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Binding and entry of animal viruses

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(Received January 4, 1988)

(Accepted July 12, 1988)

Key words: Endocytosis; Membrane fusion; Viral entry; Viral tropism; Virus binding site; Virus receptor; Virus receptor interference

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Abbreviations: EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; HTLV, human T-cell leukaemia virus; HA, haemagglutinin; HRV, human rhinovirus; MHC, major histocompatibility agent; VSV, vesicular stomatitis virus; SVP, subviral particle; SFV, Semliki Forest virus; NA, neuraminidase.

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Summary

Viruses are infectious agents capable of packaging and delivering nucleic acids and proteins to specific populations of cells. To initiate infection, viruses bind to sites, or receptors, on the cell surface and transfer their genome across the limiting membrane of the cell. The mechanisms underlying these events, and viral tropism for particular host cells, are becoming increasingly well understood. Several cell surface proteins have now been identified as viral receptors, and analyses of intact virus particles and sub-viral components are revealing the structures of the binding determinants on the viruses themselves. For many viruses, the events leading to penetration and delivery involve constitutive endocytic properties of the host cell, and the low pH environment in endocytic compartments is a crucial trigger in the penetration process. The knowledge of viral tropism, binding and entry suggests strategies which may be applied to the design of targeted therapeutic agents with appropriate specificities and effective delivery mechanisms.

I. Introduction

The aim in drug targeting is to deliver efficiently a therapeutic agent, be it an antibody, gene, drug or toxin, to a specific set of cells. Virus particles are naturally designed transport vehicles whose viability is dependent on effective intercellular transport. To this end viruses have evolved mechanisms for (1) packing, transporting and delivering nucleic acid molecules and, in some cases, transcriptional enzymes to cells; (2) for avoiding host defence mechanisms; and (3) for maintaining selectivity, or tropism for specific cell populations. During the extracellular transmission phase of the life cycle, the viral genome is packaged within and protected by either a lipid and protein membrane (enveloped viruses) or a protein shell (non-enveloped viruses). On entering a host organism, the viruses must locate susceptible cells, shed the protective coat and penetrate the cell. These events are mediated by interactions between components of the viral envelope or shell and binding sites on the target cell surface. Here we discuss some of the principles that

are emerging from a better understanding of viral recognition, tropism and penetration and consider the application of these principles to the targeting of therapeutic agents.

II. Tropism

Viral tropism refers to the ability of a virus to infect specific populations of cells. Full infection leading to the release of infectious progeny virus requires the host cell to be permissive at all stages in replication. Consequently, tropism can be determined at different steps of the virus-host cell interaction.

Frequently, tropism is established at the initial binding step of the virus-host cell association and is determined by binding components on the virus [127] and the differential expression of specific cell-surface molecules that constitute the viral receptors [2,88] (cell-surface molecules are not expressed to bind viruses, rather viruses exploit these components to facilitate their entry; the term 'receptor' is usually reserved for cell-surface molecules involved in the uptake of macromolecules such as growth factors or hormones). Cells that lack binding sites for a specific virus, or where the binding sites are blocked, are resistant to infection by that virus. These cells may, however, support replication if the viral genome is introduced through transfection, via pseudotype viruses or alternative binding sites [44,52,131].

The role of specific cell surface binding proteins in Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV) tropisms for B and T lymphocytes, respectively (the CR2 receptors for the complement component C3d and CD4 antigen, respectively) is well documented and clearly accounts for the observed tropisms of these viruses [18,23,24,87,138,56]. Similarly, the expression of as yet undetermined binding sites on cells of the nasopharynx, gut and anterior horn is believed to account for poliovirus tropism and pathogenesis [103].

The expression of receptors does not always result in productive viral infection. Cells can express binding sites for viruses that they cannot replicate. This reflects an extended receptor host range and can be demonstrated using pseudotype viruses that contain coat components of the virus in question and the genome of a virus which the cell will replicate [108]. To release infectious viruses, cells must support all steps of the viral life cycle. Defects in replication can occur at any step in replication. For example para- and orthomyxoviruses produce a lytic infection in culture and in epithelial cells of the respiratory tract. The spike glycoproteins of these viruses are proteolytically cleaved, by cellular trypsin-like enzymes, during transport to the cell surface (see below and Fig. 1). This proteolytic cleavage is essential for the fusion activity of the progeny virions (see below). Cells that lack the appropriate enzymes bind and replicate the viruses, but produce inactive virions. The viruses fail to establish an infection and the cells appear resistant to the viruses [45,51].

Tropism can thus reflect the cell or tissue type in which viruses exert a pathogenic effect. However, virus infection does not always show pathogenic effects. The human retroviruses, T-cell leukaemia virus (HTLV) 1 and 2, are regarded as having T-cell tropism. In vivo only T-lymphocytes are transformed [131] but in culture

these viruses have an extended host range and will infect human and animal lymphoid and non-lymphoid cells [132]. It is unclear whether non-T cells are infected *in vivo*. The precise mechanism of HTLV-1 and 2 transformation is unclear, a viral oncogene has not been identified and they have not been found to have common integration sites. It appears that cell-specific and virally encoded transactivating factors play a role in HTLV 1 and 2 transformation and in establishing the tissue tropism of these viruses [6].

III. The structures of viruses and their attachment sites

III.1. *Enveloped viruses*

The enveloped viruses, which include myxo- rhabdo-, flavi-, retro-, herpes, alpha-, bunya-, corona-, hepadna-, and arenaviruses, vary in size and morphology from the 65 nm diameter spherical virions of α -viruses to the bacilliform rhabdoviruses with dimensions of 50–95 nm diameter by 130–380 nm long. Enveloped viruses have been used extensively as models for membrane structure and biogenesis. Many of the viral proteins have been sequenced and their biosynthesis and structural properties studied in detail.

The virions contain DNA or RNA genomes complexed with one or more proteins to form a nucleocapsid particle. The nucleocapsids are assembled in the cytoplasm (or nucleoplasm for herpes virus) of the infected cells and are released by budding through a cellular membrane. On budding, they acquire a bilayer envelope which has a lipid composition similar to that of the host cell membrane [111]. Projecting out from the membrane are the viral envelope, or spike proteins (Fig. 1). These proteins are synthesised by the host cell and incorporated into the membrane from which the viruses bud. A virion contains multiple copies of each envelope protein. The 3D structure of Sindbis virus (an α -virus) indicates that each virion contains 240 copies of the spike heterodimers arranged as 80 trimeric units [27], in contrast influenza particles contain in excess of 1000 haemagglutinin (HA) molecules. The proteins contain a transmembrane polypeptide, or polypeptides, linking the spike to the membrane and are usually glycosylated. The spike proteins are multifunctional. They mediate (1) the binding of virions to host cell binding sites – virions which lack spike proteins, such as the 'bald' particles of the VSV ts 045 mutant do not bind to or infect permissive cells [130]; (2) they trigger membrane fusion properties of the virus; and (3) they assist in the process of virus budding (Refs. 82 and 111; see Fig. 1).

Although the viral proteins responsible for binding are in the main defined, the epitopes on these proteins which mediate attachment are largely uncharacterised. The 3D structures of the influenza virus HA has been determined to 3 Å resolution enabling a detailed analysis of the attachment sites [135]. HA, which mediates both attachment and fusion, is a trimer about 135 Å long and 15 – 40 Å in radius. During its biosynthesis the trimer must be correctly assembled in the endoplasmic reticulum of the infected cell in order to be transported to the Golgi apparatus and cell surface. In the course of transport the oligosaccharides are modified and, in

MEMBRANE PROTEINS OF ENVELOPED VIRUSES

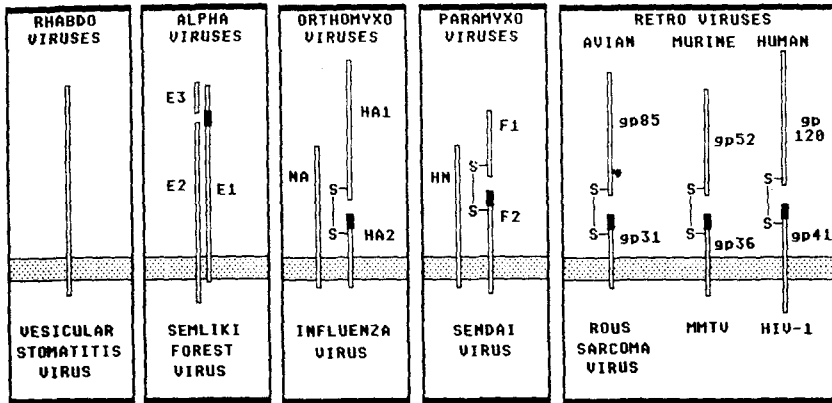


Fig. 1. Schematic representations of the subunits of the envelope proteins for five groups of enveloped animal viruses. The membrane proteins of vesicular stomatitis virus (VSV, a rhabdovirus), Semliki Forest virus (SFV, an alphavirus), influenza and Sendai virus (myxoviruses) and an avian, murine and human retrovirus are illustrated. The myxoviruses express two types of envelope protein; influenza contains a haemagglutinin/fusion protein (HA) and a binding site-destroying neuraminidase (NA), while Sendai virus contains a fusion protein (F) and a haemagglutinin/neuraminidase (HN). In contrast to the myxoviruses the rhabdo-, retro-, alpha- and flaviviruses have one type of spike protein, each subunit of which is comprised of 1-3 polypeptides. All the polypeptides are synthesised in the rough endoplasmic reticulum of infected cells and for influenza HA and VSV G at least, are assembled into trimeric spike protein units prior to transport to the Golgi apparatus and cell surface. Many are synthesised as polyproteins and are proteolytically cleaved to produce the mature proteins. The p62 subunit of SFV, for example, is cleaved to give the E2 and E3 polypeptides of the mature spike protein. Similarly, the influenza HA, Sendai F and retroviral envelope proteins are cleaved to produce the mature fusion-competent spike protein (note that the disulphide bonds which link the HA and F protein subunits in influenza and Sendai may be absent for HIV env and for at least some of the D-type retroviruses). The hydrophobic domains (indicated as filled black boxes) are believed to be involved in fusion.

many cells, the HA subunits are proteolytically cleaved to generate the fusion competent disulphide-linked heterodimer of HA1 and HA2 (see Fig. 1 and section VI [13]). HA binds to plasma membrane glycolipids and glycoproteins containing sialic acid [43,117]. Binding occurs through a region at the top of the globular head of HA1 [135]. The site consists of a surface pocket lined by a number of amino acids which are highly conserved between different strains of the virus. Three-dimensional analysis of HA complexed with sialyllactose shows sialic acid fills this conserved pocket and confirms the role of sialic acid as the influenza receptor [128]. Variations in the sequences around the pocket modify the conformation of the site such that the virus will only bind to sialic acid residues with a specific linkage configuration. For example, certain strains of virus normally bind NeuAca2-3Gal-containing surface molecules. When these viruses are grown in the presence of horse or guinea pig sera, containing NeuAca2-3Gal-, mutants arise which bind NeuAca2-6Gal-. Sequence analysis of the mutant HA genes shows a single amino acid change in the pocket region (Leu 226 → Gln or Met) is responsible for the change in binding phenotype [100].

In contrast to influenza, the 3D structure of the HIV-1 envelope protein is not resolved. Nevertheless, the attachment of this virus is relatively well understood. The gp120 fragment of the envelope glycoprotein (Fig. 1) binds with high affinity ($K_d = 4 \cdot 10^{-9}$ M) to human CD4 molecules [58]. The CD4-binding domain of gp120 appears to lie in a 42 amino acid (residues 397–439) region of the molecule containing a number of residues that are conserved in different strains of HIV-1. Deletion of a 12 residue peptide from within this domain decreases gp120-CD4 binding [58]. In addition, regions of the protein adjacent to the 42 amino acid domain also appear to influence binding [57].

The envelope proteins of the yellow fever and Murray valley fever flaviviruses, contain the tripeptide R-G-D [17,98]. This is a consensus sequence contained in the binding epitopes of some ligands for receptors of the integrin family, such as the fibro- and vitronectin receptors [47,102]. However, it remains to be demonstrated that R-G-D peptides are involved in flavivirus entry or whether these viruses utilize receptors of the integrin family.

III.2. Non-enveloped viruses

Non-enveloped viruses, which include the adeno-, birna-, parvo-, picorna-, papova-, and reoviruses, contain single or double stranded RNA or DNA genomes within an icosahedral capsid. The polypeptide subunits which form the capsid also mediate viral association with host cell surface and entry of the genome into the cell. The particles do not contain a lipid bilayer, though the VP4 polypeptide of picornaviruses (polio and rhinoviruses for example) and the VP2 polypeptide of SV40 and polyoma (papovaviruses) contain covalently bound myristic acid [9,115]. The amino acid sequences of many of the structural polypeptides of these viruses have been determined.

As with the enveloped viruses, non-enveloped virus capsids contain multiple attachment sites. Adenovirus binding is mediated by the fibre protein. The virion has 12 copies of this protein projecting from each of the penton bases. The fibre protein can be removed from the capsid by proteases rendering the particles incapable of binding [96]. The isolated fibres appear to retain activity and will compete with intact virus for cell surface binding sites [61].

Reovirus attachment is mediated by the $\sigma 1$ polypeptide located at the vertices of the icosahedron adjacent to the $\lambda 2$ (core spike) polypeptide [109]. Reoviruses have segmented double stranded RNA genomes in which recombination can occur. Mixed infections, using variants with different tropisms, give rise to progeny viruses containing reassorted genomes. The tropisms of the progeny viruses are determined by the S1 genome segment which encodes the $\sigma 1$ polypeptide [127].

The 3D structures of human rhinovirus (HRV) 14 – a common cold virus – and poliovirus type 2 [46,101], have indicated putative attachment sites on the surface of picornaviruses. In HRV 14, the two capsid proteins VP1 and VP3 form a canyon or cleft on the surface of each of the icosahedral faces of the particle. The dimensions of the canyon (25 Å deep, 12–30 Å wide) are such that an antibody, in which the Fab fragment diameter is about 35 Å, would have difficulty reaching

the canyon floor – its entrance being blocked by the canyon rim. Thus residues in the binding site would not be under selective pressure, and would enable the virus to retain binding specificity. Variations in the amino acid sequences of the VP1 and VP3 domains that line the walls of the canyon are found in picornaviruses with different binding specificities [101].

IV. Binding

IV.1. Mechanisms of virus binding

Binding occurs through complex interactions between a virion and the cell surface (Fig. 2). In tissue culture the binding reactions are influenced by environmental factors that include temperature, ionic strength, pH, composition of the medium, presence of serum, etc. [60] (note that for viruses dependent on exposure to acid conditions for entry, such as alphaviruses, increased binding at low pH should not be confused with low pH-induced membrane fusion [125, see below]).

The initial virus–cell-surface interactions may be low affinity and lead to the rapid dissociation and release of virions (viruses, such as the myxoviruses, which have



Fig. 2. Electron micrograph of SFV bound to the surface of hamster kidney cells. BHK-21 cells were grown on glass coverslips. To bind virus, the cells were incubated with 2 μ g purified SFV for 1 h at 0°C. After washing away the unbound virus the cells were fixed and processed for electron microscopy. The figure shows virions bound to the cell surface, frequently in association with microvilli. A coated pit also contains bound virus. Bar = 150 nm.

receptor-destroying neuraminidase activity, can be released enzymatically) or to the establishment of higher-affinity association. The increased affinity (avidity) can result from interaction with high affinity binding sites or through multiple interactions with lower affinity sites; as many viruses have multiple attachment sites a combination of the two is likely. Experiments with SFV illustrate this point. The SFV spike glycoproteins can be presented to cells in different multimeric forms, either as virions having 240 copies of the spike proteins or as artificial octameric complexes. The binding constants are 10^{-10} – 10^{-11} M for virus, and 10^{-7} M for the octamers. BHK cells have the capacity to bind approx. $1.3 \cdot 10^6$ complexes or $5 \cdot 10^4$ virions per cell. Thus at high virus loads the number of binding interactions per SFV virion is reduced and the binding avidity decreases [25,72].

IV.2. Cellular binding determinants for viruses

Considerable effort has been spent in trying to determine the nature of the specific cell-surface determinants involved in virus binding. In recent years refined immunological techniques, molecular biology and genetics have identified at least some of these components.

IV.2(a). Experimental methods for identifying virus binding sites

(i) *Affinity chromatography.* Intact virions or isolated viral attachment proteins have been used to identify virus binding sites by affinity isolation. For example a 110 kDa putative glycoprotein receptor was isolated from the surface of murine L cells using spike glycoproteins of Moloney murine leukaemia virus [50] and class 1 MHC antigens were identified as potential receptors for SFV [39]. The approach requires the disruption of either the virus, or the cell plasma membrane, or both. Consequently, only monomeric interactions can occur and this approach may be limited to viruses which have a high affinity for their binding sites.

(ii) *Antibodies against cell surface viral binding determinants.* For viruses with a narrow host range panels of well-defined antibodies have been used to identify virus binding sites. HIV-1 is tropic for cells bearing the CD4 antigen, primarily the helper/inducer subset of T-lymphocytes. The CD4 molecule was identified as the virus binding protein using anti-T-cell monoclonal antibodies [18,56]. Antibodies against the HIV-1 binding domain of CD4, and adjacent epitopes, block infection by HIV-1 and VSV(HIV) pseudotype virus [105]. Following binding of HIV-1 to CD4 positive cells, and detergent lysis, gp120 and CD4 can be co-immunoprecipitated using antibodies against CD4 epitopes distinct from those involved in virus binding [58,63].

In other approaches monoclonal antibodies have been made against cell-surface proteins and screened for the ability to block virus binding and infection. This method has been used to identify a 90 kDa cell-surface polypeptide as a putative binding protein for HRV 15 [124]. Alternatively, anti-idiotypic antibodies, produced against anti-viral antibodies, have been used to identify the binding determinant for reovirus type 3. These anti-idiotypic antibodies should contain a mirror image of the attachment site on the viral $\sigma 1$ protein. The anti-idiotypes inhibit reo-

virus binding to cells and elicit both T and B cell immunity. They precipitate a 67 kDa glycoprotein from rodent and primate cells [10]. Both the anti-idiotypic and radiolabelled virus bind to a 67 kDa plasma membrane protein on Western blots. Further characterisation of the 67 kDa antigen has suggested the β -adrenergic receptor is the viral binding protein [11].

(iii) *Chemical cross-linkers.* Chemical cross-linkers have been used to identify viral binding sites [33,75]. The viral attachment protein VP1 of polyoma virus can be cross-linked to cell-surface components on mouse primary kidney cells. Purified complexes injected into rabbits produce antibodies that react with VP1, stain the surface of the mouse cells and block virus binding. On Western blots the antibodies label 3 polypeptides in a plasma membrane fraction from the mouse cells. The nature of the polypeptides and their role in virus attachment remain, however, unknown [33].

(iv) *Transfections and cell hybrids.* Some limited information on the genes encoding viral cell-surface binding sites has been obtained by chromosome assignments using somatic cell hybrids. Human chromosome 19 encodes binding sites for poliovirus, echovirus 11, and two endogenous animal C type retroviruses (Refs. 4,49,79,84, 107 and 112a). Chromosome 17 encodes binding sites for HTLV-1 and 2, and the human G chromosomes (21,22 and/or Y) encode Coxsackie B virus binding sites [53]. The identification of receptor genes has been attempted for poliovirus where total human DNA has been transfected into normally resistant mouse L cells. Transfectants expressing poliovirus binding sites have been obtained [81].

IV.2(b). *Viral binding components on the cell surface*

Most analyses of viral binding have been carried out *in vitro*, often under non-physiological conditions (e.g., low temperature). In many cases it remains unclear whether the specificities observed in culture are of functional importance for virus entry *in vivo*. For several viruses (e.g., HIV, EBV), however, the specific binding determinants identified in culture correlate with the disease symptoms and known tropisms of the virus.

Table I indicates that a range of cell-surface molecules can be involved in binding viruses that infect man and other vertebrates. The molecular nature of the binding sites, and the cellular function of these molecules, have been identified for several viruses with very specific tropisms (CD4 for HIV, CR2 for EBV). It is these proteins that are best characterised with regard to virus entry. Other viruses, such as influenza, also exhibit considerable binding specificity. Unlike the cell-specific expression of CR2 and CD4, the receptors for these viruses (*N*-acetylneuraminic acid; subsection III.1) are expressed on many cell-surface glycoproteins and glycolipids and enable the viruses to bind to a range of different cell types. Such broad spectrum binding determinants may be used by other viruses. Sialic acid has been implicated in binding for paramyxo-, picorna-, papova-, reo-, and adenoviruses [2], concanavalin A binding sites for HRV-14 (see Ref. 101), and heparin sulphate proteoglycan for Herpes Simplex virus [137].

Viruses with a broad host range may bind to more than one determinant on dif-

TABLE I
IDENTIFIED AND PUTATIVE VIRUS BINDING COMPONENTS

Virus group	Virus	Binding component	References
Enveloped viruses	Retro	human immunodeficiency virus	18,56
		Moloney murine leukaemia virus	50
		radiation leukaemia virus	92
Herpes	Epstein-Barr virus	CR2	23,24,87,97,120,121,138
	Cytomegalovirus	β_2 -microglobulin/MHC	34,65
	Herpes Simplex virus	heparin sulphate proteoglycan	137
Rhabdo	Rabies virus	Acetylcholine receptor	59
	Vesicular stomatitis virus	Phosphatidyl serine	106
Hepadna Paramyxo Orthomyxo	Hepatitis B	Polyalbumin/polyalbumin receptor	85,122
	Sendai	Sialic acid (glycoprotein/glycolipid)	71
	Influenza	Sialic acid (glycoprotein/glycolipid)	43,117
	Semliki Forest virus	Class 1 MHC	39
Toga	Lactate dehydrogenase virus	Class 2 MHC	48
	Sindbis virus	90 kDa protein/catecholaminergic receptor	75,123
Pox	Vaccinia	Epidermal growth factor receptor	22
Non-enveloped viruses	Picorna	Coxsackie B	70
		Rhinovirus	124
	Reovirus	Encephalomyocarditis virus	5
		Reovirus type 3	β -adrenergic receptor
Papova Adeno	Polyomavirus	30-40 kDa glycoprotein complex	33
	Adenovirus type 2	78 kDa, 42 kDa, 34 kDa glycoproteins	42,118

ferent host cells. SFV, for example, binds to the major histocompatibility (MHC) antigens on human and murine lymphoblastoid cells but will also bind to and infect cells that do not express MHC antigens [39,91]. Although the identities of many virus binding sites remain to be determined, it is clear that a number of viruses may utilize the same binding sites. Competition experiments show that a number of nonenveloped viruses use four distinct binding sites on HeLa cells; group 1 is shared by the HRV types 2, -1A and -1B; group 2 by Coxsackie type A21 and HRV types 3,5,14,15,39,41,51; group 3 by all three serotypes of Poliovirus and group 4 by Coxsackie B3, other B Coxsackie viruses and Adenovirus type 2 and 5 [61]. Similarly, binding sites on cultured human cells are shared by different retroviruses (Table II). Although experimental evidence indicates that the same receptors are used by different viruses, it is unclear whether the viruses bind to the same epitopes on these molecules.

The binding reaction enables a virus to recognise, and associate with, a target cell. Does binding play any further role in entry? Endocytosis is required for penetration by certain viruses (see below). These viruses may need to bind to cell surface components which are constitutively endocytosed. Alternatively virus binding itself may induce the internalization of cell surface components that are not normally internalized, perhaps by aggregating or crosslinking these sites. Although different viruses can use the same cell surface binding sites (Table II; Ref. 61), at the present time, however, it is unclear that these molecules have any specific advantages for entry.

Enveloped virus membrane proteins may themselves bind other viruses. For example, influenza HA expressed on the surface of infected cells, can bind viruses containing sialated glycoproteins [28]. Vesicular stomatitis virus (VSV) and SFV normally infect cultured epithelial cells through the baso-lateral domain. Prior infection with influenza, in which the HA is expressed on the apical surface, enables VSV and SFV to enter through this normally non-permissive region of the plasma membrane. Similarly, BHK-21 cells which are resistant to infection by murine hepatitis virus (MHV-A59, a coronavirus) become susceptible to this virus when preinfected with influenza. This phenomenon may play a role in increasing the susceptibility of cells to secondary infections and has precedents *in vivo* [28,52].

In contrast, preinfection by one virus can interfere with subsequent infections by a second virus by, for example, modulating the expression of binding determinants for the second virus. Cells chronically producing retroviruses, and expressing viral envelope glycoproteins on their surface, down-regulate, shield or otherwise hide the binding site. Thereby the cells become resistant to superinfection by any retrovirus that uses the same binding sites [35,129,113].

IV.3. Indirect binding

In addition to direct binding, viruses may bind indirectly via natural ligands for specific cell-surface receptors. Cytomegalovirus is reported to bind to β_2 -microglobulin which in turn can bind to cell-surface MHC antigens [34,65] and hepatitis B virus is believed to bind to hepatocyte polymeric albumin receptors via polyal-

TABLE II

RETROVIRAL BINDING ON HUMAN CELLS

The binding determinants on human cells utilized by animal retroviruses appear to fall into eight distinct groups as determined by syncytial interference. Most retroviruses do not establish a lytic infection, instead they become chronic virus producers. Cells infected with these retroviruses down-regulate, shield or otherwise hide their cell-surface binding sites and, consequently, they become resistant to superinfection by the same virus or other viruses that utilize the same binding site. When infected cells are mixed with uninfected cells cell fusion can occur following interaction between the viral membrane proteins expressed on the infected cell and binding sites expressed on the uninfected cell. However, if cells infected with two different viruses are mixed, syncytia will only form if different binding determinants are used by each virus. If the viruses use the same binding determinants receptor interference will prevent interaction between the cells required for cell fusion [104, 112a, 113, 129, 132].

Receptor group	Virus	Abbreviation	Retroviral type
1	Cat endogenous	RD-114	C-type
1	Baboon endogenous	BaEV	C-type
1	Mason-Pfizer monkey virus	MPMV	D-type
1	Simian retrovirus 1 (SAIDS-D)	SRV-1	D-type
1	Simian retrovirus 2 (SAIDS-D)	SRV-2	D-type
2	Murine leukaemia virus ampho- tropic	MLV-A	C-type
3	Murine leukaemia virus xeno- tropic	MLV-X	C-type
4	Feline leukaemia virus subgroup C	FeLV-C	C-type
5	Feline leukaemia virus subgroup B	FeLV-B	C-type
5	Simian sarcoma associated virus	SSAV	C-type
5	Gibbon ape leukaemia virus	GALV	C-type
6	Bovine leukaemia virus	BLV	C-type
7	Human T-cell leukaemia virus 1	HTLV-1	C-type
7	Human T-cell leukaemia virus 2	HTLV-2	C-type
8	Human immunodeficiency virus 1	HIV-1	Lenti
8	Human immunodeficiency virus 2	HIV-2	Lenti

bumins [85,122]. Antiviral antibodies facilitate the infection of Fc-receptor bearing cells by flaviviruses and other viruses in culture [89,94,95], and by flaviviruses, myxoviruses, and the lentivirus subgroup of retroviruses in vivo [64,126].

V. Penetration

The binding of a virus to the cell surface initiates penetration whereby the viral nucleic acid and associated polypeptides are transferred to the cytoplasm. Enveloped virus penetration occurs by fusion between the viral and cellular membrane. For the non-enveloped viruses, however, it remains unclear how the capsid proteins interact with cellular membranes, and whether the whole capsid particle or just the nucleic acid enters the cytoplasm.

V.1. Enveloped viruses

V.1(a). Membrane fusion

Enveloped virus membrane fusion is mediated through the fusogenic spike proteins of the viral membrane (Fig. 1). These proteins are frequently the same proteins that are involved in attachment. The spike proteins are the only viral polypeptides needed for the fusion reaction, and the principal requirement for their activity is that they are anchored in a membrane [134]. The sequence and organisation of fusion proteins vary considerably between different groups of viruses; however, they can be organised into three categories.

The major group is that represented by influenza virus HA. HA is synthesised as a single polypeptide which, during transport to the cell surface, is proteolytically cleaved to form HA1 and HA2 (see subsection III.1 and Fig. 1). The cleavage results in the formation of a new terminus on HA2. This sequence is highly hydrophobic and is conserved between several different myxoviruses [134]. The hydrophobic domain is critical for the fusion activity, since HA molecules which fail to undergo proteolytic cleavage are not fusogenic. Furthermore, the introduction of charged amino acids into the hydrophobic sequence abolishes or inhibits the fusion activity [30]. The fusion proteins of paramyxoviruses and retroviruses including HIV-1, are similarly organised. In the latter case, the HIV-1 env protein has a hydrophobic domain, which is believed to be important for fusion, at the putative amino terminus of gp41. A mutant *env* gene encoding a protein with 4 or 6 amino acids inserted into the hydrophobic domain, will direct the expression of the precursor gp160 and the gp120 and gp41 polypeptides; however, the mature proteins are not fusogenic [57].

The second group of fusion proteins, represented by the α -viruses, contains two transmembrane polypeptides (P62 and E1 for SFV). P62 is proteolytically cleaved en route to the plasma membrane to form E2 and E3 (E3 is lost from some α -viruses and appears to be unimportant for virus entry). The α -viral proteins do not contain an N-terminal hydrophobic amino acid sequence; however, a conserved sequence of 17 uncharged amino acids is found in the ectodomain of E1 and may function in fusion [54].

The third group of fusion proteins is represented by the rhabdoviruses, such as VSV and rabies virus, which express one type of membrane protein. The spike proteins are assembled as trimers of this one polypeptide. Each of the polypeptides has one major hydrophobic domain, the transmembrane domain, which anchors the trimer into the membrane. The regions of the protein involved in fusion have not been identified.

Although the amino acid sequences of many viral fusion proteins have been determined, the mechanisms by which these proteins induce fusion is unclear. Studies with influenza and SFV have begun to determine some of the elements of the fusion reactions. With both viruses exposure to mildly acidic pH is required for fusion. The low pH induces irreversible conformational changes in the spike proteins which can be detected by changes in hydrophobicity, circular dichroism spectra, sedimentation and sensitivity to reducing agents or protease digestion [19,134].

For influenza HA, whose 3D structure is known, the acid-induced conformational changes occur when interchain salt bridges, that stabilize the HA trimer at neutral pH, are broken. The ectodomains of the trimer appear to separate and the hydrophobic N-termini of the HA2 subunits, which were previously buried within the molecule, are exposed. The hydrophobic domains are believed to interact with the target membrane and promote fusion by bringing the viral and cellular membranes into close proximity [19].

In addition to the orthomyxo- and α -viruses, exposure to acid conditions is important for the fusion of rhabdo-, flavi-, and some murine retroviruses (see Ref. 134). Other enveloped viruses, such as the paramyxovirus Sendai and HIV-1, do not require exposure to acid pH for fusion [62,114]. The fusion efficiency of Sendai virus is the same at neutral and mildly acid pH, and is slightly enhanced in mildly alkaline conditions. Although the organisation of the fusion proteins of Sendai and HIV-1 is similar to that of influenza HA (Fig. 1), it is not known if the proteins are multimeric, or whether the hydrophobic fusion peptides are only exposed during the fusion reaction.

In general viral fusion does not require specific lipids and can occur with membranes containing a normal complement of phospholipids, though cholesterol must be present in the target membrane for SFV fusion (see Ref. 134). SFV and influenza-induced fusion does not appear to require binding components in the target membrane, and under appropriate conditions, the viruses will fuse with liposomes devoid of protein (see Ref. 134).

V.1(b). Endocytosis

The nature of the fusion trigger requires that pH-dependent viruses encounter an acidic environment. Under normal circumstances the virions do not fuse at the cell surface and penetration occurs only after endocytosis of intact virions into acidic intracellular organelles.

The endocytic uptake of SFV has been studied in detail [40]. Following binding, the virions are internalized intact and presumably in association with the cell-surface binding components (Fig. 3). Internalization occurs through clathrin-coated pits, in coated vesicles, in a manner similar to the receptor-mediated endocytosis of physiological ligands such as low-density lipoprotein and transferrin [31]. Endocytosis through coated vesicles has a considerable capacity. In BHK-21 cells, for example, up to 3000 SFV particles can be internalized from the cell surface each minute [72]. The virions are internalized, together with other receptor-bound ligands, by the cells' constitutive endocytic activity, and uptake is not induced by the virus [72]. Other viruses, including influenza, Sendai and VSV are also internalized in coated vesicles [15,16,76,77], although with varying kinetics [76,77]. The limiting factor appears to be the size of the virus particles. SFV (65 nm diameter particles) can be contained within most endocytic coated vesicles (average diameter 100 nm) and is internalized rapidly ($t_{1/2}$ =5–10 min [72]), while larger viruses, such as influenza (100 nm diameter) and VSV (150 nm long) are internalized more slowly ($t_{1/2}$ =10–15 and 30 min, respectively [76,77]).

Clathrin-coated vesicles mediate the bulk of constitutive endocytosis in many cell

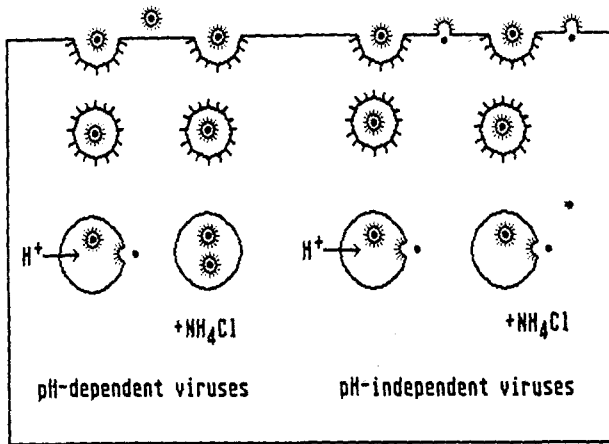


Fig. 3. Schematic representation of the pathways by which enveloped viruses enter cells. For pH-dependent viruses, such as Semliki Forest and influenza virus, membrane fusion does not occur at the cell surface. The intact virions are internalized through coated pits and vesicles and delivered to the endosome compartment. In endosomes the low pH induces a conformational change in the fusogenic spike proteins which trigger the fusion reaction. Weak bases, such as ammonium chloride, raise the pH in endosomes and prevent the conformational changes that lead to fusion. With the pH-independent viruses, such as Sendai and HIV-1, fusion can occur at the cell surface or within endocytic vesicles; however, the pH in either compartment does not influence fusion, and entry is not affected by ammonium chloride.

types and are clearly involved in the uptake of many viruses; however, other endocytic processes may internalize viruses in certain circumstances. Influenza and Sendai virions often appear wrapped in non-coated membrane [36,76], suggesting that a process similar to the phagocytic uptake of large opsonised particles may be operating. Similarly larger virions such as EBV (250 nm diameter) are reported to enter B lymphocytes in non-coated vesicles [88].

Coated vesicles rapidly lose their clathrin coat and fuse with organelles of the endosome compartment. The endosomes are the receiving station for membrane, receptors and components of the medium internalized by coated vesicles [41]. The major function of endosomes is to sort and recycle internalized membrane components to the plasma membrane and different sites within the cell [31]. The mechanisms by which this sorting is achieved involves acidification of the organelles by membrane-associated proton-translocating ATPases [78,80]. The endosome compartment appears to be the first stage on the endocytic pathway in which acidification occurs [26] and the pH decreases to approx. 5.0 by the time ligands reach the lysosomes, the terminal compartment of the pathway.

SFV, Sindbis and influenza have been shown by a combination of kinetic, morphological, biochemical and cell fractionation experiments to enter intact into the endosome compartment [73,99,119,139]. For these pH-dependent viruses, the acid conditions in endosomes trigger the reactions which lead to fusion of the viral and endosomal membranes. Internalized viruses have been shown to be infectious [38]

and fusion of SFV with endosome membranes has been visualized [37]. Following internalization the time-course of penetration varies according to the pH dependence of the fusion reaction. Viruses requiring a relatively high pH (wild type SFV, pH 6.1) fuse quickly ($t_{1/2} = 15$ min, including the time for endocytosis), while those that require more acidic conditions (SFV mutant fus-1, pH 5.3) fuse later ($t_{1/2} = 45$ min). Fusion for both viruses occurs in the endosomes prior to delivery to the lysosomes [54,55,73]. Viral components are eventually delivered to the lysosomes where degradation occurs. Following the completion of their functions in entry the viral spike proteins, and viruses that fail to undergo fusion, are delivered to the lysosomes and degraded [73].

The dependence of viral infection on exposure to acid conditions, and the consequent requirement for endocytosis, presents a point at which penetration can be blocked. Acidotropic weak bases, such as ammonium chloride, chloroquine, amantadine or methylamine accumulate in endosomes and other acid compartments causing an increase in the intravesicular pH [78,90]. Similarly, carboxylic ionophores such as monensin and nigericin will neutralise acidic organelles by exchanging H^+ for Na^+ or K^+ . Infection by a range of pH-dependent viruses, including influenza, SFV, VSV and West Nile virus, is blocked when cells are incubated with these reagents [32,38,74,76,77,99,139]. Although weak bases and carboxylic ionophores can affect a number of steps in the replication cycle, experiments with SFV and influenza show that binding, endocytosis and intracellular transport to endosomes are not inhibited by NH_4Cl or monensin. In the presence of the agents the spike proteins, however, fail to undergo the acid-induced conformational changes required for fusion. As a consequence the viruses do not fuse with the endosome membrane, the viral RNA is not released to the cytoplasm and replication does not proceed (Fig. 3). The efficacy of the reagents is related to the pH at which the viral fusion proteins undergo the conformational change, i.e., the higher the pH required for fusion the higher the concentration of drug required to block infection.

The effect of acidotropic agents can be by-passed by briefly incubating cells, with bound virus, in low pH medium and, thereby, inducing fusion at the cell surface [38,74]. Thus the reagents do not act directly on the virus itself, but indirectly through an effect on the pH of the acid compartments in which fusion usually occurs. Weak bases and carboxylic ionophores do not affect the fusion of pH-independent viruses [62,114].

V.2. *Non-enveloped viruses*

Compared to the enveloped viruses the penetration mechanisms for non-enveloped viruses is less well understood. The endocytic uptake of adeno-, reo-, polyoma- and picornaviruses has been observed both biochemically and morphologically [16,33,110] and it is now clear that some of these viruses require endocytosis for penetration.

For poliovirus type 1 entry is blocked by acidotropic amines, carboxylic ionophores and other reagents (2-deoxy-D-glucose, NaN_3) which inhibit endocytosis or

endosome acidification. This inhibition can be by-passed if the cells with bound virus are incubated briefly at low pH [67]. Low pH treatment induces changes in isolated virions (loss of VP4 and an increase in hydrophobicity) similar to changes in the virions observed during entry into cells. The changes are not found during the uptake of virions into cells treated with the inhibitors, suggesting that exposure to low pH also triggers critical conformational changes in these non-enveloped viruses [67,68]. However, low pH alone is not sufficient for entry. A pH-gradient across the membrane is required and disruption of this gradient – by, for example, acetic acid-induced acidification of the cytoplasm – inhibits poliovirus infection [68].

Although acidotropic reagents inhibit the entry of poliovirus, HRV type 2 and foot and mouth disease virus [6,69], they do not block the entry of all picornaviruses. Murine EMC virus infection is enhanced by monensin, suggesting that entry requires neutral or mildly alkaline conditions [69].

It has been suggested [101] that occupation of the binding canyon on poliovirus could facilitate disruption of the pentamer-pentamer contacts in the capsid and provide a port by which VP4 and RNA can exit the virion, possibly accompanied by a change in the isoelectric point of the virus. Thus binding may facilitate penetration by unlocking the capsid. Experiments on the pH dependency of the conformational changes observed in poliovirus type 1 support this idea. The changes are influenced by both temperature and cell association of the virus. For free virus the conformational changes are half maximal at pH 5.0, but following binding they are half maximal at pH 6.1–6.5 [68].

A low pH-dependent step has also been implicated in adenovirus penetration. Morphological experiments show that bound virions are internalized in coated vesicles and delivered to endosomes [16,93]. Subsequently, virions can be observed in the cytoplasm, suggesting that the entire capsid is translocated across the membrane. Cells treated with the weak bases chloroquine and methylamine are protected from infection, and the virions do not undergo acid-induced changes in the hydrophobicity of the penton base, hexon and fibre proteins [93].

With reovirus a variation on the theme of pH-induced changes occurs. Virions are internalized by receptor-mediated endocytosis, through coated pits and coated vesicles and are delivered to endosomes and lysosomes [116,110]. Within intracellular vesicles, the viral outer capsids are digested to produce partially uncoated subviral particles (SVPs). The growth of reovirus types 1 and 3 in mouse L cells is inhibited by ammonium chloride. The effect of NH_4Cl occurs early in infection, within 1 h of virus addition. It does not influence the binding, endocytosis or intracellular routing of the virus, but does inhibit digestion of the outer capsid proteins. If virions are digested with protease prior to infection, intermediate particles are produced that are infectious and insensitive to NH_4Cl [116]. It is unclear whether the low pH-dependent protease is viral or cellular; however, it is clear that digestion is essential for the infectious cycle.

The fact that low pH and proteolytic digestion are important for entry raises the question of how reoviruses initially enter their host. As entry appears to occur through specialized M cells of the small intestine, the viruses must pass through the acidic hydrolytic environment of the stomach and the alkaline small intestine

to reach its target site. Whether these environments influence the entry process in any way, or how the viruses survive the adverse conditions, is unclear.

V.3. *The role of endocytosis in infection*

Low pH is necessary for the penetration and entry of many viruses. Exposure to low pH can only occur within acidic vesicles following endocytosis of the virions. With the pH-independent viruses, however, penetration can occur in acid, neutral or alkaline conditions [134] and can occur at the cell surface or from intracellular vesicles. While it is clear with Sendai virus or HIV-1, for example, that fusion can occur at the cell surface [36,114], it is unclear whether this fusion results in productive infection. Experiments with SFV and HIV-1 (Ref. 93a; see also Marsh, M., unpublished results) suggest that endocytosis is important for the entry of both pH-dependent and pH-independent viruses. The reason why the pH-independent viruses should require endocytosis is unclear, but the possibility exists that for replication to occur the fusion reaction must deliver the viral genome to an appropriate site in the cytoplasm and endocytosis is the means by which this delivery is achieved.

VI. Prospects for drug development

The fundamental requirements of drug targeting are in many respects the same as those of virus transmission. Reagents must be packaged in forms suitable to ensure that (1) they are not degraded in transit and (2) any toxic effects of the cargo are minimised until delivery is complete. The carriers must have specificity to ensure their arrival at a designated target and appropriate mechanisms to release their cargo. By extending the characteristics of viruses that are essential for entry to the design of drug delivery systems it may be possible to develop carriers as effective as the viruses themselves. In several applications viruses have already been successfully exploited as delivery vehicles.

(1) *Viruses as genetic vectors.* Modified vaccinia virus incorporating genes encoding the spike proteins of enveloped viruses such as influenza and HIV-1 have been used as vaccine reagents [140]. Retroviruses are used routinely as genetic vectors for transfection in vitro [8], and are currently being adapted for use in vivo [1]. In the future retrovirus vectors may well be used in gene replacement therapies to treat a range of genetic diseases including disorders in haemoglobin (sickle cell anaemia, β -thalassaemia), severe combined immunodeficiencies and adenosine deaminase deficiency [1,20]. In addition retroviruses, with well-defined tropisms, may be modified to function as cytopathic agents by incorporating genes encoding cytotoxins such as the A chains of ricin or diphtheria toxin.

(2) *Viruses as carrier vehicles.* By replacing the nucleocapsid, within a virus particle, with an alternative cargo of nucleic acid, protein or drugs, it has been possible in culture to deliver these reagents to target cells. In addition, enveloped viruses can be used to deliver integral membrane proteins, such as receptors, to specific membrane systems. The most extensively used of these pseudo-viral par-

ticles is the Sendai virus. The virus particles can be solubilized in Triton X-100, the capsids removed and the membrane reassembled by removing the detergent. When this reconstitution is performed in the presence of exogenous proteins or nucleic acids, particles are formed which contain the protein or nucleic acid in the lumen of the vesicle. Particles can also be reconstituted in which specific membrane proteins are assembled into the virosome membrane. The reconstituted envelopes have the sialic acid binding and fusion properties of intact Sendai virus and can be used to deliver their soluble or membrane-bound load to target cells [29,103]. The specificity of these carriers can be modified by selectively destroying the sialic acid binding (haemagglutinin) activity and covalently linking specific antibodies or ligands [29,103].

Although these carriers have been used with some success to deliver receptors, DNA, RNA and antibodies in tissue culture systems, their full potential is unclear. In general reconstituted vesicles are inefficient in fusion compared to the intact virus. Much of the lost activity can be attributed to failures to efficiently reconstitute the fusion activity of detergent solubilized fusion proteins. Recent experiments with VSV, however, indicate that with appropriate reconstitution vesicles can be formed for which fusion is as efficient as that of intact virus [83]. As yet similar strategies based on non-enveloped viruses have not been used, but as the 3D structures of the viruses emerge and the interactions of the capsid sub-units are understood, development possibilities may emerge.

Further applications for virus-based carriers can be envisaged whereby the tropism or specificity of a virus is changed so as to target the virus to specific cells. Pseudotype viruses, in which the genome of one virus is coated with the shell or envelope of a different virus, have been used in culture systems to change the specificity and host range of a virus [14,21,131], and to provide viruses capable of a single round of infection. Alternatively, by understanding the structural parameters of the binding reactions, the binding epitopes on viral attachment proteins could be modified to change the binding specificity of a virus, to alter its tropism or to target carriers to specific cell populations. Such altered proteins could be utilized with the reconstituted carriers described above to target virosomes to specific cells. The requirement for low pH in the entry of some viruses might be exploited to produce carriers aimed at endocytically active cells, or at different compartments of the cell.

In order to adapt the structural features of viruses into effective drug delivery systems it is clear that many key problems remain to be solved. For example:

- (1) what are the molecular mechanisms of viral tropism?
- (2) what are the molecular identities and cellular functions of virus receptors?
- (3) do cell-surface molecules have specific properties that allow them to function as receptors for viruses?
- (4) what is the distribution of viral receptors on different cell populations?
- (5) what are the molecular mechanisms underlying viral fusion and penetration reactions?
- (6) how can isolated viral proteins be reconstituted into fully functional forms?

Analysis of the interactions of viruses with epithelial cells will also have important implications for drug delivery. For example, understanding the mechanism by which reovirus and poliovirus penetrate the epithelium of the gut may suggest mechanisms by which orally administered therapeutic agents might be delivered across this barrier [136].

The possibilities are considerable. However, it is clear that any successful exploitation of viruses in drug delivery systems must be based on a sound understanding of the interactions between a cell and a virion, and of the factors that underlie viral tropism.

Note added in proof (*received June 28, 1989*)

Since submission of this article, putative receptor proteins have been identified for several viruses. Using the transfection methods outlined in this review, a murine gene encoding a multi-spanning membrane protein has been shown to confer susceptibility to ecotropic murine leukaemia virus (Albritton, L.M., Tseng, L., Scadden, D. and Cunningham, J.M. (1989) A putative murine retrovirus receptor gene encodes a multiple membrane spanning protein and confers susceptibility to virus infection, *Cell* 57, 659–666). Similarly, ICAM-1 and a second, previously unidentified, member of the immunoglobulin gene superfamily have been proposed as receptors for human rhinovirus and poliovirus, respectively (Greve, J.M., Davis, G., Meyer, A.M., Forte, C.P., Yost, S.C., Marlor, C.W., Karmarck, M.E. and McClelland, A. (1989) The major human rhinovirus receptor is ICAM-1, *Cell* 56, 839–847; Staunton, D.E., Merluzzi, V.J., Rothlein, R., Barton, R., Marlin, S.D. and Springer, T.A. (1989) A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinovirus; *Cell* 56, 849–853; Mendelsohn, C.L., Wimmer, E. and Racaniello, V.R. (1989) Cellular receptor for poliovirus: molecular cloning, nucleotide sequence and expression of a new member of the immunoglobulin superfamily, *Cell* 56, 855–865).

Acknowledgements

We thank our colleagues – Annegret Pelchen, Mark Exley, Victoria Anne Lewis, Jane McKeating, Eddie Wawrzynczak and Robin Weiss – for helpful discussions and for reading the manuscript. The authors are supported by grants to The Institute of Cancer Research: Royal Cancer Hospital from the Cancer Research Campaign and the Medical Research Council.

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