Failure of Guanidine and 2-(α-Hydroxybenzyl)benzimidazole to Inhibit Replication of Hepatitis A Virus In vitro

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SUMMARY

Replication of hepatitis A virus (HAV) in the human hepatoma-derived PLC/PRF/5 cell line was neither inhibited in the presence of various concentrations of guanidine or D-2-(α-hydroxybenzyl)benzimidazole (D-HBB), nor were the two chemicals effective in combination. Under identical conditions, however, replication of poliovirus type 1 was inhibited. Tracer experiments with radiolabelled guanidine and D-HBB also furnished no evidence that the two antiviral substances were metabolized gradually to inactive derivatives in PLC/PRF/5 cells. Therefore, it is concluded that resistance to the action of guanidine and D-HBB is an inherent characteristic of HAV. However, the insensitivity of HAV to these drugs does not exclude the virus from the family of picornaviruses.

Hepatitis A virus (HAV) has recently been shown to share many physicochemical characteristics with well-known picornaviruses (Coulepis et al., 1980, 1981; Siegl & Frösner, 1978; Siegl et al., 1981). However, the biological properties of the virus, such as a protracted replication cycle which seems to extend over more than 24 h (G. Siegl et al., unpublished results) and the tendency to establish a persistent rather than lytic infection in most of the available in vitro cell culture systems (Provost & Hilleman, 1979; Frösner et al., 1979; Flehmig, 1980), argue against classification within one of the established genera of the picornavirus family.

Among other characteristics, the sensitivity of HAV to inhibitors like 2-(α-hydroxybenzyl)benzimidazole (HBB) and guanidine might provide valuable information with respect to its definite classification (Eggers & Tamm, 1961 a, b; Caliguiri & Tamm, 1973). Moreover, experimental analysis of virus replication would be greatly facilitated if synthesis of virus RNA could be inhibited by one or both of the two chemicals (Tamm & Eggers, 1963; Sergiescu et al., 1972). To investigate these possibilities, monolayer cultures of the cell line PLC/PRF/5, which has been derived from a human hepatocellular carcinoma (Alexander et al., 1978), were inoculated with HAV strain MBB 11/5 at a multiplicity of infection (m.o.i.) of 10⁻². Strain MBB 11/5 had been adapted to growth in these cells at 32 °C (Frösner et al., 1979; Siegl et al., 1981) and was used both at the third and seventh in vitro passage level. Both the inoculum and the culture medium contained the inhibitory substances at the following concentrations: 1 or 2 mM-guanidine.HCl; 100 or 200 μM-D-(-)-2-α-hydroxybenzyl)benzimidazole.hydrochloride (D-HBB.HCl); 1 mM-guanidine.HCl plus 100 μM-D-HBB.HCl; 2 mM-guanidine.HCl plus 200 μM-D-HBB.HCl. Stock solutions of these supplemented media were stored at +4 °C. Initially, the culture medium was changed every 4 days. In a second series of experiments, however, it was replaced by fresh medium every 24 h to guarantee the presence of a constantly high concentration of the substances. Duplicate cultures were harvested by repeated freezing and thawing at various times as indicated in Fig. 1. The amount of hepatitis A antigen (HAAg) contained in these extracts was determined by radioimmunoassay (RIA), the types of virus particles present in the harvests were determined by sedimentation in sucrose gradients, and infectivity was assayed as described by Siegl et al. (1981). In parallel to these experiments, coverslip cultures of PLC/PRF/5 cells were infected.
Fig. 1. Accumulation of HAAg in PLC/PRF/5 cells infected at an m.o.i. of 10⁻² and incubated in the presence of normal culture medium (O), or medium supplemented with 2 mM-guanidine·HCl (▲), 200 μM-D-HBB·HCl (◇), or 2 mM-guanidine·HCl plus 200 μM-D-HBB·HCl (●). At the times indicated duplicate cultures were harvested by freezing and thawing, and 0.2 ml of cell-free extracts were assayed for HAAg by RIA.

and maintained under identical conditions. These monolayers were fixed with acetone and stained by the indirect fluorescent antibody technique (Siegl et al., 1981) at 2, 5, 10 and 15 days after infection.

Fig. 1 illustrates a typical example of studies in which the accumulation of HAAg in the presence or absence of the inhibitors was monitored over a period of 4 weeks. The respective results indicate that, within the limits of significance, HAV-infected PLC/PRF/5 cell cultures treated with either guanidine or D-HBB synthesized similar quantities of HAAg as did untreated controls. Likewise, the combined addition of both guanidine and D-HBB at concentrations of 1 mM and 100 μM respectively appeared to have no influence on the accumulation of HAAg throughout the same period of observation.

Somewhat lower quantities of HAAg were only found in cultures incubated in the presence of 2 mM-guanidine plus 200 μM-D-HBB, concentrations of compounds apparently at the margin of toxicity for the cells. Sedimentation of virus in sucrose gradients also revealed that the quantitative proportion between the various forms of HAAg, i.e. between 156S virions, 70S to 80S empty capsids, and antigen sedimenting with less than 50S, was essentially the same in extracts of all cultures.

The picture evolving from the assay of virus antigen was fully corroborated by the results of infectivity titrations. For example, 10⁶ TCID₅₀/ml of HAV were present in extracts prepared 21 days after infection from both untreated controls and cultures incubated with 200 μM-D-HBB. In the same set of experiments, cultures treated with 2 mM-guanidine or with 2 mM-guanidine plus 200 μM-D-HBB yielded 10⁵.5 TCID₅₀/ml. In agreement with the apparently uninhibited synthesis of infectious HAV, fluorescent antibody staining revealed a time-dependent but otherwise unrestricted spread of infection within treated and untreated PLC/PRF/5 monolayers. However, infected cultures incubated with guanidine (1 or 2 mM) always seemed to give rise to much brighter intracytoplasmic fluorescence than the respective controls. In cells treated with guanidine plus D-HBB, on the other hand, fluorescence was always of exceptionally fine granular appearance.

It may be concluded from these findings that replication of HAV is resistant to the inhibitory action of guanidine and HBB. The reliability of this observation is emphasized by
Table 1. Inhibitory activity of D-HBB and guanidine on the replication of poliovirus in PLC/PRF/5 cells*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time of harvesting post-inoculation (h)</th>
<th>Virus yield (p.f.u./0.2 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>2</td>
<td>$5.3 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>$2.8 \times 10^8$</td>
</tr>
<tr>
<td>D-HBB. HCl 100 $\mu$M</td>
<td>9</td>
<td>$2.3 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>200 $\mu$M</td>
<td>$1.0 \times 10^6$</td>
</tr>
<tr>
<td>Guanidine. HCl 1 mM</td>
<td>9</td>
<td>$3.0 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td>$1.7 \times 10^5$</td>
</tr>
<tr>
<td>D-HBB. HCl (100 $\mu$M) plus guanidine. HCl (1 mM)</td>
<td>9</td>
<td>$2.2 \times 10^5$</td>
</tr>
</tbody>
</table>

* Single cycle replication of poliovirus 1 (strain Mahoney) in PLC/PRF/5 cells infected with 100 p.f.u./cell. Inhibitors were present from 1 h after virus inoculation throughout the replication period (Eggers & Tamm, 1962).
REFERENCES


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