with chymotrypsin, which releases amino acids 1 to 34 from glycophorin A, has little or no effect on virus attachment, whereas trypsin treatment, which releases amino acid residues 1 to 39 in glycophorin A, close to the membrane, caused a drop of more than 50% in virus attachment (Allaway & Burness, 1986; Allaway *et al.*, 1986). The second experiment used monoclonal anti-glycophorin A antibodies directed against various regions of the extracellular domain of glycophorin A (Anstee & Edwards, 1982; Ridgewell *et al.*, 1983; Gardner *et al.*, 1989). It showed that the monoclonal antibody B116 blocked the amino acid region 34 to 39 preventing attachment of virus, whereas antibodies blocking the regions 10 to 30 and 50 to 70 had no effect on virus attachment (Pardoe & Burness, 1986). The third experiment used Wr(b-) erythrocytes in which glycophorin A is highly modified between amino acids 57 and 70. EMC virus binds to Wr(b-) erythrocytes equally well compared to normal erythrocytes (Pardoe & Burness, 1986). Overall, the site of attachment of EMC virus on glycophorin

A must lie in the region of amino acids 34 to 50, and 35 to 39 may be particularly important for EMC virus binding (Allaway *et al.,* 1986; Pardoe & Burness, 1986.).

1.6.5.3. Role of Sialic Acid in the Attachment of EMC Virus to Erythrocytes

It is well known that receptors for many mammalian viruses, such as influenza virus, reovirus, and EMC virus, require sialic acid residues (Burness, 1981). Sialic acids are a family of about 30 acidic sugars (Reuter & Schauer, 1988), that are all derived from neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galactononulosonic acid), with differences in their substitution patterns on the amino and hydroxyl groups (Schauer, 1987). The amino group of neuraminic acid can be substituted either by an acetyl or glycolyl residue, whereas one or several hydroxyl groups are often methylated or esterified with acetyl, lactyl, sulfate or phosphate groups (Schauer, 1982; Corfield & Schauer, 1982). Sialic acids are usually present on terminal sugars of oligosaccharides in glycoproteins and gangliosides, but they might also bind to internal carbohydrates (Schauer, 1982; Schauer, 1987). N-acetylneuraminic acid and N-glycolyneuraminic acid are two of the most common neuraminic acids in many animals. Noticeably, mouse erythrocytes contain 27% 9-O-N-diacetyl

neuraminic acids (Reuter *et al.*, 1988), that are relatively resistant to neuraminidase (*Vibrio Cholerae*) digestion (Schauer & Faillard, 1968). The biological role of sialic acids has attracted a lot of attention in the last 10 years. Sialic acids participate in many biological and pathological processes. They have been found to be involved in constituting receptor complexes for various peptide hormones, toxins, viruses and mycoplasma species, maintaining activity of glycoprotein enzymes by influencing the glycoprotein conformation and specifying blood types and cell malignancies (Schauer, 1985).

The role of sialic acids in the attachment of EMC virus to human erythrocytes has been extensively studied in Dr. A. T. H. Burness's laboratory at this institution. As components of virus receptors, sialic acids can have two possible roles, a direct role or an indirect role. Being an integral part of the virus binding site on the receptor, sialic acids can play a direct role in virus attachment to the receptor. Alternatively, negatively charged sialic acids could play an indirect role by interacting with positively charged basic amino acid residues in the receptor polypeptide to hold the virus binding site in the correct configuration (Tavakkol & Burness, 1990). To examine the possibility of an indirect role of sialic acids in the binding of EMC virus to glycophorin A, the positively charged lysine residues and arginine residues in glycophorin

A were blocked by acetylation with acetic anhydride or reaction with butanedione. Neither of these treatments affected binding of EMC virus to its receptor on human erythrocytes, suggesting that positive charges on basic amino acids are not required for EMC virus attachment to human erythrocytes. In contrast, blocking of carboxyl groups on sialic acid residues by amidation caused a 96% inhibition of EMC virus attachment. Furthermore, removal of the three-carbon long polyhydroxy side chain in sialic acids, by mild periodate-borohydride treatment, had no effect on EMC virus attachment. Overall, the studies suggest that the carboxyl groups, not the polyhydroxy side chain of sialic acids, play a direct role in EMC virus attachment, and that positively charged lysine and arginine residues in the polypeptide are not involved in the interaction with negatively charged sialic acids to maintain the integrity of the virus attachment site on human erythrocytes (Tavakkol & Burness, 1990).

1.7. Purpose of Research

1.7.1. General Purpose of Virus Receptor Studies

Viruses induce various human diseases, from the annoying common cold to the lethal hepatitis B and AIDS. Unfortunately, vaccines, the only powerful weapon available in the battle against viral diseases, can not protect

humans from many viral infections. For example, it is almost impossible to develop a vaccine against the common cold. The disease can actually be induced by almost 200 different viruses, rhinoviruses alone has over 100 different serotypes. However, to initiate an infection, the virus must attach to specific receptors on a host cell. The attachment between the virus and its cellular receptor not only grants the virus physical access to a susceptible cell, but also contributes to the subsequent stages of viral replication (Crowell & Landau, 1983), such as virus penetration (Pastan & Willingham, 1981) and uncoating (Crowell & Siak, 1978). The specific attachment step supplies a perfect target to intercept the viral infection cycle. To develop agents, which will inhibit virus initial binding to cells, information on both the VAP and cell receptor at the molecular level is essential. In addition, the study of virus receptors is important to understand the precise mechanisms of viral replication and tissue tropism. Finally, the study of virus receptors may eventually lead to the identification of the normal physiological functions of these cell surface molecules.

1.7.2. Purpose of the Project

The receptor for EMC virus on human erythrocytes is glycophorin A

(Allaway *et al.*, 1986). However, since the erythrocyte does not support viral replication, the cell is not regarded as a host nucleated cell for the virus. Therefore, whether glycophorin A is a genuine receptor for EMC virus on host cells remains unclear. The K562 cell, a human erythroleukemic cell line allows us to study this problem. These cells like erythrocytes express glycophorin A on their surface (Gahmberg *et al.*, 1983) and they are susceptible to EMC virus infection (Pardoe *et al.*, 1990). However, recent information suggests that glycophorin A is not the receptor for EMC virus on K652 cells. The suggestion is based on the data showing that transfection of K562 cells with anti-sense glycophorin A cDNA, to block synthesis of glycophorin A, or saturating the cell with anti-glycophorin A antibodies, does not affect virus attachment to, and infection of K562 cells (Hamid & Grewal, unpublished results). Thus, the question remains, if glycophorin A is not the receptor for EMC virus on K562 cells, what is the receptor for EMC virus on K562 cells? Therefore, this project was designed to gather more information about the nature of EMC receptors on human nucleated cells.

1.7.3. Experimental strategy

In the beginning, only human erythroleukemic K562 cells and the K562 D

clone mutant cell line, which resists EMC virus infection, possibly because of a deficiency in EMC virus receptors (Pardoe *et al.*, 1990), were involved in the project. We initially considered producing monoclonal anti-receptor antibodies as the main approach to isolate the receptor until it was found that the colorimetric hybridoma screening assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and *p*-iodonitrotetrazolium violet, which was used successfully in screening monoclonal anti-receptor antibodies for the poliovirus (Shepley *et al.*, 1988), was not suitable for K562 cells, which grow in suspension. This was because several washes are required in this screening assay, which caused a significant loss of cells. Consequently, HeLa cells, a human cervical adenocarcinoma cell line, which grows in monolayers, were introduced into the project to allow for the production of monoclonal anti-receptor antibodies. Eventually, the anti-receptor monoclonal antibodies were developed by others outside this laboratory, but Hela cell studies were continued in this project for comparison.

Since anti-receptor monoclonal antibodies are usually generated at a very low frequency when whole cells are used for immunization, it was necessary to try other methods to isolate the virus receptor. The success in isolating the EMC virus receptor on mouse insulinoma cells by EMC virus-Sepharose

affinity chromatography (Baldeh & Burness, manuscript submitted), motivated us to try the same technique in this project.

The principle of this affinity chromatography technique was based on two findings from studies of interactions between glycophorin A and EMC virus. The first finding was that the bond between glycophorin A-EMC virus is a weak ionic bond which can be easily broken by 0.2 M NaCl (Allaway & Burness, 1987). The second finding was that glycophorin A binds to EMC virus on the affinity column in the presence of 6 mM sodium deoxycholate (DOC) (Baldeh, 1987). This affinity chromatography technique was shown to be suitable for the purification of EMC virus receptors from human cell lines, and was adopted in the project.

As part of the long term objective of the receptor study for EMC virus on human cell lines, some preliminary physical and biochemical characterizations of the receptor were carried out. These experiments included the following: (1) determination of the number of EMC virus binding sites and the virus-receptor equilibrium dissociation constant (Kd) in HeLa cells. Similar experiments had been done using K562 cells before this project was started (Pardoe & Burness, unpublished results); (2) effects of proteases and neuraminidase on the attachment of EMC virus to intact cells, membrane preparations and detergent solubilized membranes of K562 and HeLa cells;

(3) effect of lectins on the attachment of EMC virus to K562 and HeLa cells, (4) biosynthesis of EMC virus receptor activity on K562 and HeLa cells after trypsin treatment.

Chapter 2

Materials and Methods

2.1. Growth and Purification of EMC Virus

The K_2 strain of EMC virus was used in this project. Virus was grown in swirling cultures of Krebs ascites tumor cells (Burness *et al.*, 1974). ^{3}H-labeled EMC virus was produced by adding ^{3}H-labeled leucine (Dupont, Boston, MA, USA) to the culture medium during virus growth. The virus was purifed by the method of Ziola and Scraba (1974) and kindly provided by Ms. Pardoe from Dr. A. T. H. Burness's laboratory of this institution. The number of viral particles was estimated as described previously (Burness & Clothier, 1970). In a typical preparation of ^{3}H-labeled virus, specific activity ranged from 800 to 1000 cpm/μg protein (i.e., from 1.14×10^{-8} to 1.43×10^{-8} cpm/virus particle).

2.2. Cells

2.2.1. K562 Cells

K562 cells are human erythroleukemic cells, which were originally isolated from a chronic myelogenous leukemia patient in 1975 (Lozzio & Lozzio *et al.*, 1975). Because the cells express glycophorin A (Gahmberg *et al.*, 1979), they were often used to study glycophorin biosynthesis (Gahmberg *et al.*, 1980;

Jokinen *et al.*, 1985; Silver *et al.*, 1987; Morrow & Rubin, 1987). The expression of glycophorin A on K562 cells also provides a good opportunity to investigate the role of glycophorin A as a receptor for EMC virus in nucleated cells. Recently, a K562 cell line, D clone, which is resistant to EMC virus infection was isolated from a persistantly infected culture of K562 cells (Pardoe *et al.*, 1990). It was proposed that the resistance of infection was due to lack of receptors for EMC virus in the mutant cells, as the virus binds very poorly to these cells (Pardoe *et al.*, 1990).

Both parental K562 cells and their D clone mutant cells were grown in suspension in RPMI 1640 medium (see Appendix) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml at 37°C in a humidified 5% CO_2 incubator. RPMI 1640, fetal bovine serum and antibiotics were obtained from GIBCO BRL, Grand Island NY, USA. K562 cells were grown in 75 cm^2 cell culture flasks (Costar Co., Cambridge, MA, USA). The cells were harvested by centrifugation when their densities reached about 1 x 10^6 cells/ml. The number of cells and their viability were measured by using a hemocytometer after mixing an equal volume of cell suspension with 0.1% trypan blue in phosphate-buffered saline, pH 7.4 (PBS, see Appendix). Cells were washed with cold PBS three times before use in experiments.

2.2.2. HeLa Cells

HeLa cells were initially isolated in 1951 from a patient with carcinoma of the cervix (Gey *et al.*, 1952). Later, it was found that the carcinoma was an adenocarcinoma (Jones *et al.*, 1971). HeLa cells used in this project were kindly supplied by Dr. Banfield Younghusband in this faculty. The susceptibility of the HeLa cells to EMC virus infection has been well established, and the receptor for the virus on cells was proposed to be sialylated (Jungeblut & Kodza, 1957; Kodza & Jungeblut, 1958).

HeLa cells were grown in monolayers in Dulbecco's modified eagle medium (DMEM, see Appendix) supplemented with 5% newborn calf serum, 100 U of penicillin per ml, and 100 µg streptomycin per ml at 37°C, in a humidified 5% CO_2 incubator. DMEM, newborn calf serum and antibiotics were obtained from GIBCO BRL. The cells were harvested when monolayers were about 90% confluent in 150 x 25 mm tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ, USA) by scraping into medium with a rubber policeman. The cells used in attachment assays were grown in 35 mm tissue culture dishes (Corning Glass Works, Corning, NY, USA), and washed with cold PBS three times before the assay.

2.3. Preparation and Solubilization of K562 and HeLa Cell Membranes

2.3.1. Preparation of Cell Membranes

The cell membranes were prepared by using a method described previously (Atkinson & Summers, 1971).

Materials:

(1) 10 mM Tris-HCl buffer, pH 8.0, containing 15 mM sodium iodoacetate.

(2) Phosphate-buffered saline, pH 7.4 (PBS, see Appendix).

(3) Nuclei stabilizing buffer: 30 mM $MgCl_2$, 100 mM NaCl.

(4) 1x concentration of standard protease inhibitors: 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM N-ethylmaleimide.

Method:

(1) Cells collected from suspension cultures or monolayers were pelleted by centrifugation at 300 x g for 15 minutes, and washed twice with cold PBS to remove serum and cell debris.

(2) The pellet was resuspended in 20 times its volume of 10 mM Tris-HCl buffer, pH 8.0, containing 1x concentration of standard protease inhibitors. The cells were allowed to swell 15 minutes in an ice bath.

(3) An additional five volumes of cold distilled water was added to the cell suspension and the cells were allowed to swell for another 15 minutes on ice.

(4) The swollen cells were disrupted with 15-20 strokes in a 50 ml glass Dounce homogenizer. To avoid excessive homogenization, cell disruption was

monitored by phase contrast microscopy.

(5) The cell nuclei were stabilized by the addition of 0.1 volume of stabilizing buffer, and removed by centrifugation at 1,000 x g for 30 seconds. The supernatant containing membranes was saved. The removal of nuclei and whole cells was monitored by microscopy.

(6) To extract more membranes, the pellet was resuspended in four times its volume of Tris-HCl buffer followed by 0.1 volume of stabilizing buffer. The preparation was centrifuged at 1000 x g for 30 seconds and the supernatant was combined with the previous supernatant.

(7) The membranes were pelleted in a polyallomer tube by centrifuging at 45,000 x g for 60 minutes in a Beckman ultracentrifuge (Model L5-65) equipped with a fixed-angle type 50.2 Ti rotor.

(8) Pelleted membranes were resuspended in PBS containing 0.1x concentration of standard protease inhibitors and stored at -20^{o}C. Protein concentration was measured by the Lowry method (Lowry *et al.,* 1951) using bovine serum albumin (BSA) as standard.

2.3.2. Preparation of Solubilized Cell Membranes

Materials:

(1) Cell membranes (section 2.3.1).

(2) 0.02 M phosphate buffer, pH 8.0 (see Appendix).

(3) 0.02 M phosphate buffer containing 12 mM sodium deoxycholate (DOC).

Method:

(1) Cell membranes were solubilized in 12 mM DOC by the addition of an equal volume of 0.02 M phosphate buffer containing 24 mM DOC to a final protein concentration of about 1.5 mg/ml.

(2) Solubilization took place in an ice bath for 30 minutes. Insoluble material was pelleted in a polyallomer tube by centrifuging at 16,000 x g for 5 minutes in a SA-14 rotor in a Beckman centrifuge (Model J-21 B, Beckman Inc., Palo Alto, CA, USA).

(3) The supernatant was saved and diluted with an equal volume of 0.02 M phosphate buffer, pH 8.0 to a final DOC concentration of 6 mM. The solubilized membranes were stored in 3-4 ml amounts at -20^{O}C for further studies.

2.4. Preparation of Erythrocyte Membranes and Glycophorins

2.4.1. Human Erythrocyte Membranes

Erythrocyte membranes were prepared from recently outdated type O human erythrocytes by hypotonic lysis.

Materials:

(1) Outdated type O human erythrocytes were kindly provided by the Canadian Red Cross, St. John's, Newfoundland.

(2) Isotonic buffer: 150 mM NaCl, 5 mM Na_2HPO_4, adjusted to pH 8.0 with 85% H_3PO_4 (Mallinckrodt Inc., Kentucky, USA).

(3) Hypotonic buffer: 5 mM Na_2HPO_4 adjusted to pH 8.0 with 85% H_3PO_4.

Method:

(1) About 25 ml blood was mixed with an equal volume of cold isotonic buffer. The cells were pelleted at 4°C by centrifuging at 1,500 x g for 5 minutes. The supernatant and "buffy coat" were aspirated.

(2) Packed cells were washed at least three times by suspension in 50 ml cold isotonic buffer and centrifugation at 4°C to remove serum proteins. The volume of packed cells was recorded.

(3) The cell pellet was lysed by the addition of 40 times its volume of hypotonic buffer containing 1x concentration of standard protease inhibitors.

(4) The membranes were collected at 4°C by centrifuging at 10,000 x g for 30 minutes in a Beckman centrifuge (Model J-21B, Beckman Inc.) with JA-14 rotor. The supernatant containing hemoglobin was removed by aspiration. The membranes were washed at least three times with hypotonic buffer containing 1x concentration of standard protease inhibitors, until all the hemoglobin had been removed and the membranes were creamy to white in colour.

(5) Protein and sialic acid concentrations on the membranes were measured by the Lowry method (Lowry *et al.*, 1951) and the thiobarbituric acid method

of Warren using N-acetyl neuraminic acid as standards (Warren, 1959), respectively. The membranes were stored at -20^{0}C.

2.4.2. Preparation of Glycophorins

Glycophorins were prepared from erythrocyte membranes by lithium 3,5-diiodosalicylate-phenol extraction of the membranes (Marchesi & Andrews, 1971), and kindly provided by Ms. Pardoe.

2.5. Iodination of Proteins by the Iodogen Method.

An iodination method described by Markwell and Fox (1978) was used to iodinate EMC virus, intact cells, cell membranes, glycophorins and purified putative receptors.

Materials:

(1) Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril, Pierce Inc., Rockford, IL, USA).

(2) 2 mCi Na^{125}I in 20 µl (Amersham Canada Ltd., Lachine, Quebec, Canada) was diluted with 0.1 M phosphate buffer, pH 8.0 (see Appendix) to 200 µl (10 µCi/µl).

(3) Sephadex G-25 (Pharmacia, Uppsala, Sweden).

(4) Chloroform (Fisher Scientific Co., Fair Lawn, NJ, USA)

(5) 0.02 M phosphate buffer, pH 8.0 (see Appendix).

(6) 5% bovine serum albumin (BSA) in the 0.02 M phosphate buffer (see Appendix).

(7) Phosphate-buffered saline, pH 7.4 (PBS, see Appendix).

(8) Purified EMC virus (section 2.1.), solubilized cell membranes in 12 mM DOC (section 2.3.2.), glycophorin preparation (section 2.4.2.), and cells (section 2.2.). Purified virus receptor proteins obtained by affinity chromatography are described in section 2.13.

Method:

(1) Preparation of Iodogen coated tubes: 1 mg of Iodogen was dissolved in 1 ml chloroform and plated onto the surface of glass tubes. The solvent was allowed to evaporate under a stream of nitrogen. Tubes were coated with 10 µg and 100 µg Iodogen, respectively. The coated tubes can be stored in a desiccator for several weeks before use. The tubes were rinsed with 0.02 M phosphate buffer immediately before use to remove any loose flakes of Iodogen.

(2) Preparation of Sephadex G-25 columns: 2g Sephadex G-25 beads were swollen in 10 ml of 0.02 M phosphate buffer, pH 8.0. The swollen beads were transferred to a 10 cc disposable syringe stopped with a glass fibre cushion. The column was equilibrated with 1 ml of 5% BSA in 0.02 M phosphate buffer followed by 10 ml of 0.02 M phosphate buffer, pH 8.0.

(3) Iodination Reactions:

A: Cell surface labeling:

Cells in suspension or monolayers were collected and washed with cold PBS (Section 2.2.). About 100 µl washed cells (10^7) were transferred to a 100 µg Iodogen coated tube. Iodination was initiated by adding 200 µCi of $Na^{125}I$ to the tube. The reaction was allowed to proceed for 10 to 15 minutes at room temperature with gentle agitation every two minutes.

B: Labeling of cell membranes, glycophorins, purified putative receptors and EMC virus:

200 µg solubilized cell membranes (100 µl to 200 µl), 100 µg purified glycophorins (20 µl), 10 µg purified putative receptors (20µl) and 100 µg purified EMC virus (50 µl to 100 µl) were measured into tubes coated with 10 µg Iodogen. Iodination was initiated by adding 200 µCi of $Na^{125}I$ to each tube. The reactions were allowed to proceed for 10 to 15 minutes at room temperature with gentle agitation.

(4) Removal of Unreacted Iodine:

A: Iodinated cells:

1 ml of PBS was added to the tube immediately after the iodination was completed. The cell suspension was transferred to a 15 ml polypropylene tube. The cells were washed with 10 ml PBS, pelleted by centrifuging at 250 x g for 5 minutes and the supernatant was decanted. The washing procedure was repeated at least three times. Cell viability was examined by 0.1% trypan

blue exclusion before and after the iodination. The cells were used to prepare membranes immediately after iodination by the method described above (section 2.3.).

B: Iodinated cell membranes, glycophorins, EMC virus and purified receptors:

0.02 M phosphate buffer, pH 8.0 was added to each tube to a final volume of 1 ml immediately after the iodination was completed. The mixture was transferred to a Sephadex G-25 column. The column was washed with 9 ml of 0.02 M phosphate buffer. Fractions of 1 ml were collected and their radioactivity was measured in an automatic gamma counter (1277 Gammamaster, Parmacia). Iodinated material usually appeared between fractions 4 to 6. The iodinated material can be stored at $4^{o}C$ for several weeks.

Note: Radiation safety was followed throughout the experiments.

2.6. Measurement of Radioactivity Incorporated into Proteins

A method described by Johnston and Thorpe (1987) was used for this purpose.

Materials:

(1) Radiolabeled samples (section 2.5.).

(2) 10% trichloroacetic acid.

(3) 100% Ethanol.

Method:

(1) About 10^4 cpm of ^{125}I-labeled sample was applied to each of four glass fibre filters, 24 mm diameter (Whatman Inc., Clifton, NJ, USA) and allowed to dry.

(2) Two of the filters were transferred to test tubes, and radioactivity was measured (section 2.5.).

(3) The other two filters were each placed onto a glass filter holder, 25 mm diameter (Millipore Canada Ltd., Mississauga, Ont., Canada) and connected to a vacuum source. 2 ml of 10% trichloroacetic acid was added to the glass filter holder, and left for 10 minutes to precipitate proteins before removing by vacuum. The acid precipitation step was repeated once.

(4) The filters were washed twice, with 2 ml ethanol, by vacuum suction. The washed filters were dried under vacuum, and then transferred to test tubes and their radioactivity was measured (section 2.5.).

(5) Calculation: The Proportion of sample radioactivity bound to protein = (Mean activity after acid precipitation) ÷ (Mean activity before acid precipitation) x 100%

2.7. Attachment of ^{3}H-Labeled EMC Virus to Cells and Membranes

^{3}H-labeled EMC virus was used to measure binding of virus to cells and

isolated cell membranes by methods described previously (Allaway & Burness, 1987; Pardoe *et al.*, 1990).

Materials:

(1) K562 and HeLa cells (section 2.2.), cell membranes (section 2.3.1.) and ^{3}H-labeled EMC virus (section 2.1.).

(2) Phosphate-buffered saline, pH 7.4 (PBS, see Appendix).

(3) PBS-Tween and 3% BSA in PBS-Tween (see Appendix).

(4) 0.02 M phosphate buffer, pH 8.0 and the same buffer containing 6 mM DOC (see Appendix).

(5) 1% Triton X-100.

(6) Aquasol-2 (Dupont, Boston, MA, USA).

2.7.1. Measurement of Binding of Virus to K562 cells

Method:

(1) K562 cells were washed with cold PBS three times, and counted.

(2) About 5,000 cpm ^{3}H-labeled EMC virus was added to 2 x 10^{6} cells packed by centrifugation, and PBS was added to a final volume of 0.1 ml. Attachment of virus to cells was allowed to proceed for 30 minutes on ice.

(3) After adsorption, the sample was made up to 1 ml with PBS. The cells were pelleted by centrifugation, and the supernatant was saved. The cells were washed with another 1 ml PBS and the wash was saved. The cells were

lysed in 1 ml of 1% Triton X-100 at room temperature.

(4) The radioactivity in the supernatant, wash, and lysed cells was measured by adding 10 ml Aquasol-2 and counted in a liquid scintillation counter (LS 8100, Beckman Inc.).

(5) Calculation: % attachment of ^{3}H-labeled EMC to K562 cells = (Amount of radioactivity bound to cells) ÷ (Total radioactivity recovered) x 100%

2.7.2. Measurement of Binding of Virus to HeLa Cells

Method:

(1) About 2 x 10^{6} HeLa cells in each 35 mm tissue culture dish were rinsed with cold PBS to remove tissue culture medium.

(2) About 5,000 cpm ^{3}H-labeled EMC virus was added to each dish, and PBS was added to a final volume of 0.5 ml to disperse the virus. Unless otherwise stated, attachment of virus to cells was allowed to proceed for 30 minutes on ice.

(3) PBS was added to the dishes to give a final volume of 1 ml. Unbound radioactivity in the PBS solution was saved. The cells were washed again with 1 ml PBS, and the wash was saved. The cells were lysed in 1 ml of 1% Triton X-100 before measurement of radioactivity.

(4) Radioactivity in the bound, unbound and wash samples was measured, and attachment of ^{3}H-labeled EMC to HeLa cells was calculated as a percentage

of total radioactivity recovered (section 2.7.1.).

2.7.3. Measurement of Binding of Virus to Cell Membranes.

The method was used to investigate whether the bonds between EMC virus and its receptor on K562 and HeLa cells can be broken by 0.2 M NaCl.

Method:

(1) About 5,000 cpm of ^{3}H-labeled EMC virus was incubated with 140 µg cell membranes derived from 2 x 10^{6} cells in 0.02 M phosphate buffer, pH 8.0, in a final volume of 200 µl. Attachment of virus was allowed to proceed for 30 minutes on ice. Membranes and bound virus were pelleted by centrifugation for 5 minutes at 15,600 x g in an Eppendorf microcentrifuge and washed once with the same buffer.

(2) The membranes were resuspended in 200 µl of 0.02 M phosphate buffer containing sequential increases in NaCl concentration from 0 to 0.4 M, or directly with the same buffer containing 0.2 M NaCl, for 15 minutes. At the end of each incubation stage, membranes were pelleted by centrifugation. The released radioactivity in the supernatants was measured (section 2.7.1) and expressed as a percentage of total bound radioactivity.

2.7.4. Measurement of Binding of Virus to Immobilized Cell Membranes

This method was developed to investigate whether EMC virus binds to cell

membranes in the presence of 6 mM DOC.

Method:

(1) About 140 μg cell membranes were applied to nitrocellulose (pore size: 0.45 micron; Bio-Rad Laboratories, Richmond, CA, USA) using a Bio-Dot apparatus (Bio-Rad Laboratories). The nitrocellulose was blocked with 3% BSA in PBS-Tween (see Appendix) for 2 hours at room temperature and then washed briefly with PBS-Tween (see Appendix). Then the nitrocellulose dots containing cell membranes were punched out.

(2) About 5,000 cpm of ^{3}H-labeled EMC virus was incubated with each dot in 200 μl of 0.02 M phosphate buffer, pH 8.0 (see Appendix) or the same buffer containing 6 mM DOC for 30 minutes on ice.

(3) 0.02 M phosphate buffer, pH 8.0 was added to the tubes to give a final volume of 1 ml. Unbound radioactivity recovered in the phosphate buffer was saved. The dots were washed again with 1 ml of the phosphate buffer, and the wash was saved. Bound radioactivity on the dot was released by adding 1 ml of 1% Triton X-100. Bound and unbound radioactivities were measured (section 2.7.1). Attachment of ^{3}H-labeled EMC virus to membranes was calculated as a percentage of total recovered radioactivity (section 2.7.1).

2.8. Determination of Receptor Activity by Dot Blot Assay

Dot blots were used in this project to detect EMC virus receptor activity

of cell membranes, detergent solubilized membranes, enzyme-treated cell membranes and purified putative receptors.

Materials:

(1) Phosphate-buffered saline, pH 7.4 (PBS; see Appendix).

(2) PBS-Tween, 1% BSA in PBS-Tween and 3% BSA in PBS-Tween (see Appendix).

(3) Nitrocellulose membrane, pore size : 0.45 micron (Bio-Rad Laboratories) was soaked in PBS for 10 minutes before use.

(4) ^{125}I-labeled EMC virus (section 2.5.).

Samples analyzed for their receptor activity are indicated in each particular experiment.

Method:

(1) Samples analyzed were diluted by two-fold serial dilution with PBS, pH 7.4. Diluted samples were applied onto nitrocellulose in a dot-blot apparatus (Bio-Rad Laboratories), and left for 30 minutes. Excess liquid was removed by vacuum. All steps were carried out at room temperature.

(2) The nitrocellulose was washed briefly with PBS-Tween and then soaked in 3% BSA in PBS-Tween for 2 hours with gentle shaking to block unoccupied sites.

(3) The nitrocellulose was washed with PBS-Tween to remove the blocking buffer and then incubated with about 2 x 10^6 cpm of ^{125}I-labeled EMC virus

suspended in 10 ml of 1% BSA in PBS-Tween for 2 hours with gentle shaking. (4) The nitrocellulose was washed with several changes of PBS-Tween to remove unbound radioactivity and then air dried. The dried nitrocellulose was exposed to a Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y., USA) at -70^{0}C for 3 days, and the film developed in an automatic X-Omat processor.

2.9. Enzymatic Treatment of Cells, Cell Membranes and Purified Virus Receptor Proteins

Materials:

(1) Enzymes: *Vibrio cholerae* neuraminidase, bovine pancreas α-chymotrypsin, *Clostridium welchii* phospholipase C (CalBiochem, La Jolla, CA, USA); bovine pancreas trypsin, lima bean trypsin inhibitor (1 mg trypsin inhibitor inhibitors 3.5 mg trypsin) (Worthington Biochemical Co., Freehold, NJ, USA); papain (Sigma Chemical Co., St. Louis, MO, USA).

(2) Papain solution : 1.2 mg papain (19 units/mg) in 1 ml PBS was activated by adding 5 µl cystein-EDTA solution prepared by dissolving 3 mg of L-cysteine hydrochloride (GIBCO BRL) in 0.25 ml of 0.1 M ethylenediaminetetraacetic acid (EDTA)

(3) PBS washed cells in suspension or in monolayers (section 2.1.), ^{3}H-labeled EMC virus (section 2.2.2.), cell membranes (section 2.3.1.), surface ^{125}I-

labeled cell membranes (section 2.5) and ^{125}I-labeled affinity purified virus receptor proteins (section 2.13).

(4) Phosphate-buffered saline, pH 7.4 (PBS, see Appendix) and PBS, pH 5.6 (adjusted to pH 5.6 with 0.1 M HCl).

(5) Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS; GIBCO BRL).

(6) 0.1 M EDTA.

(7) Washing buffer consists of 2 mM EDTA, and 1 mM PMSF in PBS.

2.9.1. Enzymatic Treatment of Intact Cells

Method:

(1) 20 µg trypsin, 20 µg chymotrypsin, 40 U of phospholipase C or 1 U of activated papain in 0.5 ml PBS, pH 7.4, was added to each test tube or 35 mm dish containing 2 x 10^6 cells in suspension or in monolayer, respectively. For neuraminidase treatment, 40 mU enzyme in 0.5 ml PBS, pH 5.6, was added to the cells.

(2) Enzymatic digestions were performed at 37 ^{0}C for 60 minutes. Control samples were incubated with PBS, pH 7.4 or pH 5.6, corresponding to the enzyme buffers.

(3) The cells were washed with cold PBS three times to remove the enzyme. For protease digestions, the cells were washed with cold HBSS several times

to reduce cell aggregation caused by protease digestion.

(4) Virus attachment assays were performed as described (sections 2.7.1 and 2.7.2.). Note: For the protease digestions, which destroys the cell monolayer, the cells used in control samples were collected by scraping cells from dish with a rubber policeman. The attachment assay then followed the procedures used for the cells grown in suspension (section 2.7.1.).

2.9.2. Enzymatic Treatment of Cell Membranes

Method:

(1) For trypsin treatment, 280 µg cell membranes in each sample derived from 4×10^6 cells were incubated with 200 µg trypsin in 250 µl PBS, pH 7.4, at 37^0C for 1 hour, and the reaction was terminated by adding 200 µg trypsin inhibitor. Controls were treated similarly but without trypsin. The samples were diluted by two-fold serial dilution in PBS. The receptor activity was determined by dot-blot assay (section 2.8.).

For surface ^{125}I-labeled membranes, 3.5×10^5 cpm solubilized membranes in 6 mM DOC, approximately 30 µg protein (section 2.3.2.), were treated with 200 µg trypsin. Then, the enzyme was inactivated by adding 200 µg trypsin inhibitor. Controls were treated similarly, but without trypsin. The samples were analyzed on an EMC virus-Sepharose 4B column (section 2.13.).

(2) For neuraminidase treatment, 280 µg cell membranes in each sample were

incubated with 80 mUnits of neuraminidase in 200 µl PBS, pH 5.6, at 37°C for 1 hour. Enzyme digestion was terminated by adding 100 µl of 0.1 M EDTA. Controls were treated similarly in the absence of neuraminidase. The membranes were pelleted by centrifuging at 15,600 x g for 5 minutes. The supernatant was saved, and the membranes were resuspended in 200 µl PBS. The receptor activity in both membranes and supernatant was determined by dot-blot assay as described (section 2.8.).

For surface ^{125}I-labeled membranes, 3.5 x 10^5 cpm solubilized membranes in 6 mM DOC (section 2.3.2.) were treated with 80 mU of neuraminidase at 37°C, pH 5.6 for 1 hour. After digestion the enzyme was destroyed by boiling the samples for 5 minutes. Controls were treated similarly, but without neuraminidase. The samples were analyzed on an EMC virus-Sepharose 4B column (section 2.13.).

(3) For double neuraminidase digestion of cell membranes, after completion of the first neuraminidase digestion, as described above, the enzyme was removed by resuspending membranes in 0.5 ml wash buffer and centrifuging at 15,600 x g for 5 minutes. The wash procedure was repeated three times. Then, another 80 mU of neuraminidase was added and digestion was repeated at 37°C for 1 hour. 100 µl of 0.1 M EDTA was added to the sample to terminate enzymatic activity, and the membranes were pelleted by centrifugation. Controls were treated similarly, but without enzyme.

Receptor activity in both membranes and supernatant was determined by dot-blot assay as described (section 2.8.).

2.9.3. Enzymatic Treatment of ^{125}I-Labeled, Affinity Purified Virus Receptor Proteins

Enzymatic studies were performed using ^{125}I-labeled virus receptor proteins purified from K562 and HeLa cells by affinity chromatography on virus-Sepharose columns (section 2.13.).

Method:

(1) For trypsin and chymotrypsin treatments, about 2 x 10^5 cpm of ^{125}I-labeled purified receptor proteins from K562 and HeLa cells were treated with 20 µg trypsin or 20 µg chymotrypsin at 37°C for 1 hour. Controls were treated with PBS only. The effect of proteolytic digestions was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (sections 2.15 and 2.17).

(2) For neuraminidase treatment, about 10^5 cpm of ^{125}I-labeled purified receptor proteins from K562 and HeLa cells were incubated with 80 mUnits of neuramindase in 200 µl PBS, pH 5.6, at 37°C for 1 hour and analyzed by chromatofocusing column as described in section 2.19.

2.10. Effect of Lectins on Binding of EMC Virus to Cells

Materials:

(1) Lectins: Wheat germ agglutinin (WGA) from *Triticum vulgaris* and *Limulus polyphemus* agglutinin (LPA) from horseshoe crab (Sigma Chemical Co.).

(2) PBS-washed cells in suspension or monolayer (section 2.2.)

(3) ^{3}H-labeled EMC virus (section 2.1.).

(4) Phosphate-buffered saline, pH 7.4 (PBS see Appendix).

(5) Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS; GIBCO BRL).

Method:

(1) About 2×10^{6} cells in suspension or monolayer were used for each sample.

(2) 0.5 mg WGA or LPA in 0.5 ml PBS was added to each sample, respectively. The samples were incubated at 37 ^{o}C for 1 hour, and controls were treated similarly, but without lectins.

(3) The cells were washed with HBSS at least three times, before they were used in virus attachment assays (sections 2.7.1 and 2.7.2.).

2.11. Determination of Biosynthesis of EMC Virus Receptors after Trypsin Treatment

Materials:

(1) Bovine pancreas trypsin.

(2) Cycloheximide (Sigma Chemical Co.).

(3) Phosphate-buffered saline, pH 7.4 (PBS, see Appendix).

(4) Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS).

(5) RPMI medium for K562 cells and DMEM medium for HeLa cells (see Appendix).

(6) PBS-washed K562 and HeLa cells (section 2.2.)

(7) ^{3}H-labeled EMC virus (section 2.1.).

Method:

(1) K562 and HeLa cells washed with PBS were treated with trypsin as described (section 2.9.1.).

(2) The cells were washed with HBSS several times to reduce any cell aggregation caused by trypsin treatment. The cell viability was examined as described (section 2.2.1.).

(3) About 2×10^{6} cells in each sample were resuspended in 2 ml fresh culture medium or the medium containing various concentrations of cycloheximide, ranging from 2 μg to 12 μg, to inhibit protein synthesis.

(4) The cells were incubated in 5% CO_2 at 37°C for the time specified. Controls were treated similarly, but without trypsin or cycloheximide. Cell viability was examined as described (section 2.2.1).

(5) Attachment of ^{3}H-labeled EMC virus to the treated and control cells was determined as described (section 2.7.1., 2.7.2.).

2.12. Preparation of Virus-Sepharose Columns

Materials:

(1) Cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia).

(2) Coupling buffer: 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl.

(3) Blocking buffer: 0.2 M glycine, pH 8.0.

(4) Acetate buffer: 0.1 M CH_3COONa and 0.5 M NaCl, adjusted to pH 4.0 with 0.6 M acetic acid.

(5) 1 mM HCl.

(6) Purified unlabeled and ^3H-labeled EMC virus (section 2.1.).

Method:

(1) 1 mg CNBr-activated Sepharose 4B was swollen in 20 ml of 1 mM HCl for 15 minutes and slowly washed on a glass filter, 25 mm in diameter; (Millipore Canada Ltd., Mississauga, Ontario, Canada) with 200 ml of 1 mM HCl.

(2) The gel was washed with 5 ml coupling buffer, and then immediately transferred to a tube containing 1 mg purified EMC virus in 7 ml coupling buffer. The mixture was vertically rotated on a multi-purpose rotator (Scientific Industries Inc., Bohemia, N.Y., USA) overnight at 4^0C. Then, the gel was washed on the glass filter with 20 ml of coupling buffer.

(3) The remaining active groups of CNBr-activated Sepharose 4B were blocked by rotating the gel with 10 ml of blocking buffer for 2 hours at room temperature. The gel was washed with 10 ml of acetate buffer followed by 10

ml of coupling buffer to remove non-covalently bound material. The washing procedure was repeated three times. The virus-Sepharose was packed in a 0.9 x 22 cm Pharmacia column. The columns are stable at room temperature for several weeks (Allaway & Burness, 1987)

Note: A similar experiment using ^{3}H-labeled EMC virus was performed to determine how much virus can be covalently attached to the Sepharose matrix by the procedure used.

2.13. Virus-Sepharose Affinity Chromatography

Materials:

(1) EMC virus-Sepharose 4B column (section 2.12.)

(2) Loading buffer: 0.02 M phosphate buffer, pH 8.0 (see Appendix).

(3) Eluting buffer: 0.02 M phosphate buffer, pH 8.0, containing 0.2 M NaCl.

(4) Washing Buffer: 0.1% Triton X-100 in 0.02 phosphate buffer, pH 8.0.

Method:

(1) Unlabeled or surface ^{125}I-labeled cell membranes were solubilized in DOC as described (section 2.3.2). To investigate the effect of enzyme treatment on the binding of virus to solubilized receptors, surface-labeled solubilized cell membranes in 6 mM DOC were treated with trypsin or neuraminidase as described (section 2.9.2.). All the solubilized membranes were loaded onto the column in the presence of 6 mM DOC, and then left at

room temperature for 30 minutes.

(2) The column was washed with loading buffer, to remove unbound components, followed by eluting buffer to dissociate specifically bound components. Subsequently, the column was washed with washing buffer to remove any aggregated material and then re-equilibrated with loading buffer. Chromatography was performed at a flow rate of 0.4 ml/min. Fractions of 1 ml were collected in a fraction collector (7000 Ultrorac, LKB, Bromma, Sweden). For unlabeled cell membranes, the eluate was monitored for absorbance at 280 nm by an UV monitor (2138 UVICORD, LKB). For iodinated membranes, radioactivity of each fraction was measured in a gamma counter as described (section 2.5.).

(3) The fractions containing components eluted with 0.2 M NaCl in 0.02 M phosphate buffer, pH 8.0, were combined, and dialysed against several changes of deionised water at 4^{0}C, and then lyophilized for further analysis.

2.14. Dye-Binding Protein Assay

A dye-binding protein assay described by Winterbourne (1986) was used to estimate protein concentration in purified putative receptor preparations (section 2.13.).

Materials:

(1) Standard protein solution: 1 mg/ml BSA.

(2) Staining solution: 0.4 g Coomassie brilliant blue R-250 (BRL Inc., Gaithersburg, MD, USA) dissolved in 250 ml ethanol and 630 ml water. 44 ml of this solution was mixed with 6 ml glacial acetic acid, and filtered through Whatman no.1 paper (Whatman Inc).

(3) Destaining solution: 10% ethanol, 5% acetic acid.

(4) Desorbing solution: 1 M potassium acetate in 70% ethanol.

Method:

(1) Lyophilized receptor protein (section 2.13.) was dissolved in 20 µl to 50 µl volume of deionised water.

(2) Increasing volumes (1, 2, 5, and 8 µl) of both standard and test proteins were adsorbed separately onto 1 cm^2 pieces of Whatman 3MM filter paper and air-dried.

(3) The filters were soaked in staining solution for 1 hour, washed with several changes of destaining solution until a clear background was achieved and air-dried.

(4) The filter papers were transferred to separate test tubes and 1 ml of desorbing solution was added and left for 1 hour. All solution samples, including one without protein, were read at 590 nm to measure absorbance.

(5) Absorbances of samples containing BSA standards were plotted against protein concentrations. The protein content in the test samples were estimated from the graph.

2.15. SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis on 10% polyacrylamide gels with sodium dodecyl sulphate (SDS) was performed as originally described by Laemmli (1970).

Materials:

(1) 40% acrylamide.

(2) 1% N,N'-Methylenebisacrylamide (bis acrylamide).

(3) N,N,N', N'-Tetramethylethylenediamine (TEMED). All the above chemicals were purchased from GIBCO BRL.

(4) 20% SDS (Sigma Chemical Co.)

(5) 3 M Tris-HCl, pH 8.7, and 1 M Tris-HCl pH 6.8 (see Appendix).

(6) 10% ammonium persulphate (Sigma Chemical Co.)

(7) EDTA.

(8) Running buffer: 6.0 g Tris, 28.8 g glycine and 2.0 g SDS dissolved in 2000 ml of deionised water.

(9) 2x Sample buffer: 1 ml 0.5 M Tris-HCl buffer, pH 6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2-mercaptoethanol, and 0.2 ml of 0.05% bromophenol blue mixed together, and made up with deionised water to a final volume of 8 ml.

(9) Molecular weight markers: ^{14}C-methylated high molecular weight protein standards (Amersham Canada Ltd., Oakville, Ont., Canada); prestained high molecular weight protein standards (GIBCO BRL).

Method:

(1) For preparation of lower polyacrylamide gel, 7.5 ml of 40% acrylamide, 7.8 ml of bis-acrylamide, 3.75 ml of 3 M Tris-HCl, pH 8.7, 0.2 ml of 10% ammonium persulphate, and 10.35 ml of deionised water were mixed and degassed for 3 minutes. 0.15 ml of 20% SDS and 0.02 ml of TEMED were added to the mixture immediately before the gel was poured into a vertical slab gel with 1.5 mm spacer (Bio-Rad Laboratories). About 0.5 ml of 1-butanol was layered on top of the gel to ensure a level gel surface. Polymerization was allowed to proceed for 30 to 60 minutes after which the butanol was washed off by rinsing with deionised water.

(2) For preparation of upper polyacrylamide gel, 1.25 ml of 40% acrylamide, 1.3 ml of 1% bis-acrylamide, 1.25 ml of 1 M Tris-HCl, pH 6.8, 0.1 ml of 10% ammonium persulphate, 5.0 ml of deionised water and 1.0 ml of 100 mM EDTA were mixed, and degassed. 0.01 ml of TEMED and 0.05 ml of 20% SDS were added to the mixture before pouring over the lower gel. A plastic comb was carefully inserted into upper gel to form sample wells.

(3) Sample preparation and gel electrophoresis were performed as follows: An equal volume of 2x sample buffer was added to the sample to be analyzed. Samples were boiled for 3 minutes before loading onto the gel. Electrophoresis was carried out at constant voltage of 45 volts for 15 to 16 hours at room temperature. The molecular weight of the examined protein

bands was determined from molecular weight standards run in parallel.

2.16. Coomassie Blue Gel Staining

Materials:

(1) Fixing solution: 25% isopropyl alcohol, 10% acetic acid.

(2) Staining solution: 0.5% Coomassie brilliant blue R-250 in 50% methanol and 5% acetic acid, diluted 1/10 with deionzed water before use.

(3) Destaining solution: 10% methanol, 10% acetic acid.

Method:

The gel was soaked in fixing solution for 2 hours and then in staining solution for another 2 hours. The stained gel was washed with several changes of destaining solution until a clear background was obtained. The gel was photographed for permanent record.

2.17. Autoradiography

When radioactive material was run on SDS-PAGE, the protein bands were visualized by autoradiography after drying the gel onto a piece of 3MM filter paper (Whatman Inc.). Proteins transferred to nitrocellulose from polyacrylamide gels and probed with ^{125}I-labeled virus or ^{125}I-anti-mouse antibody were visualized similarly. The dried gel or nitrocellulose membrane was exposed to a Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester,

NY, USA) at -70°C before development as described (section 2.8.).

2.18. Western Blotting and Virus Overlay Protein Blot Assay

Materials:

(1) Transfer buffer: 48 mM Tris, 39 mM glycine, 20% methanol pH 9.2.

(2) Washing buffer: PBS-Tween (see Appendix).

(3) Blocking buffer: 3% BSA in PBS-Tween, pH 7.4 (see Appendix).

(4) Blotting buffer: 1% BSA in PBS-Tween (see Appendix).

(5) ^{125}I-labeled EMC virus (section 2.5.).

(6) Monoclonal anti-glycophorin antibody in hybridoma culture fluids (American Type Culture Collection, Rockville, MD, USA).

(7) ^{125}I-labeled sheep anti-mouse antibody (Amersham Canada Ltd., Oakville, Ont., Canada).

Method:

After electrophoresis (section 2.15.), the gel was soaked in transfer buffer for 15 minutes. Proteins on the gel were electroblotted onto nitrocellulose (pore size: 0.45 micron; Bio-Rad Laboratories) using a Trans-blot cell (Bio-Rad Laboratories) at 60 V and 0.2 A for 6 hours. After transfer, the nitrocellulose was incubated with blocking buffer for 2 hours at room temperature with agitation. The blot was briefly washed with washing buffer. For virus overlay protein blot assay (VOPBA), the nitrocellulose was probed

with about 2 x 10^6 cpm ^{125}I-labeled EMC virus in 10 ml of blotting buffer overnight at 4°C. For Western blotting, the nitrocellulose was incubated with monoclonal anti-glycophorin A antibodies diluted 1/10 in blotting buffer for 2 hours at room temperature. The blot was washed with washing buffer to remove unbound antibodies. Subsequently, the blot was probed with 1 x 10^6 cpm of ^{125}I-labeled sheep anti-mouse antibody in blotting buffer for 2 hours at room temperature. All the blots were washed with several changes of washing buffer to remove unbound radioactivity. The proteins on the blots were visualized by autoradiography (section 2.17.).

2.19. Chromatofocusing of EMC Virus Receptor Proteins

Materials:

(1) Polybuffer exchanger PBE 94 preserved in an equal volume of 24% ethanol (Pharmacia)

(2) Starting buffer: 0.025 M Imidazole-HCl, pH 7.4.

(3) Eluting buffer: Polybuffer 74 (Pharmacia) diluted 1/8 with deionised water and adjusted to pH 4.0 with 0.1 M HCl.

Method:

(1) For preparation of the chromatofocusing column, 10 ml polybuffer exchanger PBE 94 was poured into a glass filter holder (Millipore Canada Ltd.) and then equilibrated under vacuum with 80 ml of starting buffer or

until the pH of the eluate was 4.0. The gel was dispersed in an equal volume of starting buffer, degassed, and carefully packed into a 0.9 x 22 cm Pharmacia column. The column was washed with starting buffer at a flow rate of 1 ml/min.

(2) Sample application was done as follows: about 10^5 cpm of ^{125}I-labeled, affinity purified EMC virus receptors were loaded onto the column. For the desialylated receptor, the ^{125}I-labeled receptor preparation was treated with *Vibrio cholerae* neuraminidase as described (section 2.9.3.) before loading onto the column. Controls were treated similarly, but without neuraminidase. The samples were allowed to adsorb to the column for 5 minutes.

(3) Running the column: Eluting buffer was applied to the column at a flow rate of 0.4 ml/minute and 1 ml fractions were collected. The pH of each fraction was measured using a pH-meter (Expandomatic SS-2, Beckman Inc.). The radioactivity of each fraction was measured in a gamma counter as described (section 2.5.).

Chapter 3

Results

3.1. Kinetics of Attachment of EMC Virus to HeLa Cells

The attachment of EMC virus to K562 cells has previously been examined (Pardoe & Burness, unpublished results). Virus binding to K562 cells was found to be relatively rapid, with maximum binding observed within 20 minutes. Using Scatchard plot analysis to examine the data on incubation of increasing amounts of EMC virus to K562 cells, an equilibrium dissociation constant (Kd) of 2.7 nM and 3.6 x 10^5 receptor sites/cell were calculated (Pardoe, personal communication).

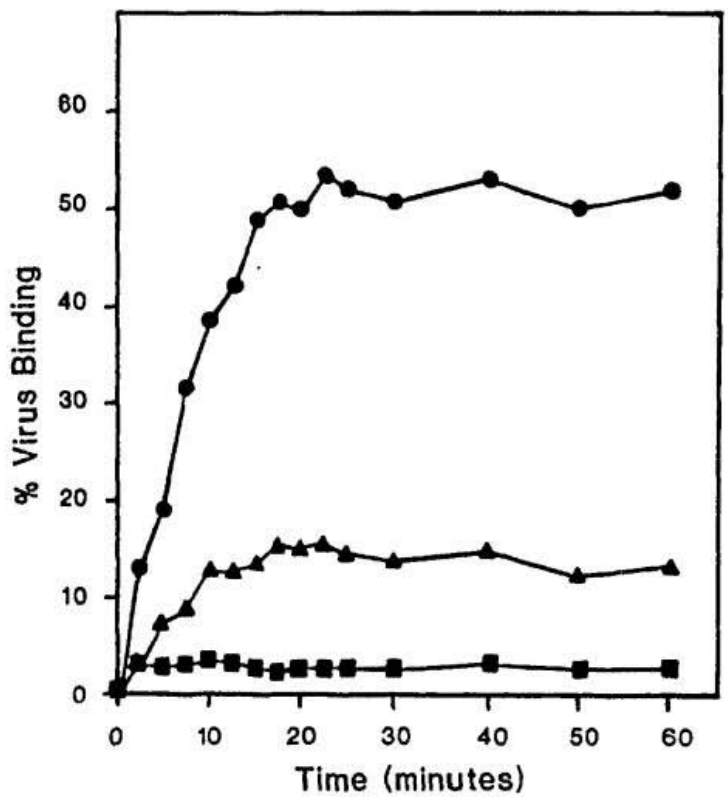
In this project, binding kinetics of EMC virus to HeLa cells were examined. Attachment studies using ^{3}H-labeled EMC virus were carried out on ice to reduce virus internalization and changes in fluidity of cell membranes. As shown in Figure 1, binding of virus increased almost linearly during the initial 10 to 12 minutes of incubation, and maximum binding was obtained between 20 to 30 minutes. The specificity of virus binding to HeLa cells was established by competition with unlabeled virus and by demonstration of its saturability. To demonstrate competition binding in the virus-receptor interaction, HeLa cells were incubated with an excess of unlabeled virus for 30 minutes before the addition of ^{3}H-labeled virus. Binding of radiolabeled

virus to HeLa cells was determined at the times indicated in Figure 1. As shown, binding of labeled virus to HeLa cells was inhibited approximately 96% by pre-incubation of the cells with a 30-fold excess of unlabeled virus. No further inhibition was observed by using 100-fold unlabeled virus (data not shown). These results show that unlabeled virus competitively inhibits the binding of labeled virus to HeLa cells.

To determine whether EMC virus receptor sites on HeLa cells are saturable, increasing amounts of ^{3}H-labeled EMC virus were incubated with a constant number (2×10^{6}) of HeLa cells. Bound and unbound virus was measured (section 2.7.2.). The number of bound virus particles per cell was plotted against the number of unbound virus particles per cell (Fig. 2A). A hyperbolic pattern of binding curve obtained suggests that EMC virus receptor sites on HeLa cells are saturable. These data, together with the results of competition experiments described above, reveal that EMC virus binds to a finite number of receptors on HeLa cells.

When the binding data in Figure 2A were replotted in the form of a Scatchard plot (Fig. 2B), a straight line was obtained, suggesting the presence of a single class of receptors for EMC virus on HeLa cells. The total number of receptor sites that can be occupied by virus (Bmax) was obtained from the intercept on the X axis of the Scatchard plot. From the value of Bmax, 1.6

Figure 1. Attachment of EMC virus to HeLa cells. 2×10^6 cells in a culture dish were washed with PBS and incubated with 5,000 cpm of ^{3}H-labeled EMC virus (●) or pre-incubated with 10-fold (▲) or 30-fold (■) excess of unlabeled virus for 30 minutes before the addition of labeled virus. Attachment of virus to cells was determined at the indicated time periods and expressed as a percentage of the total recovered radioactivity, calculated according to the formula presented in section 2.7.1. Each point represents the mean of duplicate determinations.

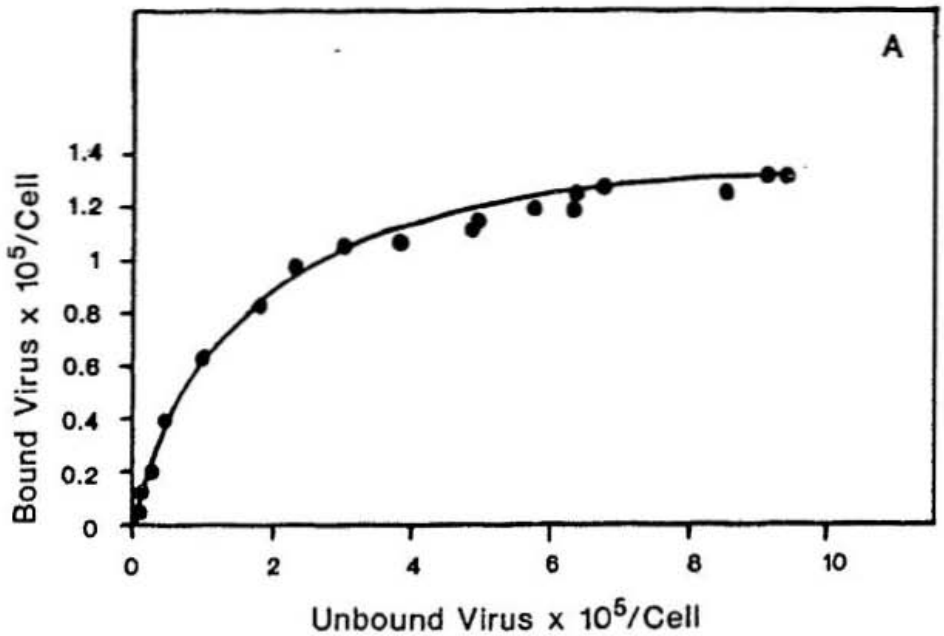

% Virus Binding
0
10
20
30
40
50
60
0
10
20
30
40
50
60
Time (minutes)

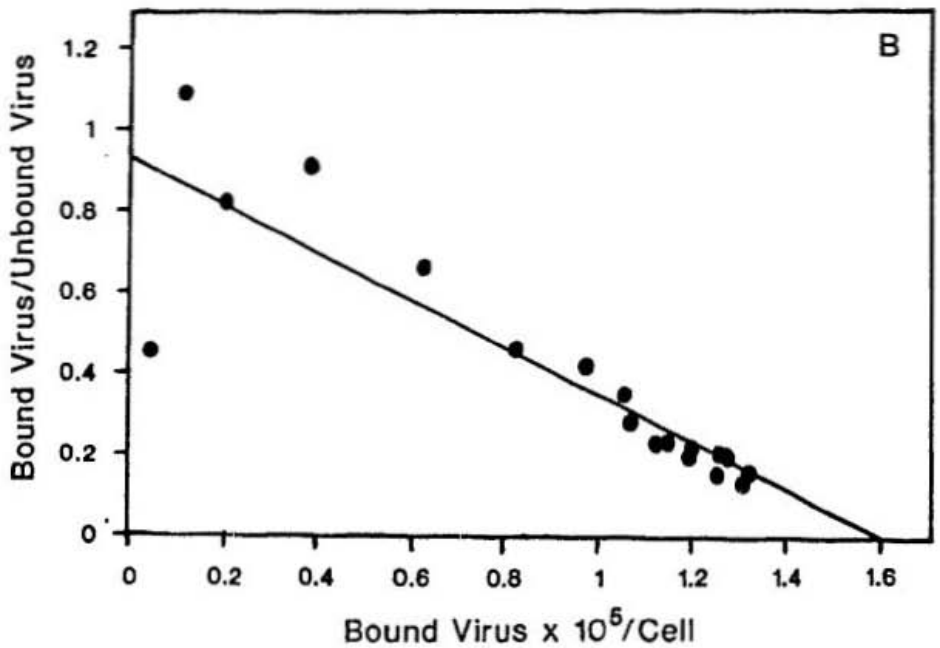
x 10^5 receptors/cell was estimated. The equilibrium dissociation constant (Kd), expressed in moles, was calculated from the slope of the Scatchard plot and was found to be approximately 1.1 nM.

3.2. Biochemical Characterization of EMC Virus Receptors on K562 and HeLa Cells

Most picornavirus receptors are believed to be cell surface glycoproteins (Zajac & Crowell, 1965; Stott & Heath, 1970; Lonberg-Holm, 1975; Krah & Crowell, 1985). For EMC virus, glycophorin A has been defined as its receptor on erythrocytes (Allaway & Burness, 1986; Allaway *et al.*, 1986). In addition, the receptors for EMC virus on two mouse cell lines, Krebs and insulinoma cells also appear to be glycoproteins (Pardoe & Burness unpublished results; Baldeh & Burness, manuscript in preparation). To characterize the biochemical nature of receptors for EMC virus on K562 and HeLa cells, cells were treated with selected proteases, sialidase, lipase and sialic acid specific lectins (sections 2.9.1 and 2.10.) and the receptor activity remaining on the cell surfaces after treatment was measured by attachment of ^{3}H-labeled virus as described (sections 2.7.1 and 2.7.2.). Trypsin and neuraminidase digestions were also performed on cell membrane preparations

Figure 2. Saturation of EMC virus receptors on HeLa cells. 2 x 10^6 cells in a culture dish were washed with PBS and incubated with increasing amounts of ^{3}H-labeled EMC virus on ice for 30 minutes. (A) Kinetics of EMC virus binding determined by plotting the number of bound virus particles against the number of unbound virus particles per cell. Each point is the mean of four determinations. (B) Scatchard plot of the binding data.


A
Bound Virus x 10^5/Cell
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Unbound Virus x 10^5/Cell


B
Bound Virus/Unbound Virus
1.2
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Bound Virus x 10^5/Cell

derived from K562 and HeLa cells as described in section 2.9.2.

3.2.1. Effect of Protease and Phospholipase C on Attachment of EMC Virus to Intact Cells

K562 and HeLa cells were treated with trypsin, chymotrypsin, papain and phospholipase C as described in section 2.9.1. Cell viability, determined by trypan blue staining, was not impaired by enzyme digestion. As shown in Table 1, treatment of cells with trypsin, chymotrypsin and papain resulted in a reduction in virus binding to HeLa cells of more than 84% compared with untreated control cells. A reduction of about 60% to 80% was observed with K562 cells, depending on the protease used. Phospholipase C, which affects lipid components of cell membranes, had no effect on virus binding to either cell type. The results suggest that the receptors for EMC virus on K562 and HeLa cells are protein in nature.

3.2.2. Effect of Neuraminidase and Lectins on Attachment of EMC Virus to Intact Cells

K562 and HeLa cells were treated with *Vibrio cholerae* neuraminidase, under conditions described in section 2.9.1, to release terminal sialic acids

from sialylated oligosaccharides. As shown in Table 1, treatment resulted in a 70% to 80% reduction in virus binding when compared with untreated control cells. The results suggest that sialic acid residues are involved in the binding of virus to receptors on K562 and HeLa cells.

Two lectins, wheat germ agglutinin (WGA) and *Limulus polyphemus* agglutinin (LPA), which both recognize and bind to sialic acid residues, were selected to see if they were able to block virus attachment. Under conditions described in section 2.10, pre-treatment of cells with the lectins resulted in a reduction of about 90% in virus binding to K562 and HeLa cells, compared to untreated controls (Table 1). This suggests that the lectins are binding to sialic acid residues and are competing with the virus by blocking the binding sites.

Sialic acids on the cell surface are attached to oligosaccharides on either glycoproteins or glycolipids. As the results from protease and phospholipase C digestions suggest that receptors are proteins not lipids, and neuraminidase digestion and lectin treatments indicate an involvement of sialic acids in EMC virus receptor activity on K562 and HeLa cells, it appears that the receptors are sialoglycoproteins.

Table 1. Effect of enzymes and lectins on EMC virus binding to K562 and HeLa Cells[1]

Treatment	% virus binding to K562 cells	% virus binding to HeLa cells
Untreated Cells	100.0	100.0
Enzyme-treated cells:		
Trypsin	25.4	13.1
Chymotrypsin	38.4	14.6
Papain	17.1	15.4
Phospholipase C	98.4	98.6
Neuraminidase	20.9	29.2
Lectin-treated cells		
WGA[2]	10.6	11.8
LPA[3]	8.1	10.6

[1]K562 or HeLa cells were treated with the indicated enzymes or lectins under conditions described in sections 2.9.1 and 2.10. Binding of ^{3}H-labeled virus to the treated cells was expressed as a percentage of the virus binding to untreated cells, calculated as described in sections 2.7.1 and 2.7.2. Each value is the mean of four determinations.

[2]Wheat germ agglutinin (WGA).

[3]*Limulus polyphemus* agglutinin (LPA).

3.2.3. Effect of Trypsin and Neuraminidase on Attachment of EMC Virus to Cell Membrane Preparations

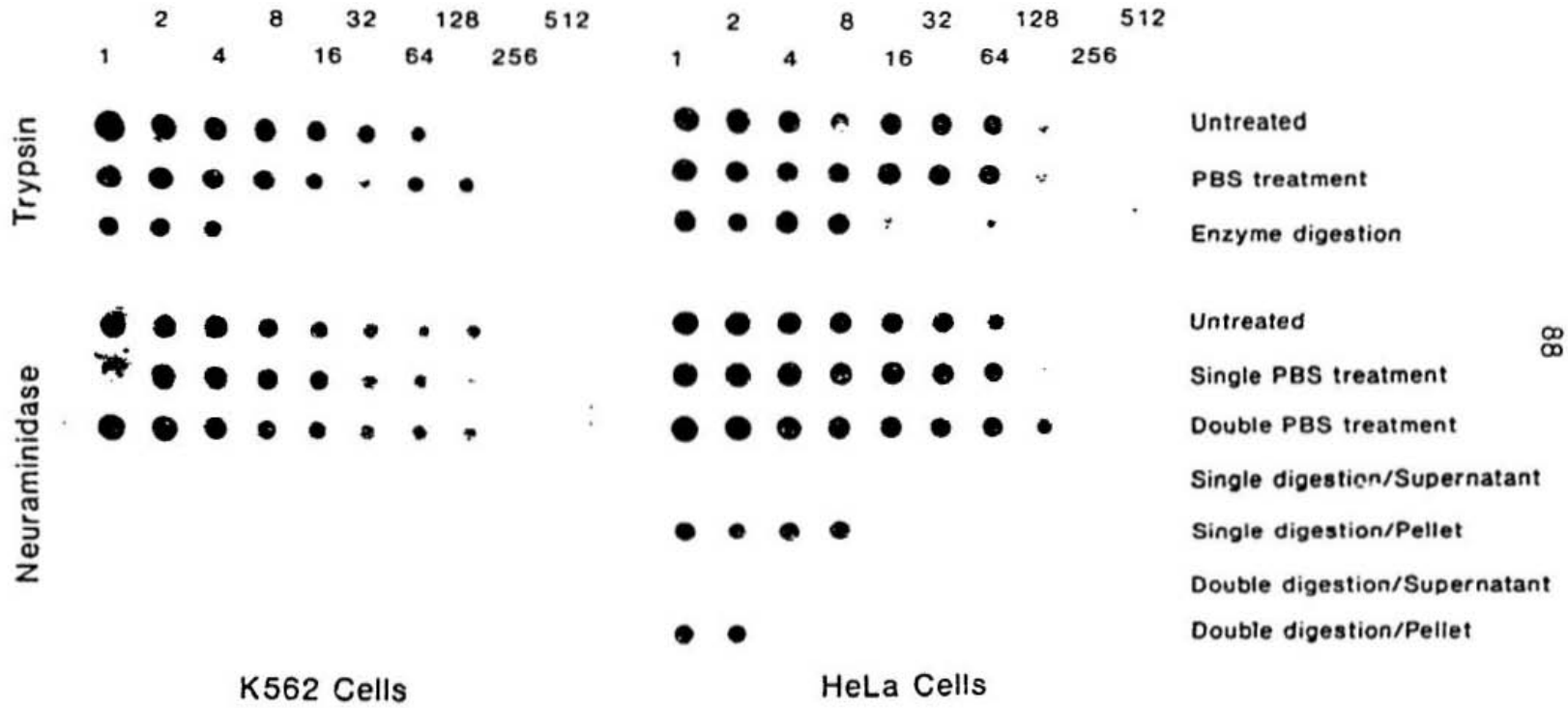
As mentioned, treatment of intact cells with proteases affect the integrity of the cell membranes in various ways (section 1.6.). To be more confident that the virus receptors are sialoglycoproteins, a similar enzymatic study was performed using cell membrane preparations. In this experiment, cell membranes were treated with trypsin or neuraminidase under conditions described in section 2.9.2. Receptor activity remaining on the cell membranes after trypsin treatment was determined by dot-blot analysis using ^{125}I-labeled EMC virus (section 2.8.). In neuraminidase-treated samples, cell membranes were pelleted by centrifugation, and receptor activity in both pelleted membranes and supernatant was determined. As shown in Figure 3, in trypsin-treated cell membranes, receptor activity decreased by 4- to 8-fold on K562 cells, and by 2- to 4-fold on HeLa cells. Single neuraminidase treatment reduced receptor activity remaining on K562 cell membranes by 128- to 256-fold, whereas HeLa cells showed a 16- to 32-fold reduction in receptor activity (Fig. 3). These findings are consistent with the previous results using intact cells, in which virus binding was significantly reduced after trypsin and neuraminidase treatment (Table 1), suggesting that the receptors are sialoglycoproteins. However, althought receptor activity on both K562 and

HeLa cell membranes was greatly decreased after single neuraminidase digestion (Fig. 3), it was not totally abolished. Therefore, double neuraminidase digestion was performed to investigate whether the remaining receptor activity could be eliminated by further neuraminidase treatment (section 2.9.2.). It was found that double neuraminidase digestion reduced the receptor activity to near background level on K562 cells and by 64- to 128-fold on HeLa cells (Fig. 3). Therefore, the activity which resisted the first treatment was still sensitive to further neuraminidase digestion. This further supports the hypothesis that the receptors are sialoglycoproteins. Moreover, as free sialic acids are able to bind to nitrocellulose (Sarris & Palade, 1979), receptor activity in the supernatants resulting from single and double neuraminidase digestion, which should contain released sialic acids, was also examed for virus binding using the dot-blot assay (section 2.8.). However, no receptor activity was detected in the supernatants suggesting that free sialic acid alone does not serve as a receptor for EMC virus. This finding is consistent with the results of previous studies (Angel & Burness, 1977).

3.3. Effect of Cycloheximide on the Regeneration of EMC Virus Receptors on Cells after Trypsin Treatment

Trypsin treatment performed under the conditions used in this project

Figure 3. Effect of trypsin and neuraminidase digestion on the binding of EMC virus to K562 and HeLa cell membranes. Membrane preparations were subjected to single trypsin digestion or, single or double neuraminidase digestion as described in section 2.9.2. Native membranes (untreated) and membranes treated with PBS (PBS treatment) were used as controls. In the trypsin digestion experiment, receptor activity associated with the digested membranes (enzyme digestion) and with the control membranes was determined using serial two-fold dilutions, presented as reciprocal values, and dot-blot radioligand binding assay as described in section 2.8. In the neuraminidase digestion experiment, samples subjected to single or double digestion were centrifuged and the receptor activity was determined in the resulting supernatants and membrane residue pellets, as well as in native (untreated) and PBS treated (single or double PBS treatment) membranes using serial two-fold dilutions.


2
8
32
128
512
1
4
16
64
256
Trypsin
Neuraminidase
Untreated
PBS treatment
Enzyme digestion
Untreated
Single PBS treatment
Double PBS treatment
Single digestion/Supernatant
Single digestion/Pellet
Double digestion/Supernatant
Double digestion/Pellet
K562 Cells
HeLa Cells

(section 2.9.1.) did not affect cell viability. Regeneration of virus receptors on K562 and HeLa cells after trypsin treatment was examined in a time course experiment. Both trypsin-treated cells and non-treated cells were incubated in fresh medium and binding of ^{3}H-labeled virus to cells was examined at the times indicated in Figure 4, as described in section 2.11. It required 6 to 8 hours for receptor activity on K562 and HeLa cells to be restored to the levels expressed by untreated cells (Fig. 4). Therefore, in the next experiment, 8 hours was allowed for receptor activity to recover on trypsin-treated cells.

To determine whether the recovery of receptor activity on trypsin-treated cells was due to synthesis of new receptors or to replacement from intracellular pools, cycloheximide, which blocks protein synthesis by inhibiting peptidyl transferase activity, was used to inhibit protein synthesis. In this experiment, various concentrations of cycloheximide were added to the medium of trypsin-treated cells (section 2.11.). After 8 hours, binding of ^{3}H-labeled EMC virus to those cells was examined and compared with control cells. In medium without cycloheximide, receptor activity was restored to the level of normal cells. In contrast, cycloheximide inhibited regeneration of receptor activity in a dose-dependent manner (Fig. 5), and 8-10 μg/ml cycloheximide nearly fully inhibited receptor regeneration. Cell viability examined before and after incubation with cycloheximide was not significantly

Figure 4. Time course of EMC virus receptor synthesis in K562 and HeLa cells. Trypsin-treated K562 (●) or HeLa (▲) cells were washed with Hanks' balanced salt solution and incubated in fresh media for the indicated time periods. Control cells were treated similarly, but without trypsin. Binding of ^{3}H-labeled EMC virus to cells was determined and expressed as a percentage of the binding to controls (section 3.4). Each point represents the average of duplicate determinations.

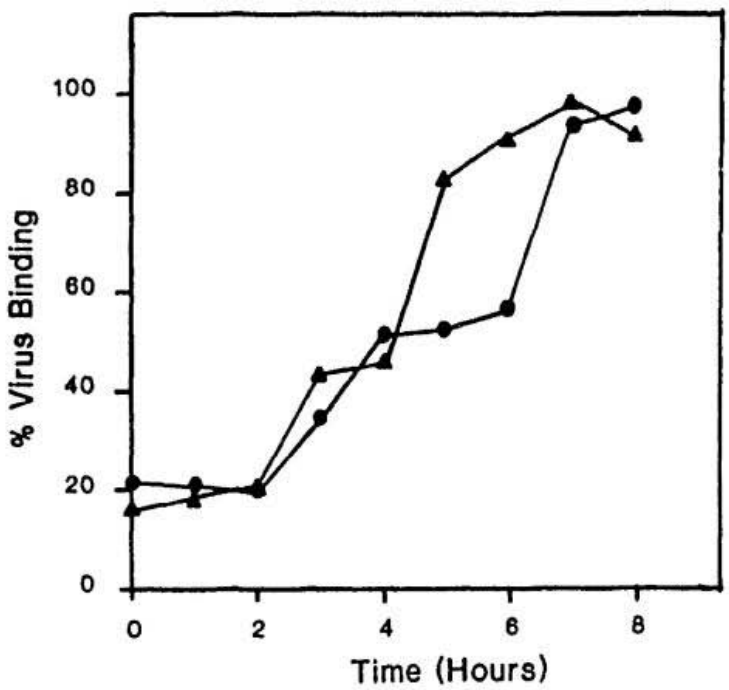
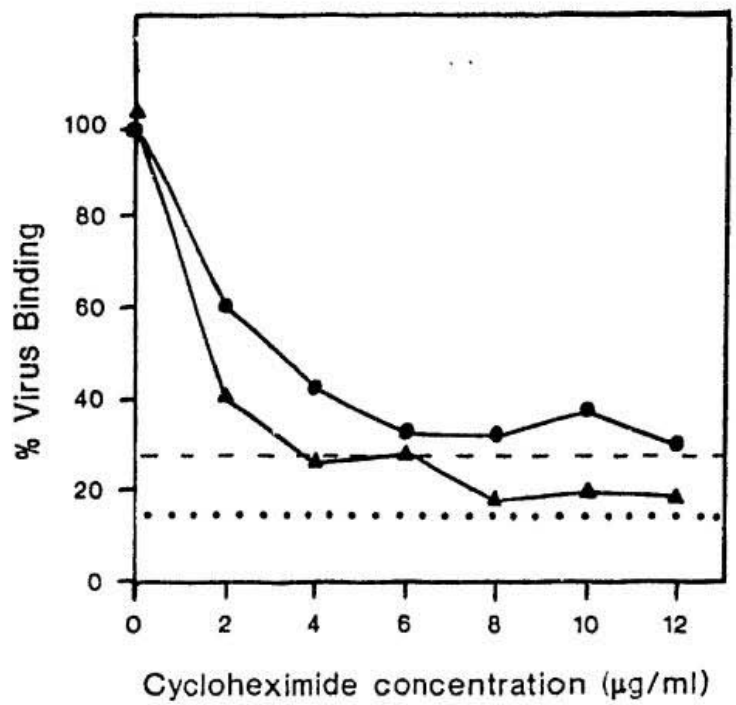

100
80
60
40
20
0
% Virus Binding
0
2
4
6
8
Time (Hours)

Figure 5. Inhibition of EMC virus receptor synthesis by cycloheximide in K562 and HeLa cells. Trypsin-treated K562 (●) or HeLa (▲) cells were washed with Hanks' balanced salt solution and incubated in fresh media containing the indicated concentrations of cycloheximide. Control cells were treated similarly, but without trypsin and cycloheximide. After 8 hours incubation, binding of ^{3}H-labeled virus to the cells was determined and expressed as a percentage of the binding to controls (section 3.3.). Points are the means of duplicate determinations. Dashed and dotted lines are the levels of receptor activity expressed by trypsin-treated K562 and HeLa cells, respectively.


% Virus Binding
100
80
60
40
20
0
0
2
4
6
8
10
12
Cycloheximide concentration (µg/ml)

reduced. The findings suggest that regeneration of virus receptors depends on protein synthesis rather than replacement from an intracellular pool.

3.4. Examination of Affinity Chromatography Conditions

In this study, EMC virus-Sepharose affinity chromatography was used to purify receptors from K562 and HeLa cells. The rationale of this affinity chromatography method was based on previous studies of the interaction between glycophorin and EMC virus (section 1.7.3.). However, those results may or may not apply to the interaction between EMC virus and its receptor on K562 and HeLa cells. Therefore, two critical conditions, the detergent concentration capable of solubilizing cell membrane receptors without inhibiting their binding activity for virus, and NaCl concentration in the eluting buffer, were re-examined before the technique was adopted.

3.4.1. Detergent Concentration.

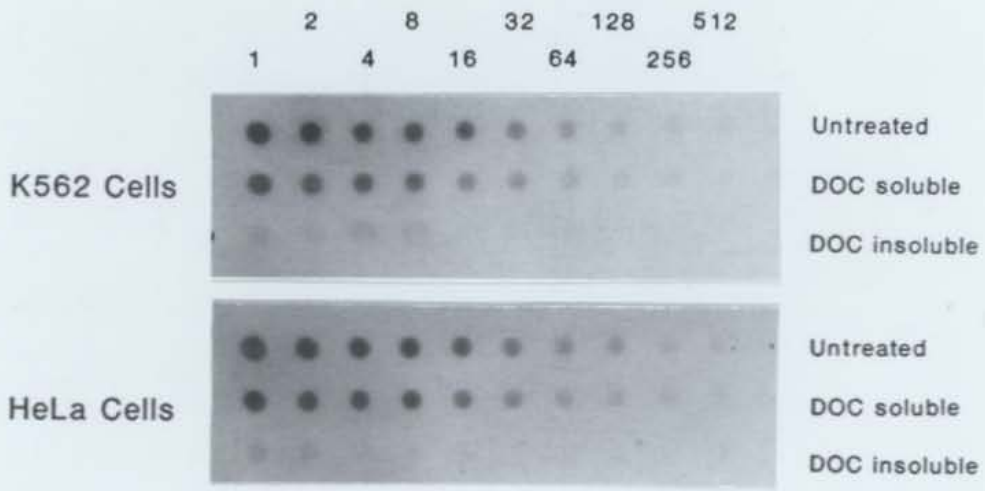
Before using virus affinity chromatography as a method for receptor purification, one has to determine an optimum detergent concentration that is able to extract most of the receptor activity from the cell membranes, but also allows the receptor to bind to the virus immobilized on the column matrix. It has been shown that diluting the DOC concentration from 12 mM

to 6 mM, following solubilization of erythrocyte membranes, allows glycophorin to bind specifically to an EMC virus-Sepharose column (Allaway & Burness, 1987; Baldeh, 1987). Whether this applied to the interaction between EMC virus and its receptor on K562 and HeLa cells was unknown. We asked the following two questions: is 12 mM DOC able to extract most of the virus receptors from K562 and HeLa cell membranes, and does the virus bind to its receptors on K562 and HeLa cells in the presence of 6 mM DOC?

To address the first question, about 140 µg cell membranes were solubilized in 12 mM DOC and then diluted to reduce the detergent concentration to 6 mM as described in section 2.3.2. Soluble and insoluble material resulting after DOC treatment were separated by centrifugation and both samples were tested for receptor activity by probing with ^{125}I-labeled EMC virus by dot-blot assay (section 2.8.). Native cell membrane preparations were also assayed as controls. Results showed that the level of receptor activity in the DOC-soluble material was very close to that in native untreated cell membranes, whereas very little receptor activity remained in the DOC insoluble material (Fig. 6). These results demonstrated that most of the EMC receptor activity can be extracted from cell membranes by 12 mM DOC and that the receptor remains soluble after diluting the detergent to 6 mM.

To answer the second question, whether virus binds to its receptors on

Figure 6. Evaluation of EMC virus receptor activity in sodium deoxycholate (DOC)-solubilized cell membranes of K562 and HeLa cells. Membranes derived from K562 or HeLa cells were solubilized in 12 mM DOC as described in section 2.3.2. and receptor activity in soluble, insoluble fractions of the membranes and native membranes (untreated) was determined using the indicated serial two-fold dilutions, presented as reciprocal values, and dot-blot radioligand binding assay, as described in section 2.8.


2
8
32
128
512
1
4
16
64
256
K562 Cells
Untreated
DOC soluble
DOC insoluble
HeLa Cells
Untreated
DOC soluble
DOC insoluble

K562 and HeLa cells in the presence of 6 mM DOC, about 140 μg cell membranes per dot were applied to nitrocellulose. Unoccupied sites on the nitrocellulose were blocked by 3% BSA in PBS-Tween and the dots containing the cell membranes were punched out. ^{3}H-labeled virus was incubated with each dot, with or without 6 mM DOC, as described in section 2.7.4. Membranes derived from K562 cell D clone and a blank nitrocellulose dot were used as controls. Table 2 shows that the amounts of virus binding to its receptor in the presence of 6 mM DOC are very close to the binding in the absence of DOC. Therefore, it appears that 6 mM DOC does not inhibit the binding of EMC virus to the receptor.

3.4.2. NaCl Concentration

Another critical condition to be considered in this virus specific affinity chromatography method is the NaCl concentration in the eluting buffer. The bond between EMC virus and glycophorin A is a weak ionic bond which can be easily broken by 0.2 M NaCl (Allaway & Burness, 1987). We designed an experiment to determine whether the bond between the virus and its receptors on K562 and HeLa cells can also be broken by buffer containing 0.2 M NaCl. K562 and HeLa cell membranes were incubated with ^{3}H-labeled EMC virus. Unbound virus was removed and the membranes with bound radiolabeled

Table 2. Binding of EMC virus to K562 and HeLa cell membrane preparations in the presence of 6 mM DOC[1]

Origin of cell membranes	Presence of 6mM DOC	% virus binding
K562 cells	–	57.8
	+	51.8
HeLa cells	–	43.0
	+	42.0
K562 cell D clone	–	5.4
No membranes	–	4.8

[1]Membranes of K562 or HeLa cells immobilized on nitrocellulose were tested for binding of EMC virus in the presence or absence of 6 mM sodium deoxycholate (DOC). Binding of ^{3}H-labeled virus was expressed as a percentage of the total recovered radioactivity calculated according to the formula presented in sections 2.7.1. Each value is the mean of duplicate determinations. Cell membranes derived from K562 cell D clone and blank nitrocellulose membranes were used as controls.

virus were incubated sequentially with buffer containing 0.05 M, 0.1 M, 0.2 M and 0.4 M NaCl (section 2.7.3.). At the end of each incubation stage, the membranes were pelleted, and radioactivity released into the supernatant was measured. Data in Table 3 shows that about 76% of the virus was released when 0.2 M NaCl buffer was added after sequential elution of the membranes with 0.05 M and 0.1 M NaCl. They also show that further increases in concentration of NaCl to 0.4 M did not significantly influence release of virus. In another experiment, membranes with bound radiolabled virus were incubated directly with buffer containing 0.2 M NaCl. This time more than 90% of the previously bound virus was released from the membranes (Table 3). The results show that the bond between EMC virus and its receptors on K562 and HeLa cells could be easily disrupted by 0.2 M NaCl.

Taken together, the obtained findings suggest that 12 mM DOC is able to solubilize most of the receptor activity from K562 and HeLa cell membranes, that diluting the DOC concentration to 6 mM, following membrane solubilization, allows the virus to bind to its receptors, and that buffer containing 0.2 M NaCl releases bound virus from membranes. Therefore, it appeared that the EMC virus-Sepharose affinity chromatography technique used for purification of glycophorin would be a feasable method to isolate virus receptors from K562 and HeLa cells.

Table 3. Elution of ^{3}H-labeled EMC virus bound to K562 and HeLa cell membranes by varied concentrations of NaCl[1]

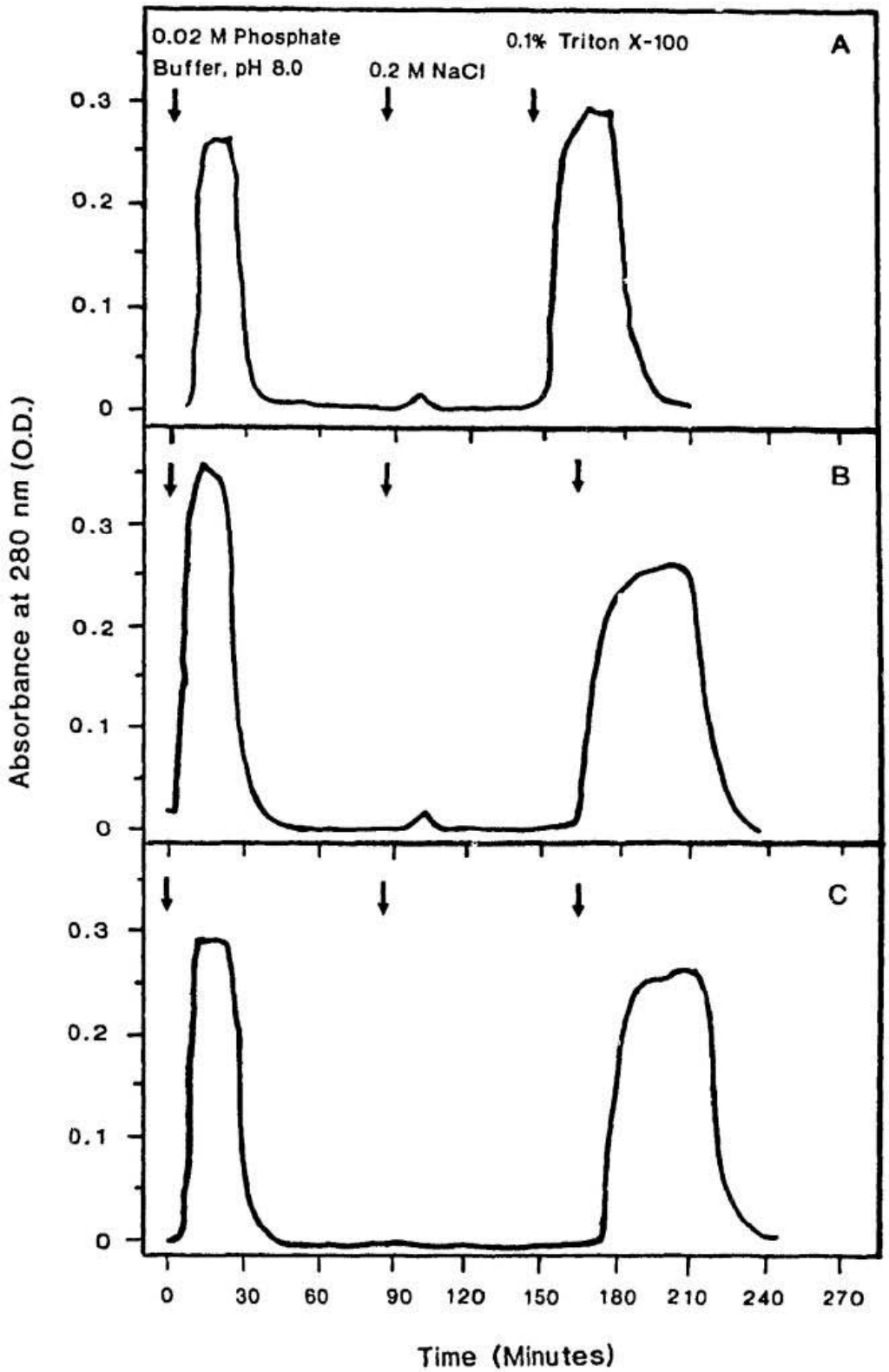
Elution protocol	% virus released from K562 cells	% virus released from HeLa cells
Sequential elution:		
0.05 M NaCl	6.6	5.3
0.01 M NaCl	5.5	4.4
0.20 M NaCl	76.5	76.7
0.40 M NaCl	5.0	5.8
Membrane residues	6.5	7.8
One-step elution:		
0.2 M NaCl	91.0	92.0
Membrane residues	9.0	7.9

[1]Cell membranes derived from K562 or HeLa cells were incubated with ^{3}H-labeled virus. After unbound radioactivity was removed by washing with phosphate buffer, radioactivity bound to the membranes was released by the incubations with increasing concentrations of NaCl as described in section 2.7.3. Released radioactivity at the end of each NaCl treatment was measured and expressed as a percentage of the sum of total radioactivity eluted from the cell membranes and radioactivity associated with the membrane residues. Each value is the mean of duplicate determinations.

3.5. Isolation of Putative EMC Virus Receptor Proteins by EMC Virus-Sepharose Affinity Chromatography

About 6 mg DOC solubilized K562 or HeLa cell membranes were chromatographed on an EMC virus-Sepharose column (section 2.13.) and absorbance of the eluate was monitored at 280 nm. The absorbance profile showed that three protein peaks were eluted from the column (Figs. 7A and 7B). The first protein peak represented unbound components of solublized cell membranes, which were washed through with 0.02 M phosphate buffer, pH 8.0. The second peak constitued bound material, which was specifically eluted by buffer containing 0.2 M NaCl. The third peak removed from the column by 0.1 % Triton X-100 in 0.02 M phosphate buffer, probably represented aggregated material . The column was re-used after extensive washing with 0.02 M phosphate buffer, pH 8.0 to remove Triton X-100. When cell membranes derived from K562 D mutants were chromatographed, no peak was eluted with 0.2 M NaCl buffer (Fig. 7C). This suggests that only cell membranes derived from receptor positive cells were able to bind to the column and were specifically eluted by the 0.2 M NaCl eluting buffer. Fractions containing bound material eluted by 0.2 M NaCl buffer were pooled and dialyzed against several changes of deionised water at 4^{0}C and then lyophilized for further analysis.

Figure 7. EMC virus-Sepharose affinity chromatography of solubilized cell membranes. 6 mg of DOC-solubilized membranes derived from K562 cells (A), HeLa cells (B) or K562 cell D clone (C) were subjected to affinity chromatography on EMC virus-Sepharose columns as described in section 2.13. Absorbance profiles of proteins eluted from the columns were determined at 280 nm by an UV monitor and presented as O.D. values. Arrows indicate points where wash buffer (0.02 M phosphate buffer, pH 8.0), eluting buffer (0.2 M NaCl in wash buffer) and regenerating buffer (0.1% Triton X-100 in wash buffer) were applied on the columns.

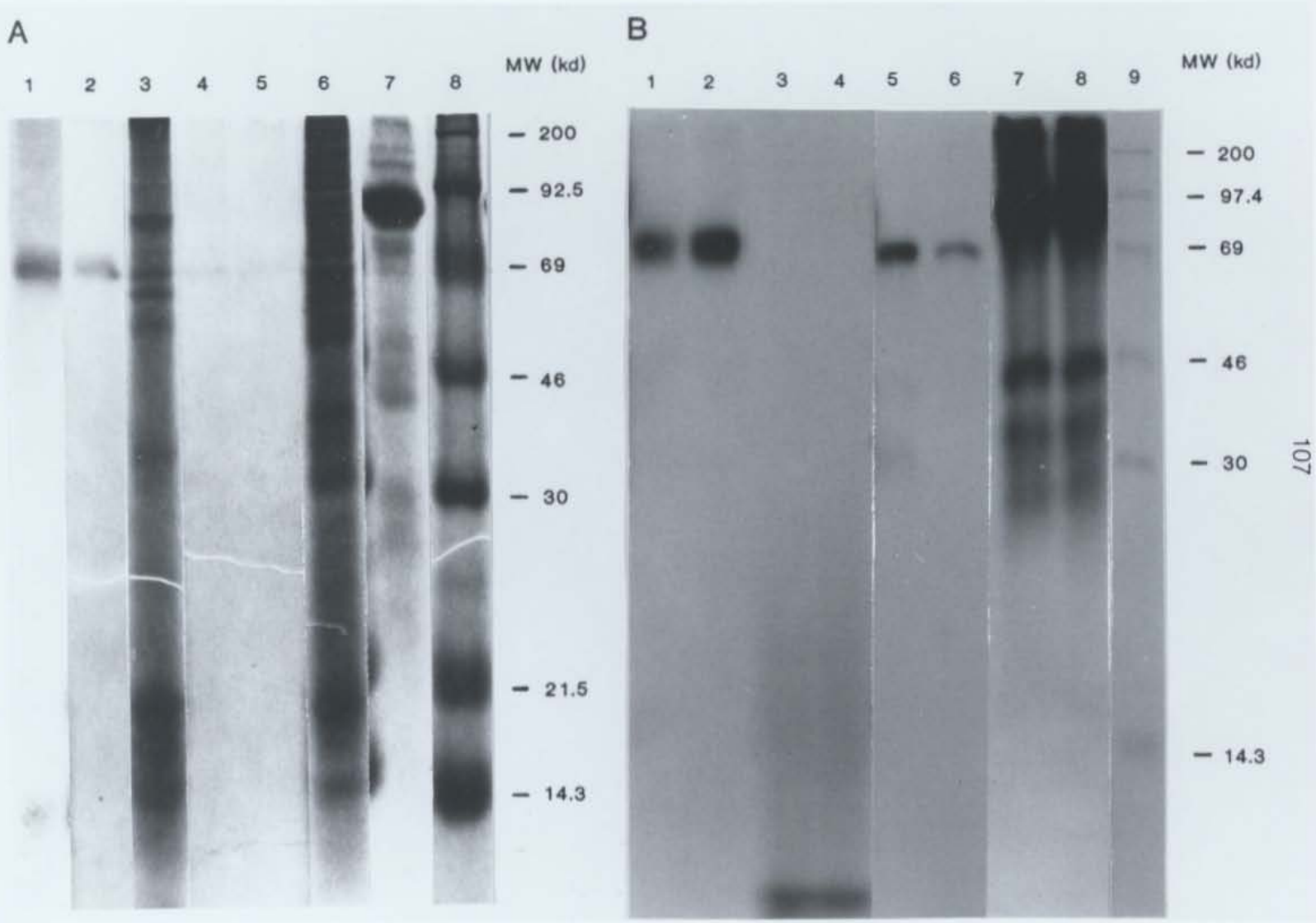

0.02 M Phosphate
Buffer, pH 8.0
0.2 M NaCl
0.1% Triton X-100
A
B
C
0.3
0.2
0.1
0
Absorbance at 280 nm (O.D.)
0
30
60
90
120
150
180
210
240
270
Time (Minutes)

Increasing the amount of solubilized K562 and HeLa cell membranes used on the column failed to increase the amount of bound material eluted with 0.2 M NaCl buffer, suggesting that the column was saturated under the conditions used (data not shown).

3.6. Molecular Weights of Purified EMC Virus Receptor Proteins

Receptor material purifed by affinity chromatography was either analyzed directly, or after radio-iodination, by 10% SDS-PAGE (section 2.15.). Protein bands on the gel were visualized by Coomassie brilliant blue staining (section 2.16.) or by autoradiography (section 2.17.). As shown in Figure 8, both these methods gave identical results. Thus, a single protein band with an approximate molecular weight of 70 kD was seen in the purified material from both K562 and HeLa cells, but not from K562 D mutants. However, the band in the K562 cell sample appeared broader than that from HeLa cells (Fig. 8), which may be due to different degrees of glycosylation. Failure to detect the 70-kD protein in K562 D mutants further proved the specificity of the column technique. It also appears that 70-kD protein does not match any glycophorin A components run in parallel lanes.

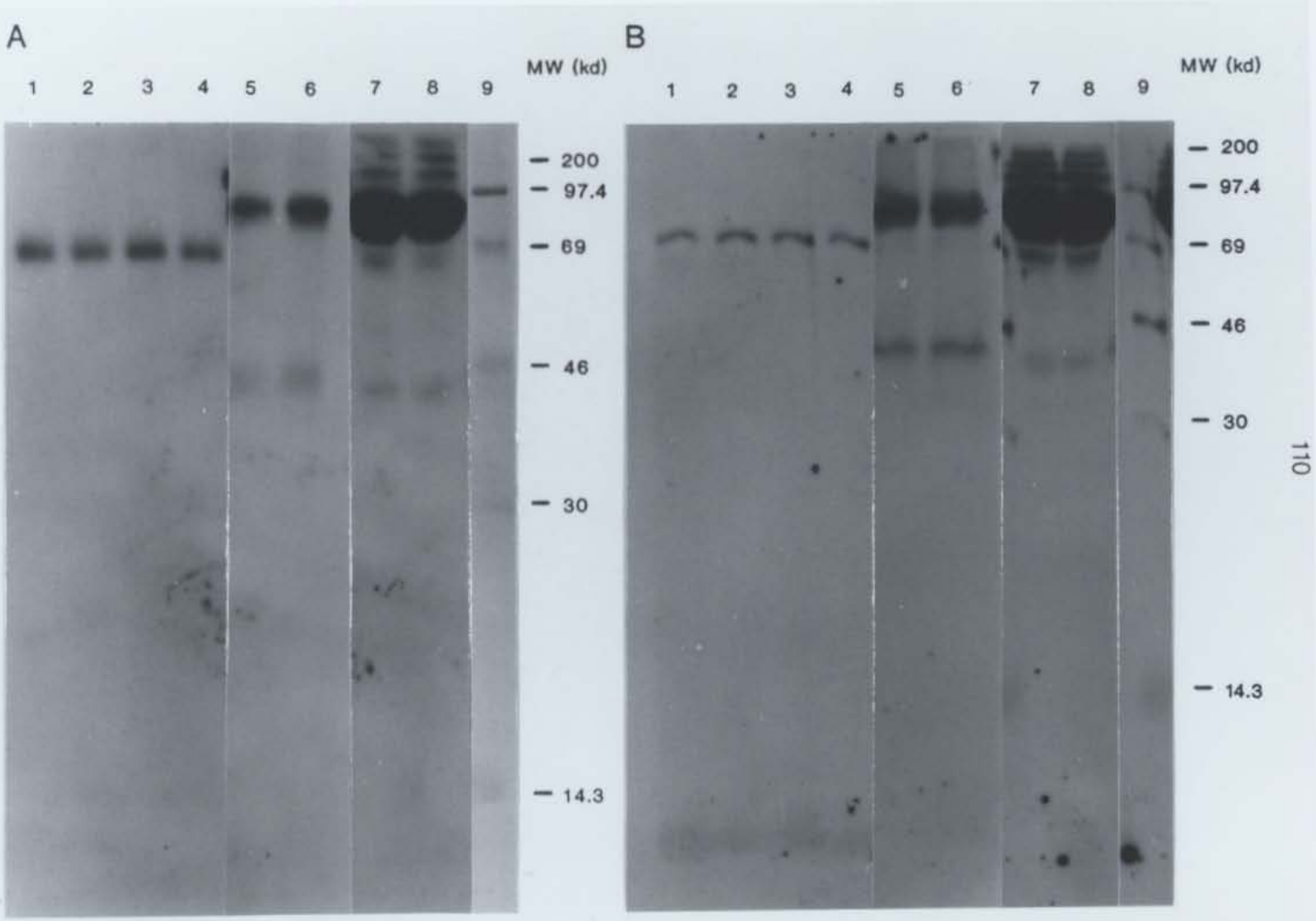
Figure 8. Molecular weight determination of EMC virus receptor purified from K562 and HeLa cells. (A) Coomassie brilliant blue staining of receptor proteins purified by EMC virus affinity chromatography from K562 (lanes 1 and 2) and HeLa (lanes 4 and 5) cells and DOC-solubilized cell membranes of K562 (lane 3) and HeLa (lane 6) cells. The preparations were separated by electrophoresis in a 10% SDS-polyacrylamide gel along with purified glycophorins (lane 7) and pre-stained protein molecular weight standards (lane 8). (B) Autoradiography of ^{125}I-labeled receptor proteins purified from K562 cells (lanes 1 and 2), K562 cell D clone (lanes 3 and 4) and HeLa cells (lanes 5 and 6) by EMC virus affinity chromatography and separated by electrophoresis in a 10% SDS-polyacrylamide gel along with ^{125}I-labeled purified glycophorins (lanes 7 and 8) and ^{14}C-labeled protein molecular weight standards (lane 9).


A
1
2
3
4
5
6
7
8
MW (kd)
200
92.5
69
46
30
21.5
14.3
B
1
2
3
4
5
6
7
8
9
MW (kd)
200
97.4
69
46
30
14.3

3.7. Specific Binding of EMC Virus to Affinity Purified Receptors

To verify whether the purified 70-kD proteins indeed express receptor activity for EMC virus, a virus overlay protein blot assay was performed. In this experiment, about 100-150 μg of 12 mM DOC-solublized cell membranes and 3 μg of the affinity purifed material from K562 and HeLa cells were electrophoresed by SDS-PAGE, and then transferred to nitrocellulose. Cell membranes and glycophorin derived from human erythrocytes were used as positive controls. The nitrocellulose was probed with 2×10^6 cpm ^{125}I-labeled EMC virus (1 μg virus) or incubated with an excess of unlabeled EMC virus (30 μg virus) prior to the addition of radiolabeled virions. As shown by autoradiography (Fig. 9), the virus recognized a similar band in the cell membrane preparations and the purified receptor material. The apparent molecular weight of the protein bands recognized by EMC virus in this experiment were identical to those shown in Figure. 8. Again, the 70-kD receptor proteins recognized by EMC virus do not match any glycophorin A components in purified glycophorin or erythrocyte membranes run in parallel lanes. In addition, the binding between the 70-kD receptors and EMC virus was inhibited by preincubation with an excess of cold virus (data not shown), indicating that the binding between EMC virus and 70-kD proteins is specific. Based on these results, the proteins purified by EMC virus affinity

Figure 9. Binding of EMC virus to receptor proteins purified from K562 and HeLa cells in a virus overlay protein blot assay. (A) K562 cells and (B) HeLa cells. DOC-solubilized membranes (lanes 1 and 2) and purified receptor proteins (lanes 3 and 4) were separated by electrophoresis in a 10% SDS-polyacrylamide gel along with cell membranes derived from human erythrocytes (lanes 5 and 6), purified glycophorins (lanes 7 and 8), and ^{14}C-labeled protein molecular weight standards (lane 9). Separated proteins were electroblotted onto nitrocellulose and probed with ^{125}I-labeled EMC virus as described in section 2.18. Binding of the virus to proteins was visualized by autoradiography.


A
1 2 3 4 5 6 7 8 9
MW (kd)
200
97.4
69
46
30
14.3
B
1 2 3 4 5 6 7 8 9
MW (kd)
200
97.4
69
46
30
14.3

chromatography appear to behave as a virus receptor.

3.8. Affinity Chromatography of Surface Labeled Cell Membranes

It was possible that the receptor preparations purifed by EMC virus affinity chromatography may contain both internal and external proteins associated with K562 and HeLa cell membranes. In order to determine whether the receptor proteins were indeed cell surface proteins, the following experiments were performed. K562 cells (including the D mutant) and HeLa cells were surface-iodinated by the method described in section 2.5. Cell viability monitored before and after iodination (section 2.2.) revealed that more than 95% of the cells were viable after iodination. Cell membranes were prepared immediately after radiolabeling (section 2.3.2.). Determination of radioactivity in each subcellular fraction during the process of membrane preparation (section 2.6) showed that over 80% of the total radioactivity was incorporated into membrane proteins. About 3.5×10^5 cpm, approximately 30 μg of solubilized cell membranes were incubated with the EMC virus-Sepharose column in the presence of 6 mM DOC and chromatographed as described in section 2.13. Fractions of 1 ml were collected and their radioactivity was measured. As shown in Figure 10, three peaks of radioactivity were eluted when solubilized cell membranes derived from K562 and HeLa cells were

examined. In contrast, only two peaks were detected when membranes from K562 cell D clone were applied to the column. These radioactivity profiles were similar to the protein profiles obtained when solublized unlabeled cell membranes were chromatographed on the EMC virus-Sepharose column (Fig. 7).

In order to secure enough material specifically eluted from the EMC virus column for SDS-PAGE analysis, about 4×10^6 cpm of solubilized membranes from cell surface labeled K562 and HeLa cells were subjected to affinity chromatography and bound material was eluted with 0.2 M NaCl. Approximately 3 to 5×10^4 cpm bound material was eluted by 0.2 M NaCl in each run, which represented about 1% of the total radioactivity applied onto the column. The radiolabeled, affinity purified material from several column runs were combined, dialyzed and lyophilized before analysis by SDS-PAGE. Unlabeled receptor proteins were radio-iodinated and electrophoresed in parallel with the surface-labeled material on the gel. Protein bands were visualized by autoradiography. As can be seen in Figure 11, identical patterns of radioactive bands were obtained when either the cell surface-labeled materials or receptor proteins first purified and then radiolabeled were examined. This result indicates that the material purified by affinity chromatography constituted cell surface molecules. Again, the 70-kD bands

Figure 10. EMC virus-Sepharose affinity chromatography of solubilized cell membranes derived from surface-labeled K562 and HeLa cells or K562 cell D clone. 3.5×10^5 cpm of DOC-solubilized cell membranes prepared from surface-labeled K562 cells (A), HeLa cells (B) or K562 cell D clone (C) were chromatographed on the virus-Sepharose columns as described in section 2.13. Radioactivity of eluates were measured and plotted against fraction number. Arrows indicate points where wash buffer (0.02 M phosphate buffer, pH 8.0), eluting buffer (0.2 M NaCl in wash buffer) and regenerating buffer (0.1% Triton X-100 in washe buffer) were applied on the columns.

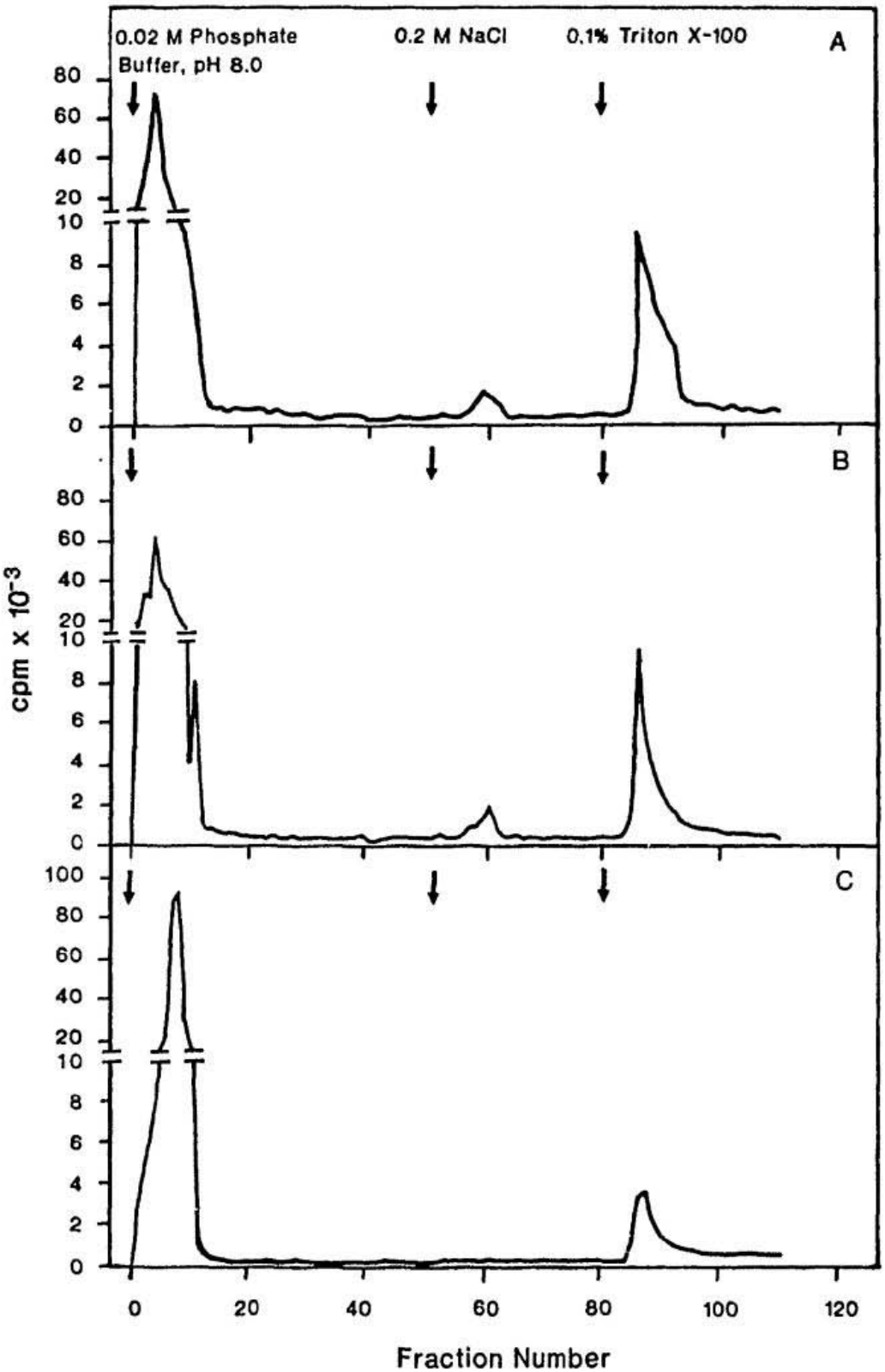
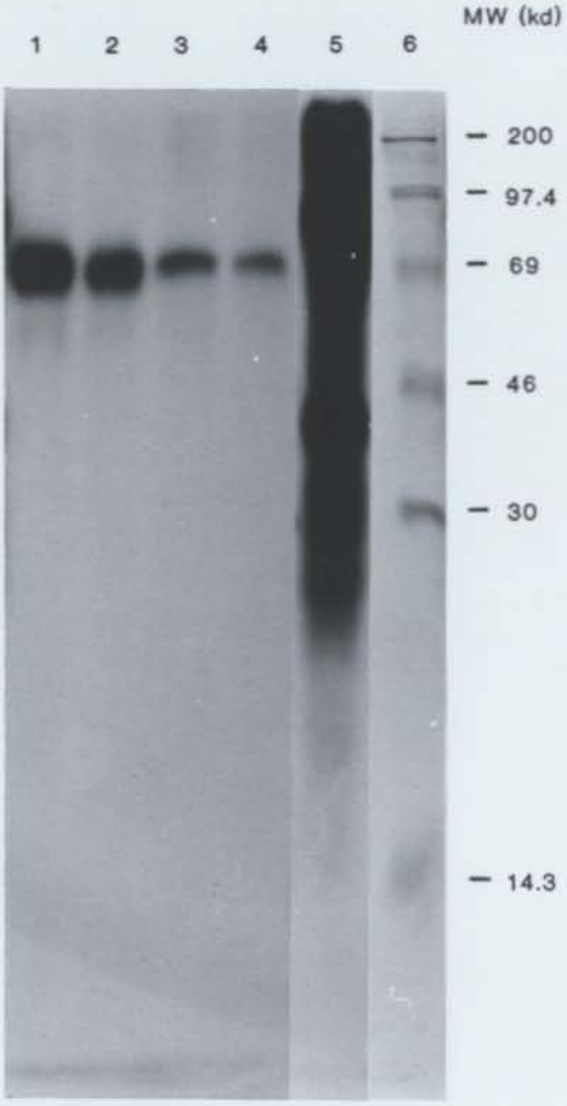

0.02 M Phosphate
Buffer, pH 8.0
0.2 M NaCl
0.1% Triton X-100
A
B
C
cpm x 10⁻³
Fraction Number

Figure 11. SDS-PAGE of EMC virus receptor proteins purified from surface labeled K562 and HeLa cells. Receptor proteins purified from surface labeled K562 (lane 1) and HeLa (lane 3) cells were separated by electrophoresis in a 10% SDS-polyacrylamide gel as described in section 2.15. Receptor proteins purified from unlabeled membranes of the same cells were labeled with ^{125}I, as described in section. 2.5, and run in parallel (lanes 2 and 4). Lanes 5 and 6 contain ^{125}I-labeled purified glycophorins and ^{14}C-labeled protein molecular weight standards, respectively. Protein bands were visualized by autoradiography.


MW (kd)
1
2
3
4
5
6
200
97.4
69
46
30
14.3

do not match any glycophorin A components run in parallel on the same gel.

3.9. Determination of Receptor Activity in the Affinity-Purified Material

Experiments were performed to determine the efficiency of purification by EMC virus-Sepharose affinity chromatography. Serial 2-fold dilutions of 100 μg cell membrane proteins and 0.1 μg purified receptor proteins were applied to nitrocellulose using a dot-blot apparatus (section 2.8.). The blots were probed with ^{125}I-labeled EMC virus and autoradiographed as described in section 2.8. As seen in Figure 12, with 3 days exposure, virus bound to as little as 3 to 6 ng of the purified receptor protein, which was about 60 times less compared with cell membrane preparations according to protein concentration. It appeared that by using EMC virus-Sepharose affinity chromatography, the specific activity of the virus receptor had been enriched about 60-fold in preparations purified from both K562 and HeLa cell membranes.

3.10. Biochemical Analysis of Solubilized EMC Virus Receptor by the Affinity Chromatography

It has been proposed that treatment of intact cells or cell membranes with enzymes, especially with proteases, may affect the integrity of the membranes,

Figure 12. Determination of receptor activity in EMC virus receptor preparations purified from K562 and HeLa cell membranes. Receptor proteins purified from DOC-solubilized K562 or HeLa cell membranes by EMC virus-Sepharose columns (purified receptors) as well as untreated cell membranes (cell membranes) were tested for receptor activity using indicated serial two-fold dilutions, presented as reciprocal values, and dot-blot radioligand binding assay as described in section 2.8. 100 μg protein of cell membranes and 0.1 μg protein of purified receptors were tested at the starting concentration (assigned the value of 1).

besides cleaving receptor polypeptides (see section 1.6.). Digestion of solubilized receptor preparations is considered to give more reliable results (Holmes, 1981; Colonno, 1987). For this reason, we performed enzymatic studies on solubilized cell membranes. The approach was to analyze the enzyme-treated cell membranes on the EMC virus-Sepharose column. For this purpose cell membranes derived from surface radio-iodinated K562 and HeLa cells were solubilized by DOC. Approximately 3.5 x 10^5 cpm in each sample was treated with trypsin or neuraminidase as described in section 2.9.2. Enzyme treated samples were chromatographed on an EMC virus-Sepharose column (section 2.13.) and radioactivity was measured in the fractions collected. The cell membranes for controls were treated similarly but without trypsin and neuraminidase. As shown in Figure 13, the peak normally eluted by buffer containing 0.2 M NaCl was absent in both trypsin- and neuraminidase-treated samples, indicating that most of, if not all, the receptor activity had been destroyed by enzyme digestion and the resulting material was unable to bind to the EMC virus-Sepharose column. Therefore, the results from enzyme studies using intact cells (section 3.2.1), cell membrane preparations (section 3.2.2) and DOC solubilized cell membranes are consistent and suggest that EMC virus receptors on K562 and HeLa cells are sialoglycoproteins in nature..

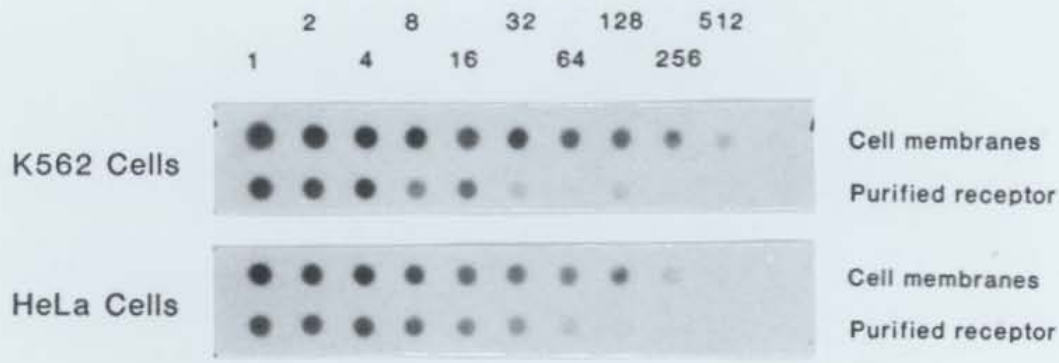
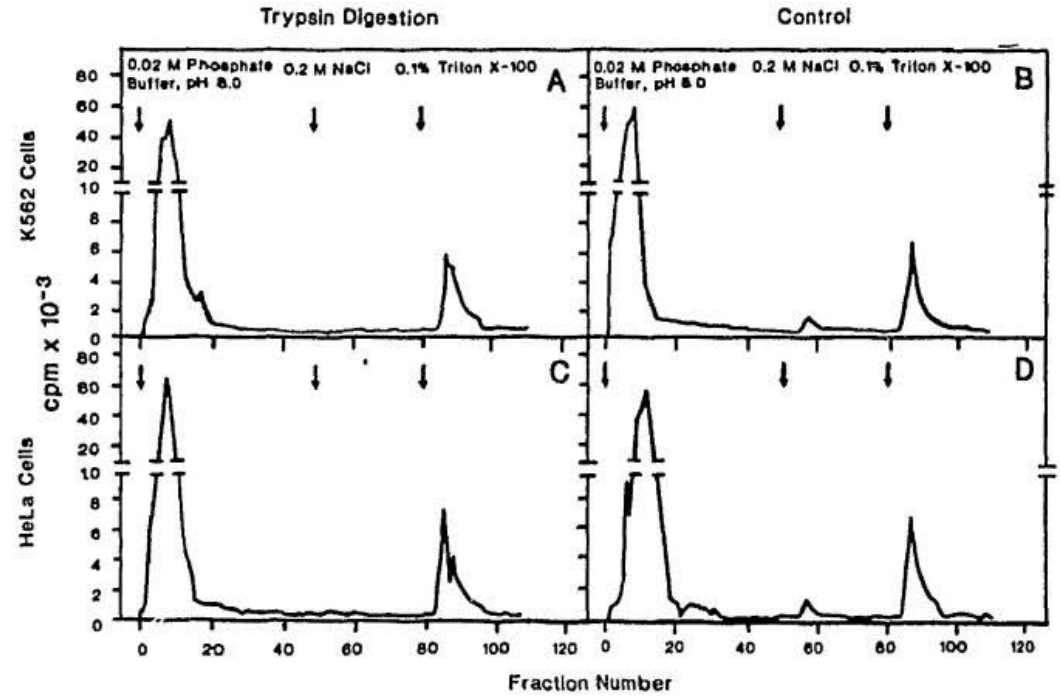
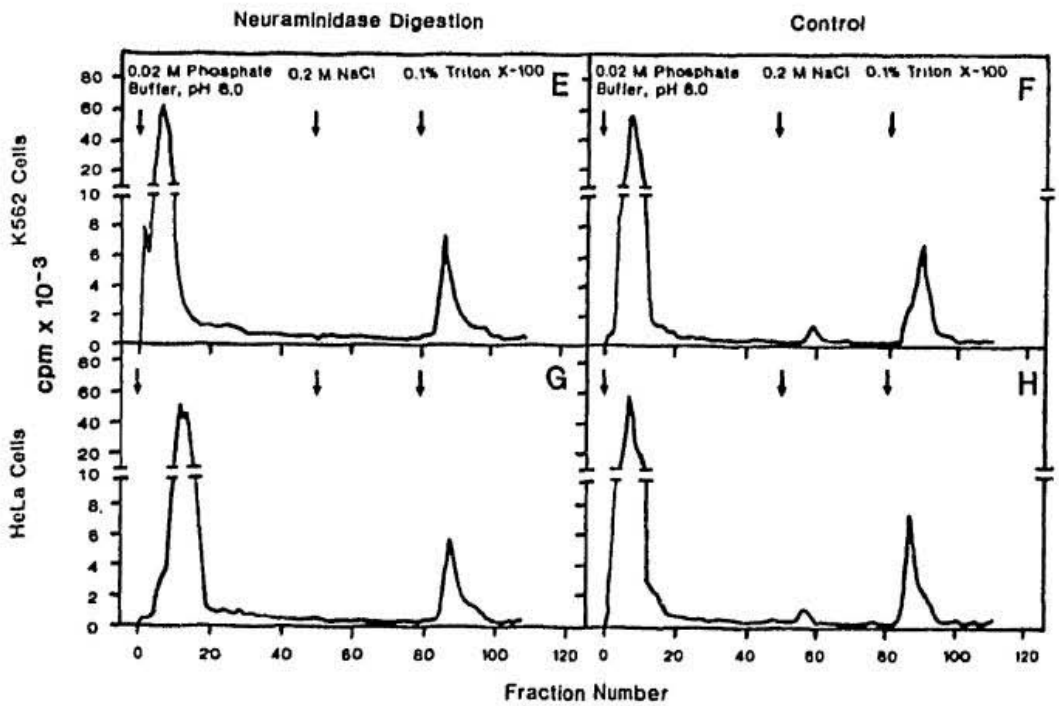

2
8
32
128
512
1
4
16
64
256
K562 Cells
Cell membranes
Purified receptor
HeLa Cells
Cell membranes
Purified receptor

Figure 13. Effect of trypsin and neuraminidase digestions on the binding of K562 and HeLa cell membranes to EMC virus-Sepharose column. 3.5×10^5 cpm of DOC-solubilized cell membranes derived from surface-labeled K562 or HeLa cells were digested with trypsin (A and C) or neuraminidase (E and G) as described in section 2.9.2. Control membranes (B, D, F and H) were treated under the same conditions, but without the respective enzymes. Samples were chromatographed as described in section 2.13. Radioactivity of the eluates were measured and plotted against fraction number.


Trypsin Digestion
Control
0.02 M Phosphate Buffer, pH 8.0
0.2 M NaCl
0.1% Triton X-100
A
B
C
D
K562 Cells
HeLa Cells
cpm x 10⁻³
Fraction Number


Neuraminidase Digestion
Control
0.02 M Phosphate Buffer, pH 8.0
0.2 M NaCl
0.1% Triton X-100
E
F
G
H
K562 Cells
HeLa Cells
cpm x 10⁻³
Fraction Number

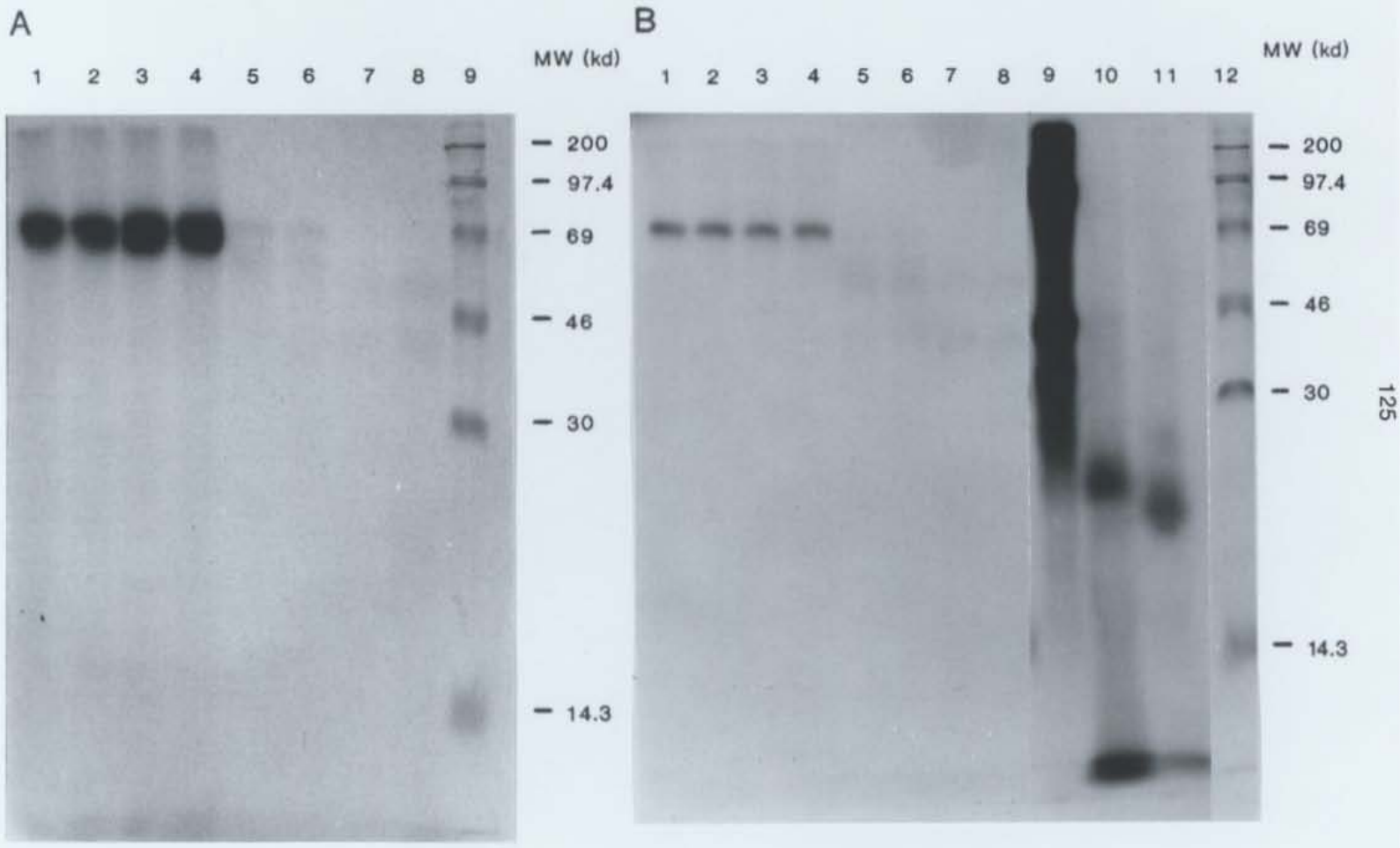
3.11. Analysis of Purified Receptor Proteins by SDS-PAGE After Protease Digestion

Treatment of intact K562 and HeLa cells with either trypsin or chymotrypsin significantly reduced binding of EMC virus to those cells. Therefore, an experiment was done to find out whether the proteases have similar effects on affinity purified EMC virus receptor proteins. ^{125}I-labeled receptor proteins purified by EMC virus-Sepharose affinity chromatography were treated with trypsin or chymotrypsin as described (section 2.9.3.) followed by analysis, along with untreated controls, by SDS-PAGE as described in section. 2.15. Labeled protein bands were visualized by autoradiography. The results demonstrated that treatment of purified receptor proteins with trypsin or chymotrypsin degraded the 70-kD bands into a few faint bands with low molecular weights (Fig. 14). These results support previous findings that the receptors for EMC virus on K562 and HeLa cells are proteins that are sensitive to trypsin and chymotrypsin digestion.

3.12. Isoelectric Points of Purified EMC Virus Receptors

As shown in Figure 8, the 70-kD protein band for the K562 cell sample was broader than that for the HeLa cell sample. It was suspected that the 70-kD band in K562 cell samples is more heterogeneously glycosylated. Also, if

Figure 14. SDS-PAGE of EMC virus receptor proteins purified from K562 and HeLa cells after proteolytic digestion. Receptor proteins purified by EMC virus affinity chromatography from K562 (A) and HeLa (B) cells were labeled with ^{125}I and treated with trypsin (A and B, lanes 5 and 6) or chymotrypsin (A and B, lanes 7 and 8) as described 2.9.3. Untreated receptor proteins (A and B, lanes 1 and 2), receptor proteins treated with PBS (A and B, lanes 3 and 4), ^{125}I-labeled purified glycophorins (B, lane 9) and glycophorins treated with trypsin (B, lane 10) or chymotrypsin (B, lane 11) were used as controls. ^{14}C-labeled protein molecular weight standards are shown in panel A, lane 9 and in panel B, lane 12. Proteins were separated by electrophoresis in a 10% SDS-polyacrylamide gel and visualized by autoradiography.


A
1 2 3 4 5 6 7 8 9
MW (kd)
200
97.4
69
46
30
14.3
B
1 2 3 4 5 6 7 8 9 10 11 12
MW (kd)
200
97.4
69
46
30
14.3

receptor proteins from K562 cell are more sialyated than those from HeLa cells, the isoelectric point (pI) of the proteins might be expected to be lower than that from HeLa cells. Chromatofocusing was used to determine the pIs of purified receptor proteins. Chromatofocusing elutes proteins from a Polybuffer ion-exchange column according to their pIs using an internally generated pH gradient. In the performed experiment, ^{125}I-labeled purified receptor proteins from K562 and HeLa cells were added to a Polybuffer PBE 94 chromatofocusing column and chromatofocused, as described in section 2.19. As shown in Figure 15, chromatofocusing of untreated receptor proteins from K562 cells resulted in most of the radioactivity being eluted as a broad peak at pH 4.8. This suggests that pI of this protein is around 4.8. The broadness of the eluting peak may reflect heterogeneity of the protein glycosylation. Similarly, the pI of the receptor protein from HeLa cells was found to be 6.4. Moreover, the peak of the receptor protein from HeLa cells was much sharper than that from K562 cells. Therefore, it appears that the receptor protein from HeLa cells may be less glycosylated than that from K562 cells.

Since the receptors appear to be sialoglycoproteins, neuraminidase treatment of purified receptor proteins, which should release negatively charged sialic acid residues, was expected to cause a change in its pI, resulting

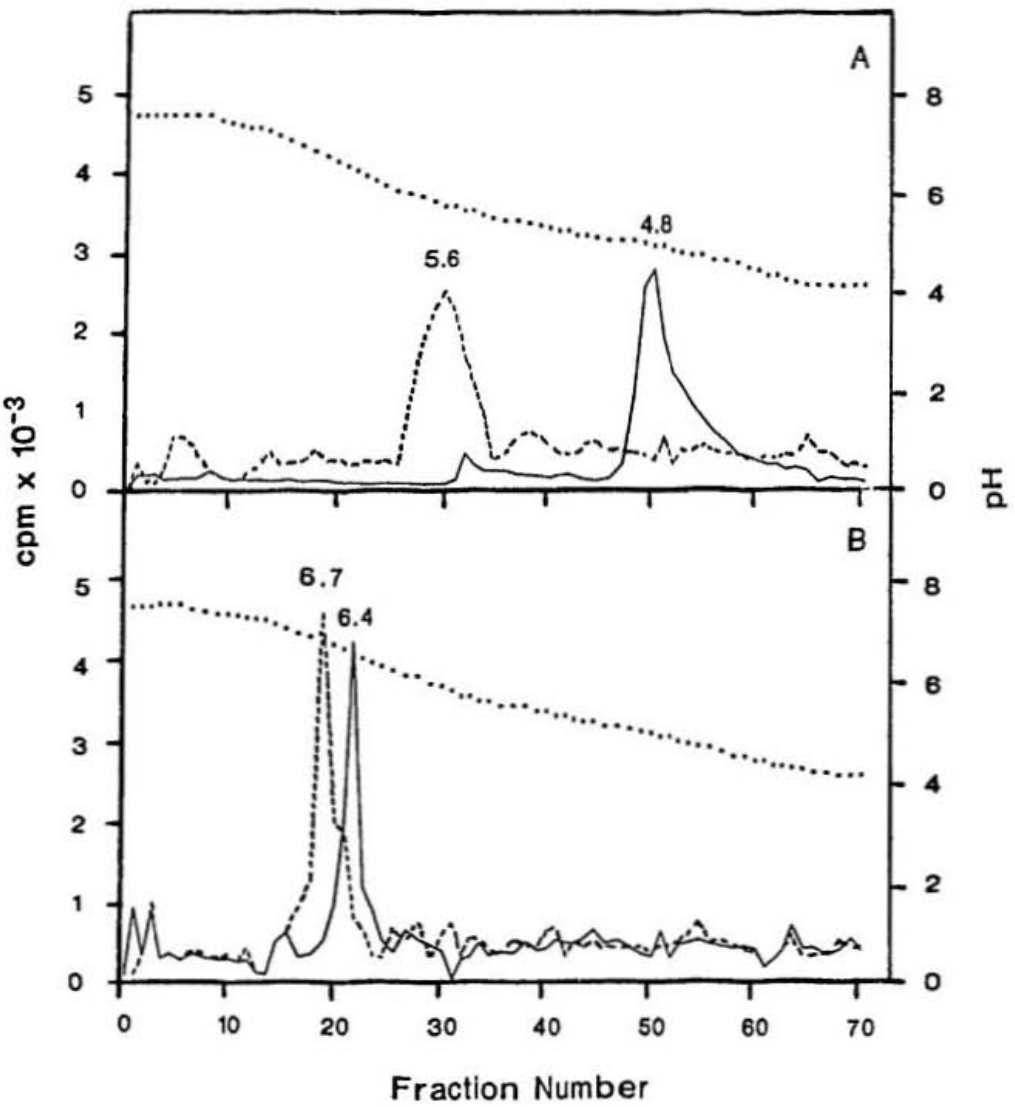
in elution at a higher pH compared to untreated samples. Therefore, the same amount of ^{125}I-labeled receptor protein was pre-treated with neuraminidase before chromatofocusing. Desialyation of the receptor protein from K562 cells indeed resulted in a shift of its pI from 4.8 to 5.6. Desialylation of the receptor protein from HeLa cells resulted in a lesser shift of its pI, from 6.4 to 6.7.

In this experiment, the pIs of purified receptor proteins were determined. The shift in pI of the receptor proteins toward neutrality by neuraminidase treatment indicates that sialic acid contributes to the acidic nature of the proteins. Also, it appears again that the receptor protein from K562 cells may be more sialylated than that from HeLa cells, as it had a lower initial pI, and a greater shift of pI occurred after neuraminidase treatment than with HeLa cell receptor proteins. This is consistent with the previous observation by SDS-PAGE that the receptor protein band from K562 cells appeared broader than that from HeLa cells (Fig. 8).

3.13. Western-Blot Analysis of EMC Virus Receptor Purified from K562 Cells with Anti-Glycophorin A Antibody.

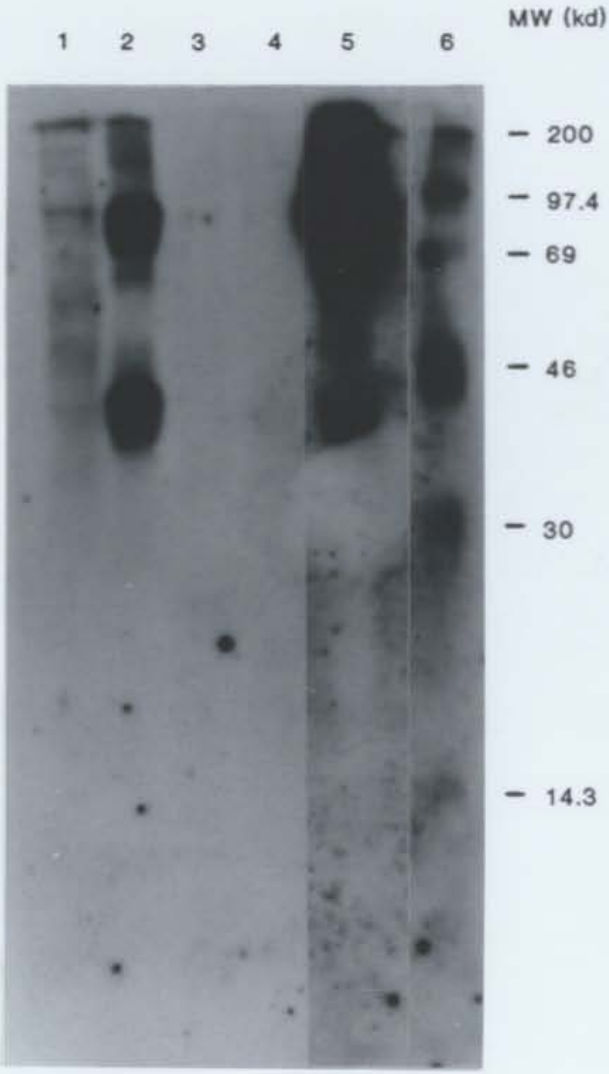
Glycophorin A, which is also expressed by K562 cells (Gahmberg *et al.*,

Figure 15. Chromatofocusing of EMC virus receptor proteins purified from K562 and HeLa cells. Approximately, 10^5 cpm of ^{125}I-labeled purified receptor proteins (——) from K562 (A) and HeLa (B) cells or neuraminidase desialylated receptor proteins (---) from the same cells were chromatofocused on a PBE 94 ion-exchange column as described in section 2.19. Radioactivity and pH (···) of each 1 ml fraction were measured and plotted against fraction number.


A
B
5.6
4.8
6.7
6.4
cpm x 10^{-3}
pH
Fraction Number
0
10
20
30
40
50
60
70
1
2
3
4
5
6
8

1983), has been reported to be a receptor for EMC virus on human erythrocytes (Allaway *et al.*, 1986; Allaway & Burness, 1986), However, it has also been suggested that glycophorin A may not be the receptor for EMC virus on K562 cells (Hamid & Grewal, unpublished results; see section 1.7.2.). The purified receptor proteins from K562 cells were tested for binding of anti-glycophorin A antibody to see whether the purified receptor proteins from K562 cells was glycophorin A. K562 cell membranes, purified receptor proteins from K562 cells, erythrocyte membranes and purified glycophorins were subjected to SDS-PAGE, and then electroblotted onto nitrocellulose. After blocking, blots were probed with monoclonal anti-glycophorin A antibodies followed with ^{125}I-labeled sheep anti-mouse antibody as described in section 2.18. The antibody binding was visualized by autoradiography. The result shows that anti-glycophorin A antibodies recognized components in the purified glycophorin A preparation and in erythrocyte and K562 cell membranes (Fig. 16). However, the antibodies did not recognize the purified 70-kD receptor protein from K562 cells. This result strongly suggests that the 70-kD receptor protein isolated by affinity chromatography may not contain glycophorin and, therefore, glycophorin A on K562 cells may not be the receptor for EMC virus.

Figure 16. Anti-glycophorin A antibody reactions in western blot analysis of EMC virus receptor purified from K562 cells. DOC-solubilized cell membranes derived from K562 cells (lane 1) and erythrocytes (lane 2), and receptor proteins purified by EMC virus affinity chromatography from K562 cells (lanes 3 and 4) were incubated with monoclonal anti-glycophorin A antibodies, incubated with ^{125}I-labeled sheep anti-mouse antibodies, and visualized by autoradiography. ^{14}C-labeled protein molecular weight standards (lane 6).


1
2
3
4
5
6
MW (kd)
200
97.4
69
46
30
14.3

Chapter 4

Discussion

To initiate infection, a virus must interact with the surface of host cells. This interaction is usually mediated by virus-specific cellular receptors. The specificity of the virus-receptor binding has long been regarded as the first and sometimes the sole determinant of virus host range and cell tropism. Therefore, identification and characterization of virus receptors is important to understand the mechanism of virus infection. This information may eventually lead to the development of new antiviral agents. For example, since CD4 was defined as a receptor for human immunodeficiency virus (Klatzmann *et al.*, 1984), an approach has been made to design CD4 immunoadhesins for AIDS therapy (Capon *et al.*, 1989). EMC virus is an important virus model for the study of several virus-induced human diseases (section 1.2). The attachment molecule for EMC virus on human erythrocytes has been defined as glycophorin A, the major sialoglycoprotein on erythrocytes (section 1.6.5). However, there is still little known about EMC virus receptors on nucleated cells. In this thesis, K562 and HeLa cells, two human nucleated cell lines, were chosen to study the attachment of EMC virus. Unlike human erythrocytes, which lack the biosynthetic machinery to support EMC virus repliction, both K562 and HeLa cells are susceptible to

EMC virus infection and are able to support the virus replication (Pardoe *et al.,* 1990; Jungeblut & Kodza, 1957; Kodza & Jungeblut, 1958). This work presents the findings on the biochemical nature of the EMC virus receptors, and describes the isolation and characterization of a new class of EMC virus receptor proteins on human nucleated cells.

4.1. Kinetics of EMC Virus Attachment.

The time required to achieve maximum binding of virus to tissue culture cells varies from a few minutes to several hours (Lonberg-Holm & Whiteley, 1976; McClintock *et al.*, 1980; Epstein *et al.*, 1984; Taylor & Cooper; 1989), and depends not only on the nature of the virus and type of targeted cells, but also on physico-chemical factors including pH, temperature, ion species and concentration, charge distribution and viscosity (Crowell & Landau; 1983). It has been suggested that the time required to achieve maximum binding of virus reflects the rate of redistribution of receptor molecules on the cell surface, to form multiple links between virus and cellular receptors (Taylor & Cooper, 1989). Relatively rapid rates of attachment have been observed in the binding of EMC virus to HeLa-S_3, mouse L-929 cells (McClintock *et al.*, 1980) and K562 cells (Pardoe & Burness, unpublished results). The time to

achieve maximum binding of EMC virus on those cells ranges from 8 to 30 minutes at 0^{o}C. In this study, we observed a similar virus binding rate to HeLa cells (Fig. 1), where maximum binding was achieved between 20 to 30 minutes.

The observation that unlabeled EMC virus competitively inhibited attachment of labeled virus to HeLa cells (Fig. 1), and that the receptors were saturated by excess virus (Fig. 2A) satisfied two major criteria for receptor specificity, i.e., competition and saturability. About 4% of the labeled virus binding, which was not inhibited by the addition of excess unlabeled virus (Fig. 1), probably represented nonspecific binding. However, the possibility that such nonspecific binding also might lead to cell infection cannot be completely ruled out.

By using Scatchard analysis, we determined that the number of cellular receptor sites for EMC virus on HeLa cells was 1.6×10^5 per cell (Fig. 2B). The number of receptor sites on HeLa cells is comparable with previous reports on the binding of EMC virus to HeLa-S_3, insulinoma and K562 cells (McClintock *et al.*, 1980; Baldeh & Burness manuscript submitted; Pardoe, unpublished results) and most reports on other picornaviruses (Crowell, 1966; Lonberg-Holm & Korant, 1972; Medrano & Green, 1973; Fotiadis *et al.*, 1991),

which range from 10^4 to 10^6per cell.

Scatchard plot analysis also revealed that the dissociation constant for EMC virus binding to HeLa cells was 1.1 nM. In comparison, EMC virus binding to insulinoma and K562 cells exhibits Kd values of 1.2 nM and 2.7 nM, respectively (Baldeh & Burness, manuscript submitted; Pardoe, unpublished results). The straight line obtained by the Scatchard plot strongly suggests that a single class of receptors is involved in the binding of EMC virus to HeLa cells (Fig. 2B). However, other possibilities exist for having a straight line in the Scatchard plot analysis, such as there being more than one kind of receptor but all having the same affinity. Although such a possibility is less likely and no such instance has ever been reported in the binding of picornaviruses to host cells, it may still be worthwhile keeping such a possibility in mind. In this respect, it has been reported that coxsackievirus B3 variant can use two distinct cellular receptors, although these receptors are located on two different cell lines (Hsu *et al.*, 1990). In addition, it is also necessary to keep in mind that Scatchard analysis is based on the assumption, that the binding of a ligand to a receptor is monovalent(i.e., one ligand molecule binds to one molecule of a receptor), thus, ideally, purified molecules of viral attachment proteins should be used to determine the

number of virus receptors. However, as the VAPs of picornavirus are located in a deep "canyon" of the viral capsid (section 1.3.1.), preparations of purified VAPs of picoranviruses are not yet available. Under this circumstance, the investigators working on picornavirus receptors have no alternative but to use purified virions in the virus receptor studies.

4.2. Biochemical Nature of the Receptor for EMC Virus

Studying the effect of various enzymes and lectins on the binding of viruses to cells or cell membranes is a useful and common method to characterize virus receptors. It must be remembered, however, that the susceptibility of the receptor to protease treatment does not necessarily mean that the protein itself is the virus receptor. Many proteases, including trypsin and papain, can affect the integrity of cell membranes and surface molecules surrounding the virus receptors, or release carbohydrate chains attached to polypeptides in addition to cleavage of a peptide of the receptor molecule. Therefore, in this project, enzymatic analysis was not only performed on intact cells and cell membrane preparations, but also on solubilized cell membranes which should reduce the side effects of enzymes to give more reliable results (Holmes, 1981; Colonno, 1987). These experiments all demonstrated that proteases and neuraminidase significantly reduced virus binding to cells and cell membrane

preparations (Table 1; Fig. 3), or destroyed the ability of cell membrane proteins to bind to the EMC virus-Sepharose column (Fig. 13), whereas phospholipase C, which affects cell membrane lipid constituents, had no effect. The incomplete removal of EMC virus receptor activity from cell membranes after single neuraminidase digestion (Fig. 3) could be due to the fact that enzymatic hydrolysis of carbohydrate residues often proceeds in a sequential manner and a sequence representing a binding site for virus may be left partially unhydrolized. Indeed, it was found that double neuraminidase digestion further reduced the receptor activity to near background level. These results, combined with the fact that binding of virus to K562 and HeLa cells was inhibited by pretreatment of the cells with two sialic acid-specific lectins, WGA and LPA (Table 1), strongly suggested that the receptors for EMC virus on K562 and HeLa cells are sialoglycoproteins.

Although the data obtained consistently showed that the receptor activity was sensitive to protease and neuraminidase treatment, different techniques used in the study showed varying degrees of receptor activity remaining after digestion. As shown in Figure 13, pre-treatment of solubilized cell membranes derived from surface-labeled cells with trypsin or neuraminidase destroyed most of the receptor activity and no peak of radioactivity was eluted from the EMC virus-Sepharose column with buffer containing 0.2 M NaCl. On the

other hand, considerable amounts of receptor activity was detected after treatment of native cell membranes with trypsin or neuraminidase using the dot-blot assay. There are several possible explanations for these differences, including, the sensitivity of the techniques, accessibility of enzyme to receptor molecules in native and solubilized cell membranes and the ratio of membrane proteins to enzyme used in each analysis. Thus, much less solubilized labeled cell membranes (about 30 μg) was used in the affinity chromatography analysis than in the dot-blot analysis, where unlabeled native cell membranes (about 280 μg) were used, but they were digested with the same amount of trypsin or neuraminidase. Undoubtedly, detergent-solubilized cell membranes may allow the enzymes better access to the receptor molecules and, therefore, more extensive enzyme digestion was observed when using solubilized membrane preparations. In addition, it has been suggested that EMC virus covalently bound to Sepharose, results in a reduced affinity of the virus for its receptors (Allaway & Burness, 1987). If so, this may further contribute to the differences in the results obtained using these two techniques. Overall, the observed variability in the effect of trypsin and neuraminidase on receptor activity is likely related to different degrees of solubilization of cell membranes and the different methods used for the assessment of receptor activity.

The length of time for receptor recovery varies from one virus receptor to another. For example, the receptor for poliovirus T1 on HeLa cells reaches half maximum recovery within 1 hour post-trypsin treatment, whereas half maximum recovery for the coxsackievirus B3 receptor, on the same cell line, requires 3 to 4 hours (Levitt & Crowell, 1967). In this study, it was observed that after trypsin digestion, K562 and HeLa cells recovered fifty percent of their receptor activity in about 4 hours, and full recovery took about 8 hours. In contrast with a rapid replacement of receptor molecules, which is characteristic for replacement of cell surface proteins from an intracellular pool, almost no receptor recovery was observed within the first 2 hours after enzyme digestion in either K562 or HeLa cells (Fig. 4). Therefore, it appears that receptor recovery requires protein synthesis. Indeed, cycloheximide, an inhibitor of protein synthesis, prevented receptor recovery in a dose-dependent manner (Fig. 5). This suggests the involvement of *de novo* protein synthesis in regeneration of the EMC virus receptor.

4.3. Properties of EMC Virus-Sepharose Columns

It has been reported that more than 90% of EMC virus can be covalently bound to CNBr-activated Sepharose 4B, and prolonged incubation with 0.05%

SDS, 0.1% Triton X-100 or 0.5% (12.1 mM) DOC released less than 5% of the bound virus (Allaway & Burness, 1987). Similar efficiency of coupling of ^{3}H-labeled EMC virus to Sepharose 4B was found in this project (section 2.14). EMC virus-Sepharose columns were stable for several weeks, without showing an appreciable decrease in amounts of cellular material binding, under the conditions used (Allaway & Burness, 1987).

As mentioned in section 3.5, the possibility of using EMC virus-Sepharose columns to purify EMC virus receptors from nucleated cells arose from the studies on the interaction between glycophorin A and EMC virus-Sepharose columns. Following solubilization of erythrocyte membranes, diluting the DOC concentration to 6 mM allows glycophorin to bind to an EMC virus-Sepharose column, and bound glycophorin can be specifically eluted with buffer containing 0.2 M NaCl (Baldeh, 1987; Allaway & Burness, 1987). The specificity of this technique has been carefully studied using glycophorin A on columns of Sepharose 4B conjugated with glycine, bovine serum albumin or fetuin instead of EMC virus (Allaway & Burness, 1987). Only about 10 to 20% of the glycophorin was retained on these non-virus columns compared with more than 80% retention on the virus column (Allaway & Burness, 1987). In addition, 0.2 M NaCl buffer eluted little if any glycophorin from those non-virus columns, whereas 0.2 M NaCl buffer eluted 80 to 90% of

bound glycophorin on the EMC virus column (Allaway & Burness, 1987). Glycophorin remaining on either the virus or non-virus columns after 0.2 M NaCl elution could be eluted by buffer containing 0.1% Triton X-100, indicating that this material was possibly aggregated and bound non-specifically (Allaway & Burness, 1987). Although the sensitivity of the EMC virus-glycophorin bond to 0.2 M NaCl buffer was unexpected, since EMC virus binds to cells under physiological conditions (0.14 M NaCl), it was confirmed that 0.2 M NaCl buffer released most of the ^{3}H-labeled virus previously bound to erythrocyte membranes (Allaway & Burness, 1987).

It is reasonable to assume that EMC virus will recognize a similar receptor epitope on different cells, as the receptor attachment site on picornaviruses is highly conserved (section 1.3.1.). In other words, the structure of receptor complexes may vary from one cell line to another, but they may all have a similar epitope within their structures. If this is true, a weak bond force similar to the EMC virus-glycophorin bond will be expected in the binding of EMC virus to K562 and HeLa cells. This assumption was confirmed by showing that ^{3}H-labeled virus bound to K562 and HeLa cell membranes was released by buffer containing 0.2 M NaCl (Table 3). Also, we showed that most of the EMC virus receptor activity on K562 and HeLa cell membranes were solubilized by 12 mM DOC and bound to EMC virus after diluting the

detergent concentration to 6 mM (Fig. 6, Table 2). Therefore, it was considered to be feasible to use this affinity chromatography technique to purify receptors from K562 and HeLa cells.

During receptor purification in this project, more evidence was found to support the specificity of the chosen affinity chromatography technique. Thus, (1) when solubilized cell membranes derived from K562 cell D clone were chromatographed, no detectable material was eluted by 0.2 M NaCl (Figs 7 and 8), (2) prolonged washing with 0.02 M phosphate buffer, pH 8.0, did not release any bound material of K562 and HeLa cells, that could be subsequently eluted with buffer containing 0.2 M NaCl (data not shown). These data are consistent with previous findings, using mouse insulinoma and Krebs cell membranes (Baldeh & Pardoe, personal communication), and (3) trypsin or neuraminidase treated membranes of K562 and HeLa cells were unable to bind to the column (Fig. 13). These results strongly dispute the possibility that the material eluted by 0.2 M NaCl buffer was non-specifically retained on the EMC virus column.

4.4. Properties of EMC Virus Receptor Purified by Affinity Chromatography

The ^{125}I-labeled or unlabeled affinity-purified receptor proteins analyzed by SDS-PAGE, revealed a protein band with an approximate molecular weight

of 70 kD, by autoradiography and Coomassie blue staining, respectively. Proof of involvement of this 70-kD protein in the specific attachment of EMC virus was obtained using the virus overlay protein assay. This technique was described by Co *et al.* (1985), to identify the receptor for reovirus type 3. Subsequently, the technique has been used successfully to identify receptor proteins for several viruses including Sendai virus (Gershoni *et al.*, 1986), mouse hepatitis virus (Boyle *et al.*, 1987), cytomegalovirus (Adlish *et al.*, 1990; Taylor & Cooper, 1990), Theiler's virus (Kilpatrick & Lipton, 1991) and visna virus (Dalziel *et al.*, 1991). Using this technique in this study, it was demonstrated that ^{125}I-labeled EMC virus recognized the same 70-kD protein band both in DOC-solubilized membrane preparations and purified receptor material from K562 and HeLa cells (Fig. 9). Furthermore, the binding to the 70-kD protein was inhibited by preincubation with an excess of unlabeled virus (data not shown), supporting the conclusion that the identified protein may mediate specific recognition of EMC virus on K562 and HeLa cells.

Although EMC virus specifically bound to the 70-kD proteins in the virus overlay protein blot assay, this does not directly prove that the proteins are the receptors used by the virus *in vivo* for cell entry. Further work is required to explore the biological role of the 70-kD protein for EMC virus to attach

and enter the cells *in vivo* (section 4.5). In addition, as shown in the virus overlay protein blot assay used in this study, EMC virus bound to the 70-kD proteins under denaturing and reducing conditions, suggesting that the virus recognizes an epitope on the 70-kD proteins independent of intramolecular disulfide bonds.

In purifying virus receptors from solubilized cell membrane preparations of K562 and HeLa cells by affinity chromatography, proteins originating from both internal and external surfaces of the membranes were exposed to the immobilized virus. It is possible that the purified 70-kD proteins are not cell surface molecules. To determine whether this 70-kD protein is in fact a surface molecule, solubilized cell membranes derived from surface labeled cells were chromatographed on the virus affinity column and the resulting eluates were analyzed by SDS-PAGE. As shown in Figure 11, similar results were obtained when either the unlabeled purified receptors, followed by iodination, or the surface labeled material was used. Thus, it is evident that the 70-kD receptor proteins purified by chromatography are cell surface molecules.

Sialic acids participate in many biological and pathological processes and have long been known to be involved in binding of many viruses to their

cellular receptors. We have shown in this study that the receptors for EMC virus on K562 and HeLa cells appear to be sialoglycoproteins. Therefore, it was important to show that the 70-kD proteins are also sialoglycoproteins. The isoelectric point for a typical sialoglycoprotein is usually lower than 4 (Burness & Pardoe, 1983), the pI for glycophorin is around 1.7 (Burness & Pardoe, 1983), the pI of human acid glycoprotein ranges from 1.8 to 2.7 (Jeanloz, 1972; Burness & Pardoe, 1983) and the pI of fetuin ranges from 2.6 to 4.1 (Graham, 1972; Burness & Pardoe, 1983). Unlike these heavily sialylated glycoproteins mentioned above, the purified receptor proteins appeared to be less sialylated as they had higher pIs. The pIs of the purified receptor proteins were 4.8 and 6.4 for K562 and HeLa cells, respectively (section 3.12.). However, the K562 cell receptor protein, with a low pI, eluted as a broader peak, from the chromatofocusing column and desialylation resulted in a greater shift of pI compared with HeLa cells, suggesting that the K562 cell receptor protein is more heavily glycosylated than that on HeLa cells. These results implied that acidic sialic acid is a component of the indentified putative receptor proteins on both cell types examined.

Also, 70-kD purified receptor proteins treated with trypsin or chymotrypsin gave several lower molecular weight fragments when analysed by SDS-PAGE (Fig. 14). This suggests that a polypeptide chain is an integral part of the 70-

analyzed by denaturing SDS-PAGE, the form of the native receptor is unknown. A faint protein band with a molecular weight of over 200-kD was occasionally observed in both K562 and HeLa cells, in addition to the 70-kD proteins by autoradiography on SDS-PAGE with longer exposure (data not shown). It is not clear whether the 70-kD protein is a subunit of the larger receptor complex, or one of its polypeptide chains, or represents a complete receptor molecule. However, other identified and purified picornavirus receptors provide some interesting observations in this matter. The receptor for poliovirus is a member of the immunoglobulin family, a single-chain of transmembrane protein with three homologous domains (Mendelsohn *et al.*, 1989), and the receptor for the major group of rhinovirus is ICAM-1, a single-chain protein with five homologous domains (Dustin *et al.*, 1986; Staunton *et al.*, 1988; Simmons *et al.*, 1988). Also, the receptor for coxsackievirus is suspected to be a protein with multimeric polypeptide domains (Mapoles *et al.*, 1985). These observations favour the possibility that the detected 70-kD proteins may be polypeptide units of a larger receptor protein. Undoubtedly, further studies are needed to explain details of the receptor structure on nucleated human cells.

Furthermore, it was found that the monoclonal anti-glycophorin A

kD receptor molecules and, therefore, further indicating that the receptor proteins are sialoglycoproteins.

Although, it appeared that affinity purified receptor proteins from K562 and HeLa cells (section 3.7.), and cells of the mouse cell lines, Krebs and insulinoma cell lines (Pardoe & Baldeh, personal communication) had similar molecular weights of approximately 70 kD as determined by SDS-PAGE analysis, additional information is required before we can be certain that these molecules are the same proteins. It may be worthwhile noting that glycosylation of proteins can affect the electrophoretic mobility on SDS-PAGE. Therefore, molecular weights may vary, depending on the degree of glycosylation.

To date, our attempts to obtain a carbohydrate-free polypeptide constituting the 70-kD receptor molecule have not been successful. Whether these 70-kD proteins on the cells examined are different proteins, but with a similar conformation for virus binding, or contain the same peptide backbone with different glycosylation remains unknown. Further work on the amino acid sequence and cDNA cloning of genes of the identified receptors will help to provide a solution to this problem. However, such studies are beyond the scope of this thesis.

Since the 70-kD proteins were purified in the presence of detergent and

antibodies, which block EMC virus attachment to glycophorin A on erythrocytes (Burness & Pardoe, 1986), did not recognize the 70-kD protein from K562 cells on Western-blots and the 70-kD protein did not match any glycophorin A components separated by SDS gel electrophoresis (Figs 8, 9, 11, 14 and 16). As mentioned in section 1.7.2, transfection of K562 cells with anti-sense glycophorin A cDNA, to block synthesis of glycophorin A, or saturating cells with anti-glycophorin A antibodies, does not affect virus binding to, and infection of K562 cells (Hamid & Grewal, unpublished results). Thus, results obtained in this study support previous indications that glycophorin A may not be a receptor for EMC virus on K562 cells. However, these results do not exclude the possibility that glycophorin A can serve as a receptor for EMC virus in nucleated cells under suitable conditions. For example, Madin-Darby bovine kidney cells do not normally express glycophorin A and are resitant to EMC virus infection. However, transfection of the cells with glycophorin A cDNA results in expresssion of glycophorin A on the cell surface and cells become susceptible to EMC virus infection (Grewal *et al.*, 1991). Thus, the result suggests that glycophorin A can serve as an EMC virus receptor in an appropriate cell line. It is not totally clear why EMC virus does not bind to glycophorin A on K562 cells. It has been

reported, however, that glycophorin A on K562 cells is less glycosylated and contains fewer sialic acid residues than it does on human erythrocytes (Silver *et al.*, 1987). This may explain, to some extent, why EMC virus does not bind to glycophorin A on K562 cells since we know that sialic acid residues are directly involved in the binding of EMC virus to glycophorin A (Tavakkol & Burness, 1990). It is also not unusual that glycosylation of glycophorin A can vary in different cell lines (Remaley *et al.*, 1991). Overall, evidence obtained in the course of this study implies that the identified 70-kD glycoproteins on the surface of human nucleated cells represent a novel, not yet described, class of EMC virus receptor molecules.

4.5. Suggestions for Further Studies

In the present study, a receptor-like protein, most likely a sialoglycoprotein, has been successfully isolated from K562 and HeLa cells using EMC virus affinity chromatography. If specific antibodies could be prepared against the putative receptor proteins that would prevent EMC virus infection of K562 and HeLa cells, it will provide direct evidence that the putative 70-kD receptor proteins play a functional role in EMC virus recognition and attachment to susceptible cells. Moreover, the antibodies could help us to

locate the virus binding epitope on the receptor molecule and provide an opportunity to investigate the physiological function of the 70-kD receptor molecules, by examining the influence of receptor antibodies on cell growth and maintenance (Tomassini & Colonno, 1986).

Another approach, no less important than the studies using specific anti-receptor antibodies, is to determine the amino acid sequence of the receptors and subsequent cloning of the receptor genes. Preliminary attempts to determine an amino acid sequence of the receptor proteins isolated from K562, Krebs and insulinoma cells have met with little success. It appears that the N-terminus of these receptor proteins may be blocked by carbohydrate moieties and glycosylation of the polypeptide chains interferes with sequence determination. However, even if a partial sequence of the identified receptor can be determined, one can search the computerized data banks to compare the sequence with others already described. If the receptor protein turns out to be a sequence previously identified, we will be in a favourable situation. For example, such was the case with the receptor for the major group of rhinoviruses, where researchers found that a sequence they had determined was identical to ICAM-1, a member of the immunoglobulin superfamily (Greve *et al.*, 1989; Staunton *et al.*, 1989). Should we be as lucky as they were,

we may then find that monoclonal antibodies already exist for the protein and hence the receptor protein for EMC virus. If the receptor is an unidentified protein, an oligonucleotide sequence would have to be synthesized based on the amino acid sequence determined. Using the oligonucleotides, we may be able to produce a cDNA for the receptor protein and be able to clone the gene into a suitable bacterial vector. Once the receptor gene is cloned, it should be easy to determine the nucleotide sequence of the receptor gene, and subsequencetly determine the complete amino acid sequence of the receptor of interest.

4.6. Summary and Conclusion

The findings obtained in the course of this study can be summarized and concluded as follows:

(1) EMC virus binds specifically to a finite number of a single class of receptor molecules on human nucleated HeLa and K562 cells. The number of receptors on HeLa cells is in the range of 1.6×10^5 per cell with a dissociation constant for virus binding of 1.1 nM.

(2) The receptors for EMC virus on both K562 and HeLa cells are intracellularly synthesized proteins carrying sialic acid residues. The apparent molecular size of these receptor proteins is in the range of 70 kD.

(3) Examination of K562 and HeLa cell surface proteins for binding of EMC virus using the virus overlay protein binding assay and radiolabled EMC virions, revealed that virus specifically recognizes only the identified 70-kD receptor molecules both in the membranes solubilized in sodium deoxycholate, and in the receptor preparations purified from these solubilized membranes by EMC virus affinity chromatography.

(4) The purified 70-kD receptor protein from K562 cells appears to be more sialylated than that from HeLa cells as shown by chromatofocusing of untreated and neuraminidase desialylated receptor preparations.

(5) The 70-kD receptors for EMC virus on K562 and HeLa cells do not display glycophorin A specificity, as demonstrated by Western blotting analysis with monoclonal anti-glycophorin A antibody. This confirms a previous finding that glycophorin A may not be the receptor for EMC virus on K562 cells and indicates that receptor proteins identified in the course of the present studies represent a novel class of not yet described receptor molecules for EMC virus.

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Appendix

Media:

1. RPMI Medium 1640

RPMI medium 1640 (Roswell Park Memorial Institute medium) was prepared from RPMI medium 1640 powder purchased from GIBCO BRL. 10.38 g of powdered medium was dissolved in 900 ml of deionized distilled water and then 2.0 g of $NaHCO_3$ was added. The medium was adjusted to 0.2-0.3 below desired pH 7.4 with 1N HCl and made up to 1000 ml with distilled water. The medium was sterilized by membrane filtration (pore size: 0.22 micron, Becton Dickinson Labware, Lincoln Park, NJ, USA) and stored at $4^{o}C$. To grow K562 cells, the medium was supplemented with 10% fetal bovine serum, 100 U of penicillin per ml and 100 µg of streptomycin per ml (GIBCO BRL).

2. Dulbecco's modified eagle medium

Dulbecco's modified eagle medium (DMEM) was prepared from DMEM powder purchased from GIBCO BRL, 13.37 g of powdered medium was dissolved in 900 ml of deionized distilled water and then 3.7 g of $NaHCO_3$ was added. The medium was adjust to 0.2-0.3 below desired pH 7.4 with 1N HCl and made up to 1000 ml with distilled water. The medium was sterilized by membrane filtration and stored at $4^{o}C$. To grow HeLa cells, the medium

diluted with deionized distilled water to the desired molarity of 0.02 M or 0.1 M.

4. Tris-HCl buffers

212.0 g Tris (hydroxymethyl) aminomethane (Bio-Rad Laboratories) was dissolved in 500 ml of deionized distilled water to prepare a 3.5 M stock solution. The buffers were titrated to desired pH of 8.7 or 6.8 with 1M HCl, and then diluted to the required molarity of 1 M or 3 M.

was supplemented with 5% newborn calf serum, 100 U of penicillin per ml and 100 µg of streptomycin per ml (GIBCO BRL).

Buffers:

1. Phosphate-buffered saline, pH 7.4

Phosphate-buffered saline, pH 7.4 (PBS, pH 7.4) was prepared in a 10x solution. 80.0 g NaCl, 2.0 g KCl, 2.0 g KH_2PO_4 and 11.50 g Na_2HPO_4 were dissolved in 900 ml deionized distilled water. The solution was adjusted to pH 7.4 with 1N HCl and the volume was made up to 1000 ml.

2. PBS-Tween

0.05% PBS-Tween solution (v/v) was prepared by adding Tween-20 (Bio-Rad Laboratories) to PBS, pH 7.4.

3. 0.5 M phosphate buffers

Solution 1: 69.0 g $NaH_2PO_4 \cdot H_2O$ was dissolved in 1000 ml deionized distilled water.

Solution 2: 71.0 g Na_2HPO_4 was dissolved in 1000 ml deionized distilled water.

Solution 1 and solution 2 were mixed to obtain the required pH and