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Early interactions between animal viruses and the host cell: relevance to viral vaccines

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Viral recognition of specific receptors in the host cell plasma membrane is the first step in virus infection. Attachment is followed by a redistribution or capping of virus particles on the cell surface which may play a role in the uptake process. Certain viruses penetrate the plasma membrane directly but many, both enveloped and non-enveloped viruses, are endocytosed at coated pits and subsequently pass into endosomes. The low pH environment of the endosome facilitates passage of the viral genome into the cytoplasm. For some viruses the mechanism of membrane penetration is now known to be linked to a pH-mediated conformational change in external viral proteins. As a consequence of infection there are alterations in the permeability of the plasma membrane which may contribute to cellular damage. Recent advances in the understanding of these processes are reviewed and their relevance to the development of new strategies for vaccines emphasised.

Keywords: Viruses; virus entry; virus uncoating

Introduction

The past seven years has seen an upsurge of interest in early virus cell interactions, as is evident in recent reviews on the subject. Even so, very little is known in precise terms about the molecular details of interactions between animal viruses and the host cell receptors. However, with the advent of rapid sequencing of virus glycoproteins involved in these interactions, our knowledge of these processes should now quickly expand. It seems a very opportune moment, therefore, to reappraise the biological data up to the present time. This review will concentrate on a few well studied viruses. It is hoped that this will illustrate some of the general mechanisms involved whilst at the same time emphasising that specific details may not apply universally even to genetically closely related viruses. Where possible we have also emphasized the practical applications to which details of virus–cell interaction may be applied, including new developments in viral vaccines and antiviral compounds. To some extent many of these applications are self evident, such as with influenza viruses where host cell selection studies could result in new vaccine virus cultivated in mammalian cells rather than in eggs.

Cell receptors for viruses

Cellular attachment and uptake of virus is dependent on the presence of specific cell surface structures recognised by the virion attachment protein. The cell surface receptor may be restricted to a single species of plasma membrane receptor, as is probably the case for adenovirus and Epstein-Barr virus. On the other hand, the recognition structure may have a more ubiquitous distribution. Sialic acids, for example, are essential components of the cell surface receptors for influenza, paramyxovirus, polyoma and encephalomyocarditis viruses and since a heterogeneous array of glycoproteins and glycolipids contain these residues, the number of potential receptor molecules is large.

Nevertheless, these viruses do exhibit strict and varied specificities for the precise sialyloligosaccharide sequences recognised. A well characterized virus receptor is glycoporin, a sialoglycoprotein binding site for influenza virus on erythrocytes. The receptor for adenovirus type 2 on HeLa cells has been identified as a 42 kdalton glycoprotein. Less well defined is the receptor for Semliki Forest virus (SFV); histocompatibility antigens have been reported to be associated with the SFV binding site. However, since the virus will attach to and grow in MHC negative cell lines, these glycoproteins may be involved in non-infectious adsorption. In this latter context, it is interesting to note that foot-and-mouth disease virus (FMDV) does not appear to require specific receptor sites for binding to BHK cells, although interactions with specific cell membrane molecules may still be required for subsequent internalization and uncoating.

Membrane lipids may serve as receptors, the binding site being a lipid or complex of lipids. Sialic-acid-containing glycolipids (gangliosides) occur naturally in the plasma membrane of cells and act as receptors for cholera toxin, interferon and some glycoprotein hor-
gangliosides and glycoproteins may be involved in contact with the plasma membrane containing gangliosides. Thus arboviruses may interact specifically with phosphatidylglycerol and polyphosphoinositides and SFV infectivity is inhibited by treatment of host cell with phospholipase C. An interesting approach to investigating the biochemical nature of these binding sites is to solubilize membrane constituents from cells and to determine which components bind to the virus and hence inhibit its binding to cells. Schlegel et al. have carried out detailed studies with vesicular stomatitis virus (VSV) and their work may be used to illustrate the general principles. Phosphatidylserine (PS) inhibited VSV plaque formation by 80–90%, whereas herpes virus plaque formation was unaffected. Moreover, PS-containing liposomes bind to VSV, suggesting that PS functions as a binding site for this virus. VSV presumably interacts with the hydrophilic polar head region of PS since it is that domain which faces the external environment. The specific binding of VSV to PS is likely to be mediated by the virus G protein and since the amino acid sequence is now known, it should be possible to define the precise region of the protein responsible for binding. The G protein–PS binding almost certainly involves more than simple ionic interactions, because otherwise anionic phospholipids would inhibit VSV binding and they do not. Finally, it is possible that PS has a role in promoting internal membrane fusion events between the virus G protein and the membrane of acidified endosomes.

In our introductory remarks it was pointed out that although some general observations may be made pertaining to early virus cell interactions, the specific details for different viruses, even for genetically related viruses, may not be the same. This is illustrated by the work of Carroll et al., who describe the different adsorption properties of the influenza A RI/5 and RI/5+ viruses with erythrocytes and attribute this to the different sialylligosaccharide receptor specificities of their HAs. Erythrocytes were treated with Vibrio cholerae neuraminidase to remove sialic acids and then modified with CMP-NeuAc and three purified sialyltransferases to contain either the NeuAc2,3Gal, NeuAc2,6Gal or NeuAc2,6GalNAc linkages on cell surface glycoproteins. The HAs of the two viruses RI/5 and RI/5+ had totally different specificities, binding respectively to NeuAc2,3Gal and NeuAc2,6Gal linkages. Similarly derivatized erythrocytes were used to study the receptor specificities of human influenza A (H3N2) isolates selected in the presence of either horse serum or guinea-pig serum and then modified with CMP-NeuAc and three purified sialyltransferases to contain either the NeuAc2,3Gal, NeuAc2,6Gal or NeuAc2,6GalNAc linkages on cell surface glycoproteins. The HAs of two viruses RI/5 and RI/5+ had totally different specificities, binding respectively to NeuAc2,3Gal and NeuAc2,6Gal linkages. Similarly derivatized erythrocytes were used to study the receptor specificities of human influenza A (H3N2) isolates selected in the presence of either horse serum or guinea-pig serum and then modified with CMP-NeuAc and three purified sialyltransferases to contain either the NeuAc2,3Gal, NeuAc2,6Gal or NeuAc2,6GalNAc linkages on cell surface glycoproteins. The HAs of the two viruses RI/5 and RI/5+ had totally different specificities, binding respectively to NeuAc2,3Gal and NeuAc2,6Gal linkages. Similarly derivatized erythrocytes were used to study the receptor specificities of human influenza A (H3N2) isolates selected in the presence of either horse serum or guinea-pig serum and then modified with CMP-NeuAc and three purified sialyltransferases to contain either the NeuAc2,3Gal, NeuAc2,6Gal or NeuAc2,6GalNAc linkages on cell surface glycoproteins. The HAs of the two viruses RI/5 and RI/5+ had totally different specificities, binding respectively to NeuAc2,3Gal and NeuAc2,6Gal linkages. Similarly derivatized erythrocytes were used to study the receptor specificities of human influenza A (H3N2) isolates selected in the presence of either horse serum or guinea-pig serum and then modified with CMP-NeuAc and three purified sialyltransferases to contain either the NeuAc2,3Gal, NeuAc2,6Gal or NeuAc2,6GalNAc linkages on cell surface glycoproteins. The HAs of the two viruses RI/5 and RI/5+ had totally different specificities, binding respectively to NeuAc2,3Gal and NeuAc2,6Gal linkages. Similarly derivatized erythrocytes were used to study the receptor specificities of human influenza A (H3N2) isolates selected in the presence of either horse serum or guinea-pig serum and then modified with CMP-NeuAc and three purified sialyltransferases to contain either the NeuAc2,3Gal, NeuAc2,6Gal or NeuAc2,6GalNAc linkages on cell surface glycoproteins. The HAs of the two viruses RI/5 and RI/5+ had totally different specificities, binding respectively to NeuAc2,3Gal and NeuAc2,6Gal linkages. Similarly derivatized erythrocytes were used to study the receptor specificities of human influenza A (H3N2) isolates selected in the presence of either horse serum or guinea-pig serum and then modified with CMP-NeuAc and three purified sialyltransferases to contain either the NeuAc2,3Gal, NeuAc2,6Gal or NeuAc2,6GalNAc linkages on cell surface glycoproteins. The HAs of the two viruses RI/5 and RI/5+ had totally different specificities, binding respectively to NeuAc2,3Gal and NeuAc2,6Gal linkages. Similarly derivatized erythrocytes were used to study the receptor specificities of human influenza A (H3N2) isolates selected in the presence of either horse serum or guinea-pig serum and then modified with CMP-NeuAc and three purified sialyltransferases to contain either the NeuAc2,3Gal, NeuAc2,6Gal or NeuAc2,6GalNAc linkages on cell surface glycoproteins. The HAs of the two viruses RI/5 and RI/5+ had totally different specificities, binding respectively to NeuAc2,3Gal and NeuAc2,6Gal linkages.

show that most anti-haemagglutinin monoclonal antibodies raised against the NeuAc2,6Gal-specific virus react equally well with both variants, but a small number fail to react with the NeuAc2,3Gal-specific virus. These studies demonstrate that host-specified sialylligosaccharides can exert selective pressure on influenza viruses, and in agreement with this it is known that influenza A (H3N2) viruses isolated from equine and avian sources are naturally NeuAc2,6Gal-specific. This selective procedure has been demonstrated and shown to vary for influenza A (H3N2) variants of avian and human origin. Whereas human isolates of either NeuAc2,6Gal- or NeuAc2,3Gal-specificity were stable when grown either in MDCK-culture or eggs, an avian NeuAc2,6Gal-specific variant was stable in MDCK-culture but reverted spontaneously on passage in eggs. This reversion was again shown to involve amino acid substitution at position 226 of HA1. Concurrently we have described the selection of subpopulations of influenza A and B viruses by cultivation of virus in MDCK cells or in embryonated hens eggs. The latter host system clones out a population of viruses with a different antigenic composition, as detected with both monoclonal and polyclonal antibodies, compared to the same parental virus cultivated in MDCK cells. The novel implication is that a non-immune selective mechanism involving host cell receptor sites may operate to select and generate antigenic variants of influenza virus, thus contributing towards antigenic drift and evolution of the virus. Since eggs are routinely employed for the preparation of influenza vaccines, these findings have important practical implications for vaccine design and efficacy. Table 1 shows haemagglutination-inhibition (HI) reactions of four influenza B viruses representative of 27 strains isolated and passed in eggs or MDCK cells from specimens collected during a school outbreak of influenza in 1982. The virus preparations were tested against anti-HA monoclonal antibodies prepared against influenza B virus cultivated in eggs or MDCK cells as shown in Table 1. Three patterns of serological reactivity were discernible. Monoclonal antibody 195 reacted to high titres with all the influenza B viruses tested. Monoclonal antibody 209 reacted with all the viruses which had been isolated and subsequently passed in MDCK cultures and with none of the viruses which had been cultivated in eggs at any stage in their passage histories. Antibody 238 reacted only with viruses which had at least one passage in eggs and with none of the viruses which had been passaged in MDCK cultures only. The mutually exclusive nature of the reactions of antibodies 209 and 238 with the 27 virus preparations tested was striking.

Viruses having different passage histories in eggs or cell cultures would be expected to react differently with monoclonal antibodies if these were directed against antigens present as carbohydrate moieties derived from host cell membranes and covalently linked to the haemagglutinin molecule. However, this is not an explanation for the findings. None of the monoclonal antibodies gave HI reactions which were entirely determined by the last culture system in which the test virus was grown. Moreover, the reactions were influenza B specific and were not affected by absorbing the antibodies with homogenates of chorioallantoic membrane or MDCK cells.
Table 1  Haemagglutinin-inhibition reactions with monoclonal antibodies to haemagglutinin of influenza B viruses of different isolation and passage histories

| Specimen No. | Cell substrate for virus isolation and passage | Monoclonal antibodies to B/Oregon/5/80 virus (prepared using egg grown virus) | Monoclonal antibody to B/England/145 virus (prepared using egg grown virus) |
|--------------|-----------------------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| 145          | M-M                                           | 25600                                                                          | <                                                                         | 320                                                                        | 209                                                                        |
|              | M-M (×3)                                       | 12800                                                                          | <                                                                         | <                                                                         | 320                                                                        |
|              | M-A1                                          | 38400                                                                          | 9600                                                                      | <                                                                         | <                                                                         |
|              | M-A1 (×3)                                      | 12800                                                                          | 9600                                                                      | <                                                                         | <                                                                         |
|              | M-A1-M (×3)                                    | > 6400                                                                         | <                                                                         | 160                                                                      | <                                                                         |
|              | Am-A1                                         | 4800                                                                           | 6400                                                                      | <                                                                         | <                                                                         |
|              | Am-A1-M (×3)                                   | 6400                                                                           | 6400                                                                      | <                                                                         | <                                                                         |
| 222          | M-M                                           | > 6400                                                                         | <                                                                         | 40                                                                       | <                                                                         |
|              | M-A1                                          | 6400                                                                           | 6400                                                                      | <                                                                         | <                                                                         |
|              | M-A1 (×3)                                      | 6400                                                                           | 6400                                                                      | NT                                                                       | <                                                                         |
|              | Am-A1                                         | 3200                                                                           | 4800                                                                      | <                                                                         | <                                                                         |
|              | Am-A1-M (×2)                                   | NT                                                                             | 12800                                                                     | <                                                                         | <                                                                         |
| 272          | M-M                                           | > 6400                                                                         | <                                                                         | > 1280                                                                   | <                                                                         |
|              | M-A1                                          | > 6400                                                                         | > 6400                                                                    | <                                                                         | <                                                                         |
|              | M-A1 (×3)                                      | 6400                                                                           | 4800                                                                      | <                                                                         | <                                                                         |

M indicates isolation or passage in MDCK cell cultures
A1 indicates passage in the allantoic cavity of embryonated eggs
Am indicates passage in the amniotic cavity of embryonated eggs
( ) indicates number of serial passages
< HI titres less than 1:50 or, for antibody 209, less than 1:10
NT Not tested

In experiments to investigate any biological differences between the antigenically distinct subpopulations, the range of erythrocytes agglutinated by the viruses was compared. Erythrocytes may be used as a model for cell-receptor specificity for influenza virus. The preparations of B/England/145/82 and B/England/222/82 listed in Table 1 were tested with erythrocytes of chicken, turkey, man (group A, AB and O cells), rhesus monkey, guinea-pig, sheep, rat and horse. The viruses, irrespective of passage history, agglutinated the cells of these species to similar titres with the exception of sheep, horse and rat erythrocytes. For cells from the latter three species, high agglutination titres were detected with virus which had been isolated or passaged in eggs but no agglutination was detected for virus passaged in MDCK cultures only.

The findings provide strong evidence that influenza B virus isolated from clinical specimens and grown in MDCK cultures comprises at least two biologically and antigenically distinct subpopulations. A minor proportion of the MDCK-grown virus is capable of growth in the allantois of fertile eggs without prior adaptation and possesses HA which is antigenically distinct from the majority of the virus which grows poorly or not at all in the allantoic cavity. The molecular basis of this MDCK/egg selection has been investigated by HA gene sequencing and shown to be due to modification of a glycosylation site in an area corresponding to antigenic site B of the H3 structure. The glycosylation site is present in all viruses isolated in MDCK-cultures, but is lost or modified in viruses adapted to growth in eggs. More recently the antigenic heterogeneity of influenza isolates has been directly demonstrated by electron microscopy of isolates labelled with monoclonal antibodies and immunogold reagents. These studies are not unique in providing evidence of distinct subpopulations of virus within a single strain. Other reports have documented biological or antigenic heterogeneity among populations of influenza A virus cultivated in eggs. However, for the first time evidence has been accumulated of strong selective pressure for antigenic variants exerted by the cultivation of viruses in eggs. A probable explanation for covariance between growth in eggs and antigenic character of influenza B virus is that one of the antigenic sites of the HA molecule is structurally and functionally related to the binding site for cell surface receptors. Analysis of influenza A haemagglutinin indicates that the epitopes with which monoclonal antibodies react are clustered into four independent antigenic sites in the three dimensional structure of the molecule and, of these, site B appears to be topographically closest to the probable binding site for cell surface receptors.

As regards relevance to influenza vaccines, it is pertinent that most influenza vaccines utilize viruses cultivated in embryonated hen's eggs. However, the serum antibody in persons who have recovered from natural influenza reacts significantly better, both in HI and neutralization tests, with virus cultivated in mammalian cells rather than in embryonated hen's eggs. We are therefore investigating the potential of influenza vaccines prepared in mammalian cells.

Distribution of virus binding sites on the cell surface

Electron microscopical techniques have been employed to examine the cell surface distribution of bound virus. At 4°C parvovirus, coronavirus and adenovirus attach randomly over the surface of cultured cells.
Early virus-cell interactions: S. Patterson and J. S. Oxford

Figure 1 Diagram summarizing the known three-dimensional structure of influenza HA and NA spikes and their relationship with the lipid bilayer and matrix protein of the virus. The paths of the polypeptide chains of HA and NA monomers are shown. The HA1 polypeptide chain is shown as a thin line and HA2 as a thick line. The diagram indicates the extension of the C-terminus of HA2 through the lipid bilayer with a known hydrophobic portion spanning the membrane and a short hydrophilic region on the internal side of the membrane. Three long helices in the HA2 chains, one from each monomer, pack together as a triple stranded coiled-coil to form the stem of the trimer. The N-terminus of HA2 (fusion activation site) is deeply embedded in the spike. HA1 forms a globular outer region of each monomer and containing the important host-receptor binding sites and the antigenic sites A, B, C and D (C may be subdivided to give a fifth epitope). The polypeptide chain then loops back again with the N-terminus of HA1 (fusion activation site) to form a nearly continuous surface across the top of the monomer and encircle the catalytic site. A hydrophobic region in the NA stalk spans the lipid bilayer and a short sequence may be present on the cytoplasmic side (for a review of HA and NA structure, see Ref. 141).

SFV, on the other hand, preferentially adsorbs to the microvilli of BHK cells. At 37°C mengovirus, coronaviruses, reovirus, VSV, adenovirus, and influenza virus (unpublished data, see Figure 2) have been shown to redistribute or 'cap' after binding to cultured cell monolayers or to cells maintained in suspension. Redistribution of virus on the cell surface is similar in many respects to ligand induced redistribution of membrane receptors. It is temperature and energy dependent and blocked by drugs that inhibit the microfilament system. To induce capping, ligands must cross-link receptors and form a two dimensional matrix on the cell surface. Thus monovalent Fab anti-receptor antibody cannot mediate capping. Since virus particles have repeating receptor binding subunits, the virion may be regarded as a multivalent ligand able to cross-link receptors and stimulate surface redistribution.

Migration on the cell surface may facilitate entry of some viruses. Philipson et al. have suggested that adenovirus particles need to contact and bind further receptors after the initial attachment event for internalization to proceed. Additional fibre-receptor contacts were considered to be brought about by lateral diffusion of receptor molecules in the fluid membrane bilayer. The importance of both the multivalency of the virus and the lateral mobility of its membrane receptors has also been emphasized recently in analyses of the cooperative binding of adenovirus 2 to HeLa cells. Further support for these ideas is provided by experiments in which the uptake of bound adenovirus was found to be impaired by blocking unoccupied receptors with free adenovirus fibre protein. At 37°C in the presence of metabolic inhibitors or cytochalasin B, membrane receptors would be free to diffuse, however, under these conditions uptake of adenovirus by HeLa cells is inhibited. These results may be interpreted in two ways. Since these inhibitors also block surface redistribution, it may be argued that contact with further receptors is achieved by active cell-mediated movement of virions rather than by passive diffusion of receptors. Alternatively, metabolic inhibitors and cytochalasin B may block the pinocytic internalization event. However, these agents do not prevent the entry of influenza virus into cytoplasmic vacuoles, but it must be remembered that adenovirus particles have only 12 fibre attachment structures per virion whilst influenza has an estimated 500-700 HA spikes per virion and thus the pinocytic uptake mechanism for the two viruses may not be identical.

Internalization into the host cell and release of virus genome ('uncoating')

The entry route of different virus groups has been comprehensively reviewed by Dales. Enveloped
viruses can enter by fusion of viral and plasma membranes, as has been described for paramyxoviruses\(^{46}\) or, as in the case of myxoviruses\(^{49,50}\) by pinocytosis. Non-enveloped viruses such as adenoviruses, have also been observed to enter by pinocytosis\(^{51}\) but, in addition, uptake by direct penetration of the plasma membrane has been described\(^{32}\).

**Enveloped viruses**

Many enveloped viruses, even those able to fuse with the plasma membrane, have been observed to enter cells by a pinocytic-like mechanism\(^{47}\). Studies with influenza virus have shown that uptake into a cytoplasmic vacuole can occur in the presence of metabolic and cytoskeletal inhibitors\(^{52}\). There is also evidence that internalization of influenza virus\(^{53}\) and even the subsequent uncoating step\(^{54-55}\) can occur at 4°C. These findings in addition to the observations of apparent ‘phagocytosis’ of Sendai virus by liposomes\(^{56}\) indicate that the pinocytic uptake of some enveloped viruses can occur by an energy independent mechanism. Thermodynamic analysis of the binding of polyvalent ligands to cell membranes\(^{57}\) suggests that binding will induce a curvature in the membrane which is concave towards the side to which the ligand is bound. Influenza virus, with its tightly packed haemagglutinin attachment molecules, may be regarded as a polyvalent ligand. Thus, invagination of the membrane as a consequence of virus attachment may be considered to be the most thermodynamically stable configuration for the membrane to adopt.

Not all enveloped viruses are pinocytosed by energy independent mechanisms. Uptake of SFV is inhibited by metabolic poisons but is not significantly affected by cytochalasin B. The possible reasons for these differences are not yet clear.

There is now evidence that pinocytic uptake of enveloped viruses can occur at specialized sites on the plasma membrane known as coated pits\(^{34}\). That these structures may be concerned with uptake of macromolecules was first proposed in the 1960s\(^{58,59}\) and subsequently it was demonstrated that certain receptors, such as that for low-density lipoprotein, were concentrated in coated pits\(^{60}\). Receptors for other molecules, for example \(\alpha_2\)-macroglobulin, only localize in coated pits after ligand binding\(^{61}\). Ultrastructurally the sites are recognised by a thickening on the cytoplasmic side of the membrane (Figure 3) which is due to a 170 kdalton protein called clathrin\(^{62}\). Endocytosis at these specialized plasma membrane sites results in the formation of a coated vesicle containing the ligand-receptor complexes. A naked endosome is soon formed by dissociation of the clathrin coat which is then recycled back to the plasma membrane. Willingham and his colleagues have proposed that the coated vesicle is not pinched off from the plasma membrane but remains connected via a long narrow neck\(^{63}\). Receptor bound ligand then migrates to the vesicle-neck junction and buds out through uncoated membrane. They call the newly formed vesicle containing the ligand-receptor complex a receptosome.

Uptake of SFV into cytoplasmic vacuoles occurs at coated pits\(^{34}\). The receptors for the virus are not concentrated in coated pits and thus the bound virion migrates from its initial attachment site, shown to be the microvilli for BHK cells, to a coated pit. The molecular mechanisms mediating migration from uncoated to coated sites appear to be different than those operating in ‘capping’. Experiments with \(\alpha_2\)-macroglobulin\(^{60}\) have shown that clustering of bound receptors into coated pits is inhibited by dansylcadaverine although the precise mode of action of this drug on the plasma membrane is unknown. Cytochalasin B does not stop SFV from reaching coated pits\(^{64}\) and metabolic inhibitors do not prevent the clustering of bound \(\alpha_2\)-macroglobulin receptors into these sites\(^{55}\). Thus, transport to coated pits seems to proceed via a dansylcadaverine-sensitive mechanism that is independent of the cytoskeletal system and insensitive to metabolic inhibitors.

After uptake into a cytoplasmic vacuole, the virus still faces the problem of penetrating a cellular membrane and releasing its nucleic acid in order to initiate infection. Two lines of evidence, derived originally from studies on influenza and SFV, suggest that fusion is involved in the uncoating process. The infectivity of influenza virus is dependent on the post-translational proteolytic cleavage of the haemagglutinin molecule into two components termed HA1 and HA2. These subunits are linked by disulphide bridges and anchored into the viral membrane by the carboxyterminal end of HA2. The finding that the NH\(_2\) terminus of HA2 is
highly conserved throughout the different influenza serotypes and is almost identical to the NH\textsubscript{2} terminus of Sendai virus F, fusion protein strongly suggests that it mediates a fusion event\textsuperscript{65}. More direct evidence for its role in a fusion event comes from recent studies involving site-specific mutagenesis of a cDNA encoding the HA of influenza\textsuperscript{66}. Three mutant sequences within the first eleven amino acids of HA2 were generated and incorporated into SV40 vectors for HA expression in Simian cells. Two of the mutants involved substitution of glutamic acid for glycine and resulted either in no fusion activity or in a raised threshold-pH and reduced efficiency of fusion, whilst an extension of the hydrophobic region by substitution of glycine for glutamic acid gave a mutant protein with fusion characteristics comparable to wild type protein.

In their studies on SFV, Helenius and his colleagues\textsuperscript{67} observed that virions entering via coated pits ended up in lysosomes. In parallel studies SFV was found to fuse with the plasma membrane of BHK cells by absorbing cells with virus at 4°C and then briefly exposing the preparations to warm (37°C) medium at pH 5.5. In addition low-pH-dependent fusion of virus with liposomes was demonstrated. From this data it was proposed that the low pH environment of the lysosome promotes fusion of the viral and lysosomal membranes resulting in the release of the viral nucleic acid into the cell cytoplasm. The antiviral action of lysosomotropic agents such as chloroquine, amantadine and NH\textsubscript{4}\textsuperscript{+}, all of which raise lysosomal pH\textsuperscript{67} and block virus infection at an early stage prior to transcription, appeared to support their hypothesis. This work was extended to other enveloped viruses including VSV\textsuperscript{68-70}, influenza\textsuperscript{71} and rabies\textsuperscript{72} and a pathway similar to that followed by SFV proposed. Furthermore, for influenza virus there is evidence that low pH causes a conformational change in the haemagglutinin molecule and exposes the domains of the molecule that mediate fusion\textsuperscript{73-76}. Bachi et al.\textsuperscript{77} have prepared a monoclonal anti HA antibody that recognises internalized virus but not virus adsorbed to the cell surface. The binding of this antibody to internalized virus was inhibited by treating cells with ammonium chloride. These findings provide further support for the hypothesis that a conformational change in the HA which initiates fusion activity occurs in an acidic cellular compartment. In addition to influenza virus, there is recent evidence that the envelope glycoprotein of another virus, La Crosse virus, a member of the Bunyaviridae, undergoes a conformational change at the pH of fusion activation\textsuperscript{78}.

Although the evidence for a fusion step in the uncoating of several enveloped viruses is quite strong, a number of points still require clarification. The data that fusion occurs in lysosomes is indirect and fusion of viral and lysosomal membranes has not been visualised. Lysosomes are not the only low pH compartment within the cell. The pH of macropHage phagocytic vacuoles and endocytic vacuoles of fibroblasts\textsuperscript{79,80} is reduced to pH<6 prior to fusion with lysosomes. Furthermore, the argument that weak bases block virus infection by raising intralysosomal pH is not wholly convincing. Studies on amantadine have shown that its antiviral activity on cultured cells is reversed by washing in drug-free medium. However, after washing treated cells, the concentration of amantadine remaining in the lysosomal compartment is still greater than that required in the extracellular medium to produce maximal antiviral activity\textsuperscript{81}. Other inconsistencies are that chloroquine inhibits Sindbis virus infection of subconfluent BHK cells but not of confluent BHK cells or Aedes albopictus cells\textsuperscript{82}. Methylamine and chloroquine raise lysosomal pH to a similar extent yet the latter is tenfold more effective at inhibiting SFV. Therefore, these data do not provide convincing evidence for lysosomal uncoating.

Since their original publications, Helenius and co-workers have modified their scheme for the uncoating of SFV\textsuperscript{83}. They have now shown that uncoating occurs within 5–7 min of entry and before passage to lysosomes, which is only observed 15–20 min after uptake. In addition, uncoating is observed at 20°C, a temperature too low for the fusion of endocytic vacuoles with lysosomes. The acidic prelysosomal vacuole is now proposed as the site of fusion. Data supporting a similar endosomal pathway for influenza virus have also been published\textsuperscript{84}. The new scheme overcomes many of the criticisms raised above.

Although the HA of influenza virus mediates membrane fusion, there are reports that the neuraminidase (NA) glycoprotein can also play a role. Liposomes containing HA and NA were found to fuse to cell membranes whilst liposomes containing only HA lacked fusion activity\textsuperscript{84}. Additionally anti-NA antibodies were observed to block virus-mediated low-pH-dependent lysis of erythrocytes\textsuperscript{85}. However, as cells expressing HA from a cloned gene can be induced to fuse at low pH in the absence of NA\textsuperscript{86} and since antibodies to NA do not neutralize infectivity\textsuperscript{87}, it is difficult to assess the precise function of NA in uncoating.

Studies on the inhibitory action of amantadine have shown that there are two concentration dependent effects\textsuperscript{88}. At levels >0.1 mM it prevents fusion at low pH by raising endosomal pH and is effective against all strains of influenza, including the influenza B viruses as well as other groups of enveloped viruses. influenza A viruses, at least, mutate readily to overcome the effects of high levels of amantadine, and the mutants possess elevated pH-dependence of the HA conformational change required for fusion. The amino acid sequence and three-dimensional location of substitutions on the HA were determined and shown to fall into two groups, those that disrupt intersubunit contacts in the HA trimer and those that destabilize the pH 7.0 location of the NH\textsubscript{2} terminus of HA2\textsuperscript{89}. A more specific effect is observed when amounts of amantadine between 0.1 and 5 µM are used in that only certain strains of A viruses are susceptible. The gene for the matrix protein appears to play a significant role in determining low dose resistance to the drug\textsuperscript{90,91}. More recently it has been shown that a spliced transcript of the matrix gene, RNA segment 7, coding for a second protein, M\textsubscript{2}, confers sensitivity to amantadine\textsuperscript{92}. By generating amantadine resistant mutants, the key region was mapped to four amino acids in a hydrophobic region of M\textsubscript{2}. The M\textsubscript{2} polypeptide has been detected at the surface of infected cells\textsuperscript{93} but has not yet been found in free virus particles.

The inhibitory mechanism of low doses of amantadine on sensitive influenza A virus strains is not clear, but the experiments of Bukrinskaya and colleagues\textsuperscript{94-96} suggest one possible mode of action. They have
employed an amantadine derivative, rimantadine, as a tool to study influenza virus uncoating. Nuclear associated cytoplasm was separated from infected cells and analysed by density gradient centrifugation. Peaks of viral material were observed at 50 S and 80 S and these corresponded to ribonucleoprotein (RNP) and RNP plus matrix respectively. Only the former was found in the nucleus and no haemagglutinin or neuraminidase was detected in either fraction. Examination of the 80 S fraction by electron microscopy revealed core-like bodies with an apparent helical structure. In the presence of rimantadine only the 80 S but not the 50 S component was detected suggesting that the drug prevented dissociation of viral RNP and matrix protein. Two steps in the uncoating of influenza virus were proposed; fusion in an endocytic vesicle which releases an RNP-matrix complex into the cytoplasm and a second rimantadine-sensitive step occurring in the perinuclear zone which results in the dissociation of matrix from the RNP and the passage of RNP into the nucleus.

Non-enveloped viruses

Adenovirus. Two routes of adenovirus uptake have been described, direct penetration of the plasma membrane and entry via a pinocytic vesicle followed by penetration of the vacuolar membrane. Thin section electron microscopy has failed to provide images of virions halfway across the plasma membrane but penetration may be a very rapid event and since only a very limited proportion of plasma membrane is observed in thin sections, the chances of seeing penetration could be very low. In contrast, examination of adenovirus-infected KB cells by the freeze-fracture technique, which permits the observation of large expanses of membrane, has shown virions apparently lodged between the bilayers of the plasma membrane. Henmache et al. found that destabilization of HeLa cell plasma membranes with citrate buffer enhanced the attachment of adenovirus without affecting cell viability or sensitivity to infection. Freeze-fracture of citrate-treated cells that had been adsorbed with virus at 4°C revealed protrusions on the E fracture face of the plasma membrane that were devoid of intramembranous particles. Such areas were not observed on uninfected controls. Similar results were also obtained by treating cells with adenovirus fibre protein covalently attached to Sepharose beads. Intramembranous particle-free areas rapidly disappeared on warming. It is tempting to speculate that particle clearance facilitates penetration since clearance of intramembranous particles has also been observed in the opposing granule and plasma membranes of mast cells immediately prior to fusion and exocytosis.

Uptake of adenovirus particles into cytoplasmic vacuoles can occur at coated pits, although pinocytic uptake may not occur exclusively at these sites. The coated pit receptosome/endosome entry route has been examined by Fitzgerald et al. Cells were incubated simultaneously with gold-labelled epidermal growth factor (EGF), known to enter via the coated pit receptosome route, and with virus. Virus and EGF were observed together in coated pits and subsequently in uncoated receptosomes/endosomes. At a later stage, gold and virus particles were found lying free in the cytoplasm, suggesting that the adenovirus particles had escaped from the vacuole by membrane dissolution. This view was supported by the finding that the effect of Pseudomonas toxin, which acts at a cytoplasmic site, was enhanced by incubation with adenovirus. Currently little is known about the adenovirus components that mediate membrane penetration or dissolution. However, clues to possible mechanisms are provided by the investigation of Svensson. It was shown that at neutral pH adenovirus bound to cells via the fibre protein, whilst at low pH attachment was mediated by the penton base. In addition lysosomotropic agents were found to cause a partial inhibition of particle release from endosomes. These findings suggest that one route of cytoplasmic entry is dependent on the low pH environment of the endosome but since penetration of the plasma membrane has also been described, there are presumably other mechanisms.

Cells infected with radiolabelled adenovirus have been disrupted and analysed by density gradient centrifugation techniques by Lonberg-Holm and Philipson. Three subviral fractions were isolated and termed, in order of increasing density and sequence of appearance, C, D and E. The particles in the C fraction were considered to be the first intermediates of uncoating. Unlike native virus they were sensitive to DNAase and this was considered to be due to loss of some of the penton base and fibre units from the vertices of the virus. Analysis of early uncoating intermediates have demonstrated particles with reduced amounts of penton base and fibre protein. These findings have also been correlated with the less angular morphological appearance of penetrated particles. Studies by Ogier et al. indicate that loss of pentons leading to nuclease sensitivity can occur at the plasma membrane. More recently it was shown that sensitivity to DNAase at the plasma membrane level is blocked by EDTA, EGTA, dithiothreitol and dansylcadaverine and led to the proposal that destabilization of attached virions follows reorganization in the plasma membrane. Cytoplastic and nuclear fractions obtained by Lonberg-Holm and Philipson's experiments yielded D particles which were more sensitive to DNAase than C particles and considered to represent viral cores. The E subcomponent was the last to appear, it had a predominantly nuclear location and probably represents free viral DNA.

After penetration into the cytoplasm, the adenovirus C particle is transported to the nucleus. Microtubules seem to be involved in this process since colchicine, a microtubule depolymerizing agent, delays or decreases virus production and in studies in vitro adenovirus was found to associate specifically with microtubules. In addition Miles et al. showed that a ts mutant virus associated with microtubules at the permissive, but not at the non-permissive temperature. The final destination of the C particle is the nuclear pore (Figure 3) where extrusion of the viral nucleoprotein core (D particle) into the nucleus occurs. Histochimical studies have revealed increased ATPase activity in the nuclear membrane associated with nuclear pores. Since the ATPase inhibitor p-hydroxymercaptoacetate prevented association of virus with the nuclear pore, it was proposed that extrusion of the nucleoprotein into the nucleus is an energy-dependent process.

Weber and Mirza have exploited the adenovirus type 2 ts mutant (Ad2 ts1) to unravel some of the finer details of uncoating. Cells infected with mutant...
Early virus-cell interactions: S. Patterson and J. S. Oxford

Virus at the non-permissive temperature produce a normal yield of particles, which have a full complement of viral proteins. However, three of the core proteins, VI, VII and VIII, are present in their uncleaved precursor form. Although containing infectious DNA, these particles are non-infectious. Examination of cells infected with non-infectious Ad2 ts1 progeny virus indicate that there is a block at a late stage in uncoating resulting in the accumulation of a 150 S subviral intermediate which is composed of DNA and peptides V, pVII and trace amounts of VII11,12. Although not detected in cells infected with wild-type virus, the 150 S component was considered to be a short-lived intermediate of normal uncoating. Peptides VI and VII are basic, bind to DNA and are thought to be involved in packaging of viral DNA13. Complete dissociation of core proteins from DNA during uncoating thus seems to require proper post-translational processing. Previous investigations suggested that release of the viral core occurs within close proximity of the nuclear pore52. However, Mirza and Weber111 found that much of the 150 S component was not closely associated with the nucleus and therefore proposed that release of the core can also occur at a non-nuclear associated site.

Picornavirus uncoating. The early steps in infection by this group of viruses are of particular interest because they provided the first evidence for conformational changes in structural viral proteins during the entry process. Each virion is composed of 60 copies of four polypeptides termed Vp1, 2, 3 and 4. Studies on poliovirus14, coxsackie virus15, echovirus16, and rhinovirus17 have shown that at 37°C a proportion of cell bound virions elute and are unable to reattach. Eluted virions lack Vp4 and are antigenically distinct from native virus. Viruses can enter cells in the presence of the antiviral drug rhodamine, 2-thio-4-oxothiazolidine, but fail to shed Vp4 and do not uncoat18,19. This suggests that loss of Vp4 is not a prerequisite for virus uptake but is required before uncoating can commence.

The conversion of native virus into the modified ‘A’ particles which lack Vp4 is accompanied by a marked alteration in antigenic properties20,21 and is thought to reflect conformational changes in the virion proteins. These changes result in the exposure of hydrophobic groups, since ‘A’ particles will bind to liposomes22. Lonberg-Holm and Whiteley22 have proposed that the ‘A’ particle can intercalate with the plasma membrane and represents the penultimate stage in uncoating. However, the observation that ‘A’ particles only become susceptible to nuclease after treatment with chymotrypsin suggests that additional steps are required. Protease treatment also causes loss of Vp2 and converts the 135 S ‘A’ particle into a 70-80 S ‘C’ particle23. Thus the ‘A’ particles seem to require processing by a cell-derived protease in order to release their RNA. The detection of 70-80 S particles lacking Vp4 and Vp2 in infected cells supports these ideas.

Experiments with isolated plasma membranes have indicated that loss of Vp4 and Vp2 can occur at the plasma membrane24. These changes may also take place after entry since virions internalized in the presence of rhodamine become uncoated after removal of the drug. Density gradient fractionation of HeLa cell plasma membranes have revealed four bands with virus binding activity25. The heaviest bands, 3 and 4, contain 70% of the activity. Both bands are able to convert native particles to ‘A’ particles but only band 3 mediated the subsequent processing to ‘C’ particles. Rather surprisingly an extract from band 4 was found to inhibit the processing of ‘A’ particles into ‘C’ particles by band 3. It was suggested that the inhibitory fraction may prevent further uncoating and arrest the ‘A’ particle at the plasma membrane or alternatively that it may enhance infection by delaying the second uncoating step until after entry into the cell.

Although most picornviruses are uncoated in several steps as outlined, FMDV provides an exception. FMDV that elutes from cells at 37°C can, in contrast to other picornviruses, reattach and shows no loss of Vp425,26. If cells are infected with virus in the presence of low salt buffer, the virus disrupts at the membrane, releasing its RNA and 12 S subunits. Although these conditions are unphysiological, they may mirror events occurring after cellular entry, since approximately 90% of cell-associated FMDV is disrupted into 12 S subunits within 20 min of entry. Intermediate lacking Vp4 have not been found. These data suggest that FMDV uncoating is a single rather than a multistep process.

There have been comparatively few electron microscopical studies on the mechanism of cellular entry of picornviruses. Dunnebake129 observed polioviruses lying free in the cytoplasm as well as in vacuoles two minutes after infection and proposed that the virus could directly penetrate the plasma membrane. This idea is compatible with data showing that ‘A’ particles bind to liposomes125. However, the most recent publications on poliovirus uptake indicate that entry is via coated pits into endosomes and possibly lysosomes130. At reduced pH there is a conformational change which results in the exposure of hydrophobic regions of the virion that facilitates passage of the viral genome through the membrane into the cytoplasm131,132.

Permeability changes in virus infected cells

Compared to the number of studies which have examined morphological interactions between viruses and cells, much less data has been published about the immediate physiological effects of these interactions, although these may have profound effects on the cell or organ infected. Immediate practical aspects include a possible differential entry of antiviral compounds in virus infected cells compared to normal cells. Ishida and Homma133 demonstrated that u.v.-inactivated Sendai virus interacted with cells to produce leakage of certain phosphorylated metabolites such as phosphatidylcholine, even though the cells were not lysed and 35S-labelled intracellular components did not leak out. Pasternak and Micklem134 have hypothesised that this leakage results from incorporation of the inherent leaky viral envelope in the cell plasma membrane. The fusion event introduces non-specific hydrophilic channels into the cell membrane and results in leakage of low molecular weight compounds such as nucleotides, sugar phosphates and Na+ and K+ ions into and out of cells. Extracellular Ca2+ and some other divalent cations reversibly prevent leakage and thus virally induced channels operationally resemble intercellular communicating junctions (‘gap junctions’) between cells135. These effects are noted particularly with certain para-
myxoviruses, although ‘early harvest’ virus which is unable to cause fusion and haemolysis is also unable to induce leakiness in cell membranes. No cell specificity is noted, which is not surprising since paramyxovirus receptors occur on a wide variety of cell types and similar changes are also noted in organ cultures. Carrasco and Esteban have described early (1 h postinfection) permeability changes in cell membranes induced by vacccinia virus. For example, the modified membrane permits the entry of the translation inhibitor hygromycin B, and the protein toxin α-sarcin. Adeno-virus infection also promotes the entry of high molecular weight toxin into the cell. It should be added that changes in permeability to ions also occur during later stages of actual virus replication in cells and this phenomenon is not restricted to paramyxoviruses but also includes viruses not possessing a lipid envelope. At late times after virus infection, translation inhibitors which do not normally penetrate uninfected cells can pass into cells. Defective cell permeability would be expected to contribute to cellular damage in several ways and, for example, Carrasco has argued that an increase in intracellular Na+ might be a contributing factor in the switch from synthesis of host proteins to the synthesis of viral proteins. An increased entry of Ca2+ is likely to have adverse consequences on general cellular metabolism, whilst a loss of intracellular nucleotides especially ATP and cyclic nucleotides is likely to be even more damaging. All cells have a tendency to swell because of osmosis but water entry is normally prevented by an active extrusion of Na+ from cells through operation of the Na+ pump. If the pump malfunctions through lack of ATP, for example, or if the surface plasma membrane is made so leaky that entry of Na+ exceeds the capacity of the Na+ pump to remove it, a net entry of Na+ accompanied by water occurs with resultant cell swelling. This leads to cell lysis in the case of erythrocytes infected with paramyxoviruses (and influenza virus) whereas other cells are able to increase their volume without rupture and giant cells may be formed.

In conclusion, we emphasised in the introduction to this brief review, and it is obvious throughout the text, that most studies of early interaction of viruses and cells to date have been rather biological, often employing direct observation with electron microscopy. We must recognize an important pitfall, namely that an observed virus–cell interaction may not actually lead to infection. Future studies using interactions of a variety of viral proteins of known amino acid sequences, possibly with model virus–liposome systems, for example, should lead to a rapid expansion of new and detailed knowledge. We would re-emphasise that in addition to its intrinsic value, we would expect such data to be useful in the design of specific inhibitors of virus replication for chemoprophylaxis of virus disease and also for new approaches to immunoprophylaxis and vaccine design encompassing, for example, epitopes inducing antibody to receptor sites on virus proteins.

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Vaccine, Vol. 4, June 1986 87
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