Electron Microscopic Observations on the Entry of Influenza Virus into Susceptible Cells

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SUMMARY

Influenza A2 virus particles were observed attached to the surface of chick CAM after 1 h at 4 °C. After incubation at 35 °C, virus particles were seen inside cytoplasmic vesicles (viropexis). Subsequently, the virus particles penetrated the vacuolar membranes and uncoating was observed within the cytoplasm without the intervention of lysosomal enzyme. Fusion of virus coat with cell membrane was not thought to play a part in virus entry. The pinocytosis of virus was shown to be an active process, triggered by the attachment of virus to the cell membrane.

Viropexis occurred in cells treated with amantadine, cytochalasin B and colchicine and in normal cells to which heated virus had been added. Virus was seen eluting after treatment with antihaemagglutinin antibody, but after treatment with antineuraminidase antibody, the virus did not elute; in neither case did the virus enter the cells in the presence of antibody.

INTRODUCTION

In a previous study (Dourmashkin & Tyrrell, 1970) we examined the early stages of entry of influenza and parainfluenza (Sendai) virus into the ciliated epithelium of guinea pig trachea maintained in vitro by the technique of organ cultures (Hoorn & Tyrrell, 1969). When studying the attachment of parainfluenza virus to cilia we noted that cell membranes and virus envelopes fused and the virus nucleocapsids were then released beneath the membrane. Similar experiments with influenza virus showed no fusion of the virus with the cell surface nor was any manner of virus entry observed. We thought that the reason for this might be that the influenza virus used was relatively inefficient in entering guinea pig tracheal cells.

A preliminary experiment showed that roller tubes containing pieces of chorioallantoic membrane were infected by about 100-fold less virus of the type used than similar cultures using rings of guinea pig trachea (Harnett & Hooper, 1968). It was possible, therefore, that there would be 100-fold greater chance of seeing the early events in the infectious process in chorioallantoic membrane (CAM) than in guinea pig trachea. This paper reports the experiments which were then done in this model system.
METHODS

Virus preparations. Egg-adapted strains of influenza A2 were used, namely A2/Eng/12/64 or A2/Hong Kong/1/68. In many experiments a recombinant (6c) of the latter strain with AO/PR8 (MacCahon & Schild, 1972), was used in order to obtain high titres. Standard and RIF-free (SPF) embryonated eggs were used.

In most experiments 10- or 11-day-old chick embryos were inoculated allantoically with a 10^{-5} dilution of infected allantoic fluid. They were incubated at 33 °C and about 24 h later the eggs were chilled and harvested. Fluids with a titre of 500 or more were satisfactory. The fluids were usually stored at −70 °C until the morning of the experiment when they were thawed, clarified by sedimentation at low speed and then the virus was deposited by sedimentation at 20000 rev/min for 30 min in an angle head. The supernatant fluid was poured off and the pellets were allowed to soften for about 1 h at 4 °C in the supernatant fluid, which drained back. They were then resuspended by pipetting in the same fluid, and were used immediately.

Chorioallantoic membrane preparations. The embryos and fluid were poured from 11-day incubated eggs and the chorioallantoic membrane attached to the shell was cut into roughly triangular pieces, with sides about 0.5 cm long. The pieces were kept immersed in Eagle’s medium and handled by the corners. Each piece was put in a few drops of medium in a depression in a plastic tray which was then closed with paraffin film. In some experiments the membrane was incubated like this in medium containing various drugs. For virus adsorption the tray was put in the cold room, the medium was removed with a pasteur pipette and replaced with 2 drops of virus suspension. If necessary this was replaced with further medium.

To study virus entry a cup and the film covering it were cut from the plastic tray with scissors after 1 h at 4 °C and floated on a water bath at 35 °C; buffered glutaraldehyde was added as soon as the required time had elapsed. Samples were usually taken for fixation after 5 min, 10 min, 30 min, 1 h and 2 h.

Drugs and sera. Cytochalasin B was kindly provided by Dr P. Davies. It was dissolved in dimethyl-sulphoxide and this was diluted 1:100 in Eagle’s medium immediately before use, to a final concentration of 30 μg/ml.

Colchicine and amantadine were prepared freshly for each experiment by dissolving them in Eagle's medium. The CAM cultures were incubated with each drug for 1 h before introduction of the virus, and the media used were prepared with the drugs present.

Rabbit immune sera were kindly supplied by Dr G. C. Schild and consisted of (a) a high-titre monospecific rabbit serum against influenza A2/HK haemagglutinin, (b) a similar serum against the influenza A2/HK neuraminidase, (c) a convalescent ferret serum obtained from an animal which had been infected by intranasal inoculation with allantoic fluid. Sera (a) and (b) were said not to cross-react and contained no antichick antibodies. After virus adsorption, the membranes were washed with Eagle’s medium and a drop of antiserum was added. The membranes were held at 4 °C for a further 1 h, washed again and warmed for 35 °C for 30 min.

Electron microscopic techniques. After completion of the various experiments, the chorioallantoic membranes were fixed in ice-cold 3 % glutaraldehyde freshly diluted in 0.15 M-phosphate buffer, pH 7.4. After fixation in the cold for 1 to 2 h the membranes were stripped from the shell, washed in isotonic phosphate buffer, fixed in 1 % osmic acid prepared in Millonig's buffer for 1 h and dehydrated in acetone. Following the stage of glutaraldehyde fixation, it was found that the membranes retained their flat shape,
Entry of influenza virus and subsequently on embedding in Epon or Araldite, they could be oriented in any way desired. The embedding was carried out in flat trays on a cushion of resin-infiltrated agar. Sections were stained with saturated uranylacetate in 50% alcohol for 30 to 45 min and then with concentrated lead citrate (Reynold’s) for 1 min. They were stabilized with a light coat of carbon and examined in a Philips EM 300. Electron micrographs were taken on Ilford 4 E 50 cut film.

Staining for acid phosphatase was carried out on glutaraldehyde-fixed pieces of CAM, using a modification of the technique described by Ericsson & Trump (1965). Both sodium β-glycerophosphate and cytidine monophosphate were used as substrate.

RESULTS

Attachment of virus

Examination of cultures incubated in the cold with infective virus, showed virus particles closely attached to the allantoic cell membrane, by means of their surface projections. No intracellular particles were seen without warming the preparations (Fig. 1).

Occasionally appearances were seen suggesting the fusion of virus with cell membrane, as stated by Morgan & Rose (1968) (Fig. 2). Experiments making use of the 60° tilt available on the Philips goniometer stage showed that such appearances are the result of tangential sectioning. Appropriate tilting of the section can show that the virus membrane remains distinct from the cell membrane during the stages of virus attachment and pinocytosis of virus by vacuoles (Fig. 3a, b).

Entry of virus into allantoic cells

After warming the cultures for 5 min, numerous virus particles were seen within clear cytoplasmic vacuoles, near the cell surface. This appeared to be the usual manner of virus entry (Fig. 4). If ferritin was added to CAM cultures to which influenza virus had previously been adsorbed in the cold, and the cultures were warmed for 5 to 10 min, ferritin was found in the vacuoles together with the virus particles (Fig. 5a, b). Ferritin was not seen in vacuoles in areas where virus uptake was not taking place. Similar vacuoles were seen in cells that were not incubated with influenza virus; however, these did not take up ferritin even after 2 h warming in the presence of ferritin.

After approx. 10 min warming, many examples could be found of virus particles either fusing with the membrane of the vacuoles, or penetrating the membrane, so that particles lay within the leaves of the bilayer membrane (Fig. 6a, b; 7b). Often the particles appeared to draw away from the vacuole (Fig. 6a); also, sections were found in which the virus was found wrapped tightly by only a single layer of membrane. These were interpreted to be cut in such a way as to exclude the rest of the vacuole.

In addition to these findings, many particles were found to be in the process of being dismembered. These were often seen in association with the remains of the dissolved membranes of a vacuole (Fig. 7a, b, c). Frequently all that was left of the virus particles were small cores from which surrounding dense material radiated into the cytoplasm (Fig 7c). Histochemical studies using both sodium β-glycerophosphate and cytidine monophosphate as substrates, showed no staining for acid phosphatase of the vacuoles containing virus. On the other hand, lysosomes which stained readily were found elsewhere in the cell (Fig 8a). Only very occasionally were lysosomes found close to the virus-containing vacuoles. The best illustrations of virus uncoating were seen in RIF-free eggs.
Fig. 1. Electron micrograph of CAM adsorbed with active influenza virus, and maintained at 0 °C for 1 h. There are large numbers of attached virus particles.

Fig. 2. Heated virus on CAM warmed to 35 °C for 30 min. Attached virus particles at high magnification, showing appearance of fusion (→).

Fig. 3. Active virus on CAM, warmed to 35 °C for 5 min. In (a) virus appears to be penetrating membrane. After tilting through 45°, the same particle in (b) is seen to be on surface of membrane.
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Fig. 4. Active virus on CAM, warmed to 35 °C for 5 min. Many particles are taken up in cytoplasmic vacuoles.

Fig. 5(a). Active virus on CAM, incubated with ferritin, and warmed to 35 °C for 5 min. Virus particles are seen to enter vacuoles together with ferritin. (b) As in (a), warmed to 35 °C for 10 min.
Fig. 6. (a) Active virus on CAM, warmed to 35 °C for 30 min. Virus particles are seen penetrating wall of vacuole (V). There is a continuous, single layer of membrane surrounding the virus. (b) As in (a); particles are positioned around walls of vacuoles (V) and are in a partial state of degradation; some particles are not associated with vacuoles in the plane of section (→).

After 2 h warming, virus particles could still be found in the cytoplasm, usually surrounded by a membrane (Fig. 9a to d). Ferritin taken up in vacuoles together with virus had disappeared entirely from the cells after 2 h and was found adjacent to the basement membrane of the allantois.
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Effect of heat-inactivation of virus and treatment of CAM by drugs that interfere with cellular mechanisms

The stages of virus entry that have been described above, up to the point of virus uncoating, were seen after adsorption of heat-inactivated virus to normal cells and of untreated virus to cells treated with cytochalasin, colchicine, or amantadine.
Effect of antisera on virus entry

The sera directed against the whole virus, neuraminidase and haemagglutinin each prevented virus entry completely. However, their effect on the virus differed in detail. The antineuraminidase antiserum allowed the virus to remain closely adherent to the cell
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Fig. 9. Active virus on CAM warmed to 35 °C for 2 h. Particles surrounded by a membrane (a, b, c) also apparently free in cytoplasm (d).

membrane (Fig. 10) whereas the antihaemagglutinin caused the particles to be clumped together at some distance from the membrane (Fig. 11). In both instances the virus particles were coated by a fuzzy layer, presumably representing immunoglobulin.

DISCUSSION

There has been disagreement as to how influenza virus particles enter cells. Dales & Choppin (1962) described particles being taken up into vacuoles; Morgan & Rose (1968) saw particles apparently fusing with cell membranes and thought that the occasional uptake of particles by vacuoles to be unimportant. The findings of Krizanova et al. (1971) supported this theory. Influenza virus was incubated with plasma membranes from CAM cells, following which virus g-antigen was released, presumably on fusion of the virus envelope with the cell membrane. None of the E.M. observers found that intact influenza particles could enter the cytoplasm; in the case of herpes virus, entry of capsids containing cores was subsequently observed by Miyamoto & Morgan (1971). As regards influenza virus, in contradistinction to what happens with Sendai virus, the virus particles do not become one with the membrane at the cell surface and spill their contents into the cytoplasm (Morgan & Rose, 1968). Rather they are taken into vacuoles (viropexis, Fazekas,
Fig. 10. Virus on CAM treated with antineuraminidase antibody and warmed to 35 °C for 5 min. Particles closely attached to membrane and clumped together by immunoglobulin.

Fig. 11. Virus on CAM treated with antihaemagglutinin antibody and warmed to 35 °C for 5 min. Particles eluted from membrane and clumped to each other. In neither of the two types of antisera used were any particles seen within the cytoplasm.
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1948) and find their way into the cytoplasm from there by dissolution of both vacuolar and virus membranes. The difference between our present results and those of other workers and of ourselves in previous experiments (Dourmashkin & Tyrrell, 1970) is probably due to using highly susceptible cells which were kept in as good a condition as possible, with the minimum of handling and exposed only to Eagle's medium or allantoic fluid.

In examining the question of whether, in addition to entry by viropexis, the influenza virus particle coat may fuse with the cell membrane, we have taken into account the usual artifacts of thin sectioning. By tilting the specimen, we found that images suggesting fusion of membranes could be resolved into two closely apposed membranes. Conversely we would find such images in situations where fusion would not be expected to occur, e.g. after attachment of virus in the cold. We can conclude that we have no evidence in favour of virus fusion as a means of entry.

Attention has recently been paid to the general problem of the uptake of particles by cells. Ingestion of large particles, such as bacteria or erythrocytes, has been termed phagocytosis, while the uptake of small particles or macro-molecules into cytoplasmic vesicles has been called pinocytosis. These phenomena have been differentiated recently by Wills et al. (1974) and G. D. B. Klaus (personal communication), who showed that phagocytosis by macrophages was found to be inhibited by cytochalasin B, whereas pinocytosis was unaffected. It is possible that cytochalasin B acts by exerting an effect on microfilaments; but in any case, the results support the concept that the mechanism of virus uptake is different from that of phagocytosis. Similarly, colchicine, which damages microtubules, did not affect virus uptake, so these organelles are unlikely to be involved in the mechanism.
By adding ferritin concurrently with the virus to the chorioallantoic membrane, we were able to show that the attachment of influenza virus to the allantoic cell membrane acts as a stimulus to switch on uptake of particles near the cell surface. The finding that the uptake of ferritin is activated by virus adsorption and entry, recalls an analogous situation found in recent immunological studies. The presence of antigen–antibody complexes on the surface of lymphocytes may stimulate their uptake by a conformational change in the cell membrane, which is not inhibited by cytochalasin B (Taylor et al. 1971).

Because the uptake of virus by allantoic cells differs from the usual function of pinocytosis and also from phagocytosis as observed in polymorphonuclear leukocytes or macrophages, (Hackemann, Denman & Tyrrell, 1973), we have preferred to retain the term, viropexis (Fazekas, 1948).

Our observation that the uncoating of virus particles takes place in direct contact with the host cytoplasm, without the intervention of lysosomal enzymes, suggests a pathway for virus replication that is consistent with the notion that lysosomal enzymes would be generally destructive for virus nucleic acid and are too blunt an instrument for the dissection of the virus particle.

Although we have found that fusion of the virus particle coat with the cell membrane, such as occurs with paramyxoviruses, does not take place, a type of fusion does occur with the membrane of the vacuole containing virus. This is somewhat dissimilar to cell-membrane fusion, in that the virus coat and the membrane appear to dissolve simultaneously. We may speculate as to whether this is enzymic or detergent in nature.

The release of virus material into the cytoplasm occurred frequently after 10 to 30 min incubation, but was not seen after only 5 min warming. We have therefore interpreted this to be a true event rather than an E.M. artifact. However, it must not be forgotten that some virus particles do remain in vacuoles and become absorbed there.

Another aspect to our study on virus entry was the effect of antisera directed against various components of the virus coat. Antiserum against the virus haemagglutinin caused the virus particles to clump at a distance from the cell membrane. The elution of adsorbed virus was probably due to the continuing activity of the neuraminidase. Not surprisingly, no virus uptake was seen. However, when anti-neuraminidase antiserum was used, many particles were seen closely adherent to the cell membrane, even more than if no antiserum were added. We would interpret this as due to the continuing activity of haemagglutinin. However, there was no uptake of virus into vesicles after treatment with antineuraminidase antiserum; this suggests that both neuraminidase and haemagglutinin are required for virus uptake.

Anti-neuraminidase antiserum in high titre has been shown to neutralize certain strains of influenza virus (Webster, Laver & Kilbourne, 1968) and it would appear that it interferes with virus entry.

Heat inactivation of the virus, which under the conditions of the experiment did not affect the stability of the virus haemagglutinin or neuraminidase, was found not to abolish any of the observable stages of virus entry. This indicates that heat inactivation does not interrupt viropexis.

Davies et al. (1964) suggested that amantadine prevented the initial steps of virus entry into cells. Our results show that the stage of viropexis is not interrupted by amantadine. Our findings are compatible with those of Kato & Eggers (1969), who did not think that penetration was inhibited, but found that the drug depressed the loss of photosensitivity of neutral red-labelled virus in infected cells. This can be interpreted as suggesting that the virus RNA, although not identifiable morphologically, is still present as a package of
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some sort after amantadine treatment, and does not become released into the cytoplasm. Long & Olusanya (1972) have suggested that the release of virus RNA into cells may consist of two steps, one of which is interrupted by amantadine: (1) uncoating of the virus envelope and (2) release of the RNA from the nucleocapsid. We have not investigated the question of whether the E.M. appearance of virus uncoating is interfered with by amantadine.

Virus particles were found within small vesicles for as long as 2 h after warming the preparations. It is possible that these particles are still at an early stage of processing, as the initiation of the events of virus dismemberment and replication are asynchronous (Cairns, 1957).

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REFERENCES


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