The specific inhibition of influenza A virus maturation by amantadine: an electron microscopic examination

R. W. H. Ruigrok,† E. M. A. Hirst and A. J. Hay*


Amantadine specifically inhibits the release of virus particles from cells infected with the Rostock (H7N1) strain of influenza A virus, apparently as a consequence of a membrane protein M2-mediated conversion of haemagglutinin (HA) to its low pH conformation. Electron microscopic observations, together with immunogold labelling, showed that amantadine action does not alter the distribution of HA on the cell surface nor does it prevent the formation of budding virus particles. It was not possible, however, to discern whether low pH HA inhibited the final stage in virus maturation, i.e. pinching off, or simply prevented release of fully formed particles.

Amantadine (1-amino adamantane hydrochloride) and rimantadine (α-methyl-l-adamantanemethylamine hydrochloride) are effective in the prophylaxis and treatment of influenza A infections (Wingfield et al., 1969; Van Voris et al., 1981; Dolin et al., 1982; Hall et al., 1987; Tominack & Hayden, 1987). Their actions are highly selective both in vivo and in vitro. In cell culture, micromolar concentrations of these drugs specifically inhibit the replication of influenza A viruses but are inactive against influenza B infections. Characterization of drug-resistant mutants has identified the primary target of the action as the transmembrane domain of the M2 membrane protein (Hay et al., 1985; Belshe et al., 1988; Bean et al., 1989; Hayden et al., 1989). With many influenza virus strains inhibition occurs at an early stage in virus infection, preventing virus uncoating (Kato & Eggers, 1969; Bukrinskaya et al., 1982). For certain influenza H7 virus infections on the other hand, inhibition occurs principally at a later stage during replication and prevents virus maturation without affecting significantly the synthesis of most virus components (Hay et al., 1986). Analyses of genetic reassortants between amantadine-resistant and -sensitive viruses indicated the importance of haemagglutinin (HA) in influencing susceptibility to this latter action (Scholtissek and Faulkner, 1979; Hay et al., 1986). Furthermore, it appears that the block to virus release is the direct consequence of an amantadine-induced, M2 protein-mediated alteration of HA to its low pH conformation (Skehel et al., 1982) during its transport to the surface of drug-treated infected cells (Sugrue et al., 1990). The present study examined the morphological aspects of virus maturation affected by amantadine action and, in particular, addressed whether budding occurred in the absence of virus release and whether amantadine treatment caused any alteration in the distribution of HA on the cell surface.

Parallel experiments confirmed that amantadine (5 μM) added to MDCK cells 1 h after infection with the Rostock strain of influenza A virus (A/chicken/Germany/34, H7N1) at a multiplicity of approximately 50 reduced the yield of virus 6 h after infection by greater than 90%, as assayed by either HA production or isolation of 35S-labelled virus, (Hay et al., 1986). In contrast, drug treatment caused no detectable decrease in the yield of virus from cells infected with an amantadine-resistant variant, RostockR. Attempts to obtain sufficient virus from amantadine-treated cells for morphological examination by electron microscopy were unsuccessful. Electron microscopic observation of negatively stained specimens of culture medium or virus pellets (200000 g, 60 min) also failed to show that virus particles were produced by amantadine-treated infected cells; this was in contrast to virus production by control infected cells.

For electron microscopy of thin sections, MDCK cell monolayers were fixed overnight in 1% paraformaldehyde, 1·25% glutaraldehyde in 0·1 M-sodium cacodylate pH 7·2. Cells were pelleted, post-fixed for 1 h in 1% osmium tetroxide, block-stained with 1% uranyl acetate, dehydrated and embedded in Araldite. Sections (70 nm) were stained with ethanolic uranyl acetate and Reynold's lead citrate and viewed with a Jeol JEM 1200EX electron microscope. Examination of thin sections of Rostock virus-infected MDCK cells (Fig. 1a and b) clearly

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showed that virus bud formation was not impaired by
amantadine treatment and neither was the polarized
distribution of budding virus on the apical surface.
Estimates of the number of virus particles per unit length
of sectioned cell surface indicated that they were three- to
four-fold greater on the surface of amantadine-treated
cells at 6 h post-infection as compared to the control (Fig.
1e and f). Similar results were obtained whether cells
were infected for 4 or 7 h. Furthermore, similar effects of
amantadine were also noted with infected chick embryo
fibroblasts, although the general distribution of budding
particles on these cells made quantification difficult.

Differences in the morphology of HA were evident at
higher magnification (Fig. 1c and d). In contrast to the
regular array of HA spikes on particles budding from
control infected cells (Fig. 1c), the spike layer on
particles budding from amantadine-treated cells is
irregular and diffuse (Fig. 1d), similar in appearance to
low pH HA on virus particles exposed briefly to pH 5
(Ruigrok et al., 1984, 1986).

Whether the block to virus production occurs at the
final stage in virus maturation, which involves pinching-
off, or whether amantadine simply prevents release of
fully formed virus particles was not resolved. The
proportion (5 to 10%) of virus particles clearly attached
to the cell surface via a thin membrane stalk was not
discernibly different in drug-treated and control cells.
When 200 nm thick sections were tilted in the micros-
cope, more stalks were observed but for about 80% of the
virions the nature of their attachment was not evident.
In the thick sections virus particles were always observed in
association with microvilli. Apparently, in the thin
(70 nm) sections, these microvilli are often outside the
plane of sectioning and virus therefore appears to be
aggregated (Fig. 1b). This was clearly different from the
extensive aggregation observed in the absence of an
active neuraminidase (Palese et al., 1974; Palese &
Compans, 1976) and treatment with neuraminidase did
not promote virus release. Moreover, there is no genetic
evidence to implicate the virus neuraminidase in
amantadine action (Lubeck et al., 1978; Hay et al., 1979,
1985; Scholtissek & Faulkner, 1979; Belshe et al., 1988;
Bean et al., 1989). Various other treatments of drug-
treated infected cells also failed to promote the release of
virus particles. These included incubation for 15 min
with medium at alkaline pH (up to pH 8.9), NaCl
concentrations of up to 0.3 M and digestion with trypsin,
bromelain or thermolysin. Treatment with 20 mM-DTT,
known to remove most HA1 from low pH HA on
amantadine-treated cells (Sugrue et al., 1990), also had no
effect, indicating that interaction via this component is
not responsible for attachment.

The distribution of HA on the surface of infected cells
was investigated by immunogold labelling. Five hours
after infection cells were fixed with 3% paraformalde-
hyde in phosphate-buffered saline (PBS) for 15 min at
room temperature, washed and incubated for 1 h at room
temperature with a 10-fold dilution of monoclonal
antibody (MAb) in PBS with 0.5% bovine serum
albumin (BSA). Cells were then washed and incubated
for 1 h at room temperature with a 10-fold dilution of
Auoprobe (EM Protein A G10; Janssen) in PBS with
0.5% BSA. Thin sections were prepared as described
above. Three MAbs were used: HC2 which recognizes
site A (Wiley et al., 1981) and reacts equally well with
uncleaved and cleaved forms of native and low pH HA;
Table 1. Effect of amantadine on HA expression

<table>
<thead>
<tr>
<th>Antibody</th>
<th>ELISA (A450)</th>
<th>Immunogold labelling*</th>
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<tbody>
<tr>
<td></td>
<td>~A</td>
<td>+A†</td>
</tr>
<tr>
<td>HC58</td>
<td>0.56</td>
<td>0.04</td>
</tr>
<tr>
<td>H9</td>
<td>0.05</td>
<td>0.49</td>
</tr>
<tr>
<td>HC2</td>
<td>0.61</td>
<td>0.62</td>
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* Average number of gold particles per budding virion. Within parentheses, total number of virus particles counted. Counts were from electron microscopy negatives, including only viruses that were sectioned through the middle.
† Plus 5 μM-amantadine added 1 h post-infection.

HC58, which recognizes the interface between the HA subunits (site D) and reacts only with native HA; and H9, prepared against pH 5-treated HA, which recognizes only the low pH form (Sugrue et al., 1990). An ELISA (Table 1) done in parallel as described by Sugrue et al. (1990) showed that MAbs HC58 and H9 readily distinguished HA on control and amantadine-treated cells, respectively, whereas the data for MAb HC2 indicate that drug treatment caused no significant difference in the amount of HA produced. Electron micrographs of immunogold-labelled infected cells are shown in Fig. 2 and the numbers of gold particles per virion are given in Table 1. There was little difference in MAb HC2 labelling of control and drug-treated cells, whereas MAbs HC58 and H9 labelled predominantly control and amantadine-treated infected cells, respectively. In all cases more than 90% of the label was associated with virus or buds and there appeared to be no difference in the distribution of HA on the viral and cell surfaces between control and drug-treated cells.

In conclusion, therefore, it is apparent that the efficiency and morphology of virus bud formation is not impaired by amantadine action, which results in an accumulation of budding virus particles. The precise nature and location of the block was, however, not ascertained. It was not possible to discern whether the pinching off of virus particles was inhibited or whether fully formed particles simply remained attached to cells. In this context, it should be noted that, in contrast to many influenza A viruses, Rostock virus particles show a particular tendency to aggregate following exposure to pH 5 and this aggregation is not reversed by treatment with high pH or trypsin (Ruigrok et al., 1984). To date, studies using different virus–cell systems and various conditions which reverse amantadine action (Sugrue et al., 1990; A. Hay, unpublished data) have shown a strict correlation between the alteration of HA and inhibition of virus production. Furthermore, addition of amantadine later than 20 min after the start of HA synthesis causes neither the conformational change in HA nor prevents its incorporation into virus particles (Hay et al., 1986; Sugrue et al., 1990). It appears most likely, therefore, that the block to virus release is a consequence of the particular properties of low pH HA rather than the result of inhibition of some other function of M2 involved directly in virion assembly (Zebedee & Lamb, 1989).

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References


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