Effect of daunorubicin on the growth of adenovirus

K. F. Shortridge*

Department of Bacteriology, University College Hospital Medical School,
University Street, London WC 1E 6JJ, England

and S. Squires†

May & Baker Ltd., Dagenham, Essex RM10 7XS, England

Treatment of adenovirus type 5-infected cells with daunorubicin (D) at a concentration of 0.05 μg/ml reduced the \textit{in vitro} virus infectivity by six logs. Mature virus particles were not detected in extracts of D-treated cells by density gradient centrifugation and the low level of infectivity observed appeared to be due to free nucleoprotein. Virus components produced in the D-treated cells were not released into the extracellular phase.

Introduction

Human adenoviruses are best known as causative agents of a number of respiratory diseases (Fenner & White, 1971). Although they may be isolated from faeces with relative ease, their relationship to disease in the alimentary tract is still unclear. More recently, adenovirus type 11 has been strongly associated with acute haemorrhagic cystitis in children in Japan (Numazaki \textit{et al.}, 1973) and in the U.S.A. (Mufson, Belshe, Horrigan & Zollar, 1973), and in such cases the virus has been readily isolated from urine.

The eye is also susceptible to adenovirus infection and type 8 virus is well documented as the causative agent of epidemic keratoconjunctivitis. Recent work suggests a causal association between adenovirus type 11 and certain forms of eye disease recognized in the Far East—epidemic conjunctivitis in Taiwan (Tai, Chu, Chi, Wei & Hierholzer, 1974) and epidemic haemorrhagic conjunctivitis in Singapore (Yin-Murphy, Lim & Chua, 1974).

Sparse information is available on inhibitors of adenovirus at laboratory and clinical levels. Methisazone (1-methylisatin thiosemicarbazone) has been used successfully in the prophylaxis of smallpox (Bauer, St Vincent, Kempe & Downey, 1963) and \textit{in vitro} studies show that it is capable of inhibiting adenovirus replication (Bauer & Apostolov, 1966). However, limited clinical trials of methisazone to treat adenovirus infections have not been encouraging.

*Present address: Department of Microbiology, University of Hong Kong, Pathology Building, Queen Mary Hospital Compound, Hong Kong.
†To whom reprint requests should be sent.
Daunorubicin [=daunomycin (D)], an antibiotic of the anthracycline group which appears to act chiefly on DNA and DNA-dependent RNA synthesis has been shown to be capable of inhibiting the in vitro infectivity of the DNA viruses, herpes and vaccinia (Cohen, Harley & Rees, 1969). Limited studies by these authors on the production of haemagglutinin by adenovirus type 11 have shown that this component is inhibited by daunorubicin. The structural composition of the adenovirus, based principally on serotypes 2 and 5, is now well established (e.g. Valentine & Pereira, 1965; Maizel, White & Scarff, 1968; Russell & Knight, 1967; Shortridge & Biddle, 1970). Specific information on the structural detail of the higher numbered serotypes such as type 11, which now appears to be of increasing clinical importance, mainly concerns an appraisal of the nature of its soluble antigens (Norrby, 1968). Against this background we have taken the opportunity of examining further the behaviour of daunorubicin on the production of the components of a model virus, viz. adenovirus type 5, and report here that the drug significantly reduces in vitro virus infectivity.

**Materials and methods**

**Virus**

Adenovirus type 5, strain Ad.75 (Ad 5) was propagated in mycoplasma-free HeLa cell monolayers grown in medium 199 plus 5% calf serum and medium 199 without serum for maintenance. Three days post-infection Ad5 particles were purified by fluorocarbon disruption of the infected cells (Valentine & Pereira, 1965) followed by two cycles of isopycnic banding on CsCl density gradients (Shortridge & Biddle, 1970).

*Daunorubicin*

This was supplied in the form of a freeze-dried powder which was reconstituted with sterile distilled water to give a stock solution of 100 µg/ml. The stock solution was kept at 4°C and diluted as required to appropriate concentrations.

**Anti-viral assay**

This was carried out in monolayers of mycoplasma-free HeLa cells cultivated as above in Flow tissue culture tubes. The cells were infected with 10⁴ TCID₅₀/ml purified Ad5 particles and incubated at 37°C for 1 h to allow for their absorption and penetration. The infected cells were then washed three times with medium 199 to remove any unabsorbed particles. D at concentrations of 5, 0.5 or 0.05 µg/ml was added 1 h pre-infection and at 1 or 3 h post-infection. When added pre-infection, the drug was also incorporated into the virus inoculum and washing medium at the required concentration. All cell cultures were maintained in medium 199 containing the appropriate concentration of D. Control Ad5 infected, D treated, and uninfected, untreated, cell cultures were included. The cultures were incubated for 4 days by which time the cytopathic effect of the control virus infected cultures was advanced and the cells were then removed from the glass by shaking or gentle scraping prior to low-speed centrifugation using a bench centrifuge. The supernatant constituted the extracellular source of virus component. The centrifuged cell deposit was taken up in 1 ml fresh medium 199, homogenised with fluorocarbon (Valentine & Pereira, 1965) and the aqueous phase constituting the intracellular source of virus component was separated by low speed centrifugation.

* Cerubidin, May & Baker Ltd.
**Detection of virus components**

Microtitre procedures were used as reported (Shortridge & Biddle, 1970). Complement-fixing (CF) activities were detected using antisera prepared against purified hexon component and the whole virus or virion. The former antiserum detects hexons, the predominant surface component of the virus (Valentine & Pereira, 1965), whereas the latter detects both surface and internal components. The combined use of these two antisera in combination renders possible the detection of internal components (Shortridge & Biddle, 1970). The complete haemagglutinating antigen or penton component was detected by the microtitre procedure using rat erythrocytes (Shortridge & Biddle, 1970). Hexons and pentons may occur free as 'soluble antigens' unincorporated into the virus particle (Wilcox, Ginsberg & Anderson, 1963).

**Infectivity titrations.** These were performed in triplicate in HeLa cell monolayers in tubes incubated at 37°C for 7 days and 50% end-points determined according to the method of Reed and Muench (1938).

**Density gradient centrifugation**

Cell extracts were layered on to 40 to 80% CsCl (w/v) linear density gradients and centrifuged in the SW39 rotor of a Spinco model L ultracentrifuge for 5 h at 100,000 x g (mean) at 4°C as previously reported (Shortridge & Biddle, 1970). These conditions were chosen as they are isopycnic for the virus the mean buoyant density of which is 1.34 g/ml (Green & Piña, 1963, 1964). They are however rate zonal for more dense nucleoprotein and less dense soluble antigens which occur in advance and to the rear of the whole virus respectively (Shortridge & Biddle, 1970). The tube bases were pierced with a pin and the issuing fractions monitored for radioactivity, refractive index using the 'Abbe' refractometer (for conversion to density using International Critical Tables) and, where indicated, CF activities.

**Radioactive labelling**

Nucleic acid or protein was labelled radioactively with [3H] thymidine of [14C] *Chlorella vulgaris* protein hydrolysate*, respectively, and monitored as previously reported (Shortridge & Biddle, 1970).

**Results**

**Response of infected cells to daunorubicin**

Daunorubicin, at concentrations of 5 and 0.5 µg/ml, appeared to be toxic for the HeLa cells which rounded up and became detached from the glass after 24 h; this effect was not apparent macroscopically or microscopically in unstained preparations when the drug concentration was reduced to 0.05 µg/ml. Cells treated with D, 1 h pre-infection with Ad5 and 1 or 3 h post-infection, gave essentially the same results and those for 1 h post-infection are given in Table 1. Macroscopically recognizable cytotoxicity appeared to be associated with an absence of detectable Ad5 components or infectivity.

On the other hand, virus components and low levels of infectivity were detected at a drug concentration of 0.05 µg/ml but they were exclusively intracellular in contrast to the intra- and extracellular localization of these activities in cells that were not treated with daunorubicin. Comparable levels of haemagglutinating penton component were recorded intracellularly in daunorubicin-treated and untreated cells. Production of

* Radiochemical Centre, Amersham, Bucks.
hexon component was lower in cells treated with the drug; the threefold CF titre increment for whole virus antiserum over hexon antiserum suggests the presence of unincorporated internal virus protein (Shortridge & Biddle, 1970). This observation along with the fact that the intracellular level of infectivity was six logs lower than that recorded for the non-daunorubicin treated cells, without a comparable decrease in detectable virus surface components, suggested that the low level of infectivity in the drug-treated cells might be due to infectious nucleoprotein. The DNA of mammalian adenovirus is considered to be non-infectious (Green et al., 1967). Further assessment was approached by the ultracentrifugation procedures given below.

Table I. Effect of daunorubicin applied 1 h post-infection on the growth of adenovirus type 5 in HeLa cells

<table>
<thead>
<tr>
<th>Daunorubicin dose (µg/ml)</th>
<th>Cell location</th>
<th>HA</th>
<th>Virus product titres CF titre against</th>
<th>Infecitivity log₁₀</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Hexon</td>
<td>Whole virus</td>
</tr>
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<td>5·0</td>
<td>Intracellular</td>
<td>&lt;4</td>
<td>&lt;2</td>
<td>&lt;2</td>
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<tr>
<td></td>
<td>Extracellular</td>
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<tr>
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<td>&lt;4</td>
<td>&lt;2</td>
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<tr>
<td></td>
<td>Extracellular</td>
<td>&lt;4</td>
<td>&lt;2</td>
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<tr>
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<tr>
<td></td>
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<td>64</td>
<td>96</td>
</tr>
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</table>

* Not tested.

Density gradient centrifugation

The intracellular extracts of various Ad5-infected, drug-treated (0·05 µg/ml applied 1 h post-infection) and control cells labelled for protein (¹⁴C) or nucleic acid (³H) were processed and ultracentrifuged as given in Materials and Methods. Cells infected with virus yielded only a single peak of ³H-radioactivity at a density of 1·34 g/ml corresponding to mature virus particles that were produced as a result of the infection [Figure 1(a)]. Infectivity was associated exclusively with this peak. The radioactivity tailing off towards the top of the gradient was believed to be due to small cellular debris resulting from fluorocarbon extraction. No radioactivity was observed in the untreated, uninfected and daunorubicin-treated cells in the bottom half of the gradient, indicating an absence of non-specific radioactivity in the virus and nucleoprotein positions [Figure 1(b) and (c)] (Shortridge & Biddle, 1970). This was confirmed by an absence of detectable infectivity in these and the remaining fractions. In contrast D-treated Ad5 infected cells showed low levels of radioactivity for nucleic acid and protein in the bottom half of the gradient [Figure 1(d)]. Complement-fixing activity of fractions 1 to 7 was detected with whole virus antiserum only, each fraction to titre 6 indicating the presence of nucleoprotein. This distribution of radioactivity (and appropriate CF activity) was suggestive of the presence of nucleoprotein fragments of decreasing nucleic acid content from fractions 1 through 6; infectivity was confined to fraction 2. Thus, the low level of infectivity recorded in the daunorubicin-treated cells would appear to be associated with a population nucleoprotein fragments.
Degradation studies on the adenovirus have shown the presence of internal protein (Russel & Knight, 1967; Maizel, White & Scarff, 1968; Shortridge & Biddle, 1970). Using whole virus and hexon antisera, Biddle & Shortridge (1970) detected by CF an internal protein of density approximately 1.3 g/ml to the rear of virus particles in the density gradient system. Not only was there no clearly recognizable peak of $^{14}$C protein radioactivity in this position [Figure 1(d)], but there was also no detectable CF activity indicative of internal component.

![Figure 1. Density gradient centrifugation of HeLa cell extracts submitted to adenovirus type 5 (Ad5) infection or daunorubicin (0.05 μg/ml) treatment as indicated. Centrifugation was for 5 h at 100,000 × g (mean) at 4°C in the Spinco SW39 rotor. $^{14}$C protein (o) and $^3$H thymidine (x) radioactivity levels adenovirus cytopathic infectivity are indicated. All fractions were examined for infectivity but only the positive ones are illustrated. (a) Ad5 virus-infected only; (b) uninfected, untreated; (c) daunorubicin-treated, uninfected; (d) Ad5-infected daunorubicin-treated.](image-url)
Discussion

Daunorubicin, at concentrations of 5 and 0.5 µg/ml, appeared to be toxic for the HeLa cells but this effect was not evident at a level of 0.05 µg/ml, a concentration which appeared to prevent the assembly of mature particles or virions from the available components. As there is strong evidence to suggest that daunorubicin, in addition to affecting DNA synthesis, also prevents messenger RNA transcription necessary for the synthesis of virus-specific enzymes and proteins (Bernard, Paul, Boiron, Jacquillat & Maral, 1969), it might be reasonable to infer that particle assembly failed to occur because certain internal component(s) were not synthesized. This consideration is based chiefly on the contention that particle maturation takes place by condensation of internal component(s) around a nucleoprotein core followed by the addition of surface or capsid elements. Although the synthesized nucleoprotein manifested minimal infectivity capacity, the possibility also exists that it may be structurally deficient in some way as a result of daunorubicin treatment. Its ability to act as a central core or condensing nucleus could therefore be diminished.

In an earlier study Cohen, Harley & Rees (1969) found that the culture fluids (extracellular fluids) of adenovirus type 11 infected, daunorubicin treated HeLa cells were devoid of detectable haemagglutinin. Although these authors did not investigate the intracellular environment, their findings are in accord with those observed here with Ad 5 for which the extracellular fluids were not only devoid of haemagglutinin but other virus components as well.

The failure of daunorubicin-treated cells to release Ad5 components into the extracellular environment is in marked contrast to the behaviour of untreated, infected cells. This could be due to the fact that lower levels of Ad5 components were produced by the drug-treated cells and these did not 'spill-over' or were not released into the extracellular phase. Although the drug is known to act preferentially on the cell nucleus, the ability of the cell to take up eosin stain is markedly altered (Bernard, Paul, Boiron, Jacquillat & Maral, 1969). Release may not have occurred due to decreased cell permeability.

Daunorubicin at a concentration of 0.05 µg/ml prevented (1) the production of mature virus particles and (2) the release of virus components from the Ad 5 infected cell. The apparent absence of toxicity by daunorubicin for HeLa cells at 0.05 µg/ml in contrast to the toxic effect at 0.5 µg/ml suggests that the drug might have a low therapeutic index. Thus it is possible that daunorubicin, judiciously applied, might find clinical use by local application in such adenovirus mediated infections as keratoconjunctivitis and some of the more recently reported manifestations of conjunctivitis.

References


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