Effect of Chloroquine on African Swine Fever Virus Infection

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SUMMARY

When present during the whole infective cycle, the lysosomotropic drug, chloroquine, inhibited cytopathic changes and production of African swine fever virus (ASFV) in Vero cells. This inhibition decreased when the drug was added from 1 h to 4 h after infection. Chloroquine had no direct effect on the virus nor on viral adsorption and internalization. Electron microscopy showed that, in the presence of the drug, the virions were retained in large vacuoles having a lysosomal appearance. This inhibition was fully reversible, even when the drug was removed as late as 72 h after infection. The results support the hypothesis that ASFV enters the cells by adsorptive endocytosis and not by fusion with the plasma membrane.

African swine fever virus (ASFV) is an enveloped DNA virus provisionally classified as a member of the family Iridoviridae (Matthews, 1982) and whose pathway of penetration into cells has not yet been established.

Enveloped viruses may enter cells by fusion with the plasma membrane (e.g. Sendai virus; Poste & Pasternak, 1978). However, in the case of other groups of enveloped viruses, such as toga-, orthomyxo- and rhabdoviruses, the virions are first internalized by endocytosis and fusion occurs later with the membranes of endocytic vacuoles (for reviews, see Dales, 1973; Helenius et al., 1980b; Marsh, 1984). The fusion activity is triggered by an acidic environment (pH 5 to 6) and this process can be inhibited by lysosomotropic substances which raise the pH of endosomes and lysosomes (Marsh et al., 1983), such as weak bases like chloroquine, ammonium chloride and amantadine (Ohkuma & Poole, 1978). The use of these inhibitors is a convenient way to confirm that penetration occurs intracellularly and not by fusion with the plasma membrane. The present work was undertaken to determine the effect of chloroquine on the infection of Vero cells by ASFV and to establish whether acidic endocytic vacuoles were involved in the infection.

Vero cell monolayers were infected with ASFV, strain Lisbon 60 (Manso Ribeiro & Rosa Azevedo, 1961) adapted to Vero cells, at a variable multiplicity of infection (m.o.i.) (see figure legends). The virus was kindly supplied by Dr J. D. Vigario. After adsorption for 2 h at 37 °C or 4 °C, the cells were washed three times with DME2 (Dulbecco's modified Eagle's MEM, 2% newborn calf serum and 50 μg/ml gentamicin) at the same temperature and incubated at 37 °C with the same medium. The infection was measured from this time. All these steps were carried out in the presence or absence of chloroquine (see figure legends).

The effect of chloroquine ('Aralen', Winthrop, U.S.A.) on the infection was studied by plaque titration of progeny virus and/or by a quantitative determination of c.p.e. by a dye uptake method (Finter, 1969). This method is based on the capacity for uptake of neutral red by infected cells compared with control uninfected cells. In our system and for the range of 0.07 to 1.0 p.f.u./cell, the relationship between the quantity of virus inoculated and the c.p.e. measured by this method is highly reproducible and represented by a logarithmic-like curve which is not converted to a linear form by plotting on semi-log paper. Depending on the m.o.i., titrations and/or dye uptake assay for c.p.e. were performed 24 to 48 h post-infection, when infected control cells showed extensive c.p.e. (90 to 100%) by microscopy.

For electron microscopy (performed at the National Laboratory for Veterinary Research, Lisbon), the cells were fixed for 1.5 h with half-strength, ice-cold Karnowsky solution.
Fig. 1. Relationship between the time of addition of chloroquine and c.p.e. Vero cells were infected at a m.o.i. of 10 at 4 °C. At different times after infection, chloroquine (32 μM) was added. The c.p.e. was measured at 24 h.

Table 1. Inhibition of ASFV infection by chloroquine*

<table>
<thead>
<tr>
<th>Drug concentration (μM)</th>
<th>Dye uptake (% c.p.e.)</th>
<th>Virus titre (log_{10} p.f.u./ml)</th>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>6.70</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>ND†</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>9</td>
<td>4.48</td>
</tr>
<tr>
<td>32</td>
<td>6</td>
<td>ND</td>
</tr>
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</table>

* The conditions of the experiments were as follows: m.o.i. = 10; adsorption at 37 °C; the drug was present during adsorption and throughout the experiments; titrations and dye uptake 24 h post-infection.
† ND. Not done.

(Karnowsky, 1965), scraped from the glass surface and pelleted by low-speed centrifugation. The pellets were post-fixed with osmium tetroxide and block-stained with uranyl acetate. Thin sections obtained after dehydration and embedding in Epon–Araldite were double-stained with uranyl acetate and lead citrate and examined in a Jeol 100 C electron microscope.

The results in Table 1 show the effect of different concentrations of chloroquine on virus infection. In this case, the drug was present during adsorption and throughout the experiment. As can be seen, a concentration as low as 16 μM inhibited c.p.e. by 90% and the yield by 99%. The concentration that reduced the c.p.e. to 50% was about 2 μM. These concentrations are much lower than those reported in other systems. This difference may be due to the cell physiology, as the maximum concentration that is not toxic to Vero cells, as measured by their capacity for dye uptake (64 μM), is much lower than those reported for other cells (Helenius et al., 1980a; Krzystyniak & Dupuy, 1984).

The inhibitory effect of chloroquine depended on the time when the drug was added to the infected cells. As can be seen in Fig. 1, the protective effect of chloroquine began to decrease significantly at about 1 h and reached a plateau by 4 h after infection. The level of this plateau was lower when the m.o.i. was higher. Although we can not rule out other explanations, this plateau may be due to a failure to infect 100% of cells even at m.o.i. = 10, as well as the added...
Fig. 2. Electron micrographs of cells infected with ASFV in the presence or absence of chloroquine. Cells were infected at a m.o.i. of 10 at 4 °C for 2 h. Fixation was done after a further 1.5 h at 37 °C. Chloroquine (32 μM) was present throughout the experiments. (a) No chloroquine; (b, c, d) chloroquine present: intact viral particles (arrows) in large vacuoles of lysosomal appearance. Bar markers represent 200 nm.

chloroquine protecting the uninfected cells from the virus produced during the first cycle of replication.

These results and the fact that the drug did not appear to affect viral adsorption and internalization (no inhibitory effect was observed when the drug was present only during adsorption at either 37 °C or 4 °C) indicate that chloroquine mainly affects an early event during the infective cycle of ASFV.

We observed by electron microscopy 80 viral particles from three experiments carried out in the presence of chloroquine. These observations were done after infection for 2 h at 4 °C and 1.5 h at 37 °C and showed that all the viral particles were in large vacuoles of lysosomal appearance (Fig. 2b,c,d) and most of them were intact. Probably due to the relatively low m.o.i. used, we rarely saw more than one particle per vacuole and per cell section. At this time post-infection, no more viral particles were seen in the infected control cells (Fig. 2a). These results suggest that, in the presence of chloroquine, the virions were retained in lysosomes and that the step inhibited by the drug is the uncoating of the virion and the release of its DNA into the cytoplasm.

Finally, the inhibitory effect of chloroquine was reversible. The removal of the drug, even at
Table 2. Effect of drug removal on infection*

<table>
<thead>
<tr>
<th></th>
<th>Virus titre (48 h post-infection)</th>
<th>Virus titre after removal of the drug</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.68</td>
<td>6.74</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>4.46</td>
<td>6.74</td>
</tr>
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</table>

* The conditions of the experiments were as follows: m.o.i. = 5; adsorption at 37 °C; chloroquine (16 μM) was present during adsorption and up to 72 h post-infection; removal of the drug by washing three times with medium; titrations 48 h after removal of the drug. The titres are given as log₁₀ p.f.u./ml.

72 h after infection, allowed the infective cycle to start again and to proceed in a manner similar to that of the untreated cultures (Table 2).

In conclusion, our results suggest that chloroquine inhibits ASFV infection in Vero cells as reported for several enveloped viruses (Helenius et al., 1980a, 1982; Matlin et al., 1982; Krzystyniak & Dupuy, 1984). Our results also indicate that ASFV probably enters cells by adsorptive endocytosis with the participation of acidic intracellular vesicles.

REFERENCES


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