The Effect of Ethidium Bromide on Lcells and Encephalomyocarditis Virus Replication

By MARIANNE SEMMEL, MARIE-ANGE VERJUS AND J. HUPPERT

Institut Gustave-Roussy, Villejuif (94), France

(Accepted 15 February 1973)

SUMMARY

Ethidium bromide inhibits multiplication of encephalomyocarditis virus and synthesis of infectious single-stranded virus RNA. Infectious double-stranded virus RNA and cellular RNA synthesis are inhibited to a lesser degree. It is suggested that the effect is due mainly to binding of the drug to double-stranded replicative virus RNA.

INTRODUCTION

Ethidium bromide (EB), a phenantridine dye, binds to nucleic acids by intercalation (Le Pecq & Paoletti, 1967). This mode of binding alters the tertiary structure of DNA and in particular that of circular DNA (Waring, 1970). Hence, RNA replication mediated by circular DNA, such as mitochondrial DNA, is specifically inhibited (Zylber, Vesco & Penman, 1969). Nuclear RNA synthesis is also affected: EB stimulates this synthesis at low concentrations and inhibits it at high concentrations (Meyer, Probst & Keller, 1972). The morphology of mitochondria and mitochondrial DNA is modified by EB (Nass, 1970; Smith, Jordan & Vinograd, 1972). In trypanosomes, EB causes dyskinetoplasy and abnormal circular DNA (Riou & Delain, 1969). In Acetabularia, RNA synthesis by mitochondria and chloroplasts is inhibited (Heilporn & Limbosch, 1971). In every case, modification of the tertiary structure of circular DNA seemed to be the primary cause of the specific biological action of EB.

The replication of herpes simplex virus and vaccinia virus is inhibited by EB, provided the drug is added during the initial stages of virus synthesis. The dose necessary to inhibit virus multiplication is considerably higher than that needed to inhibit mitochondrial RNA synthesis (Villagines, 1970a, b). Again, the primary cause of inhibition seems to be binding of EB to DNA.

Low doses of EB inhibit transformation of chick fibroblasts by Rous sarcoma virus but do not inhibit virus production or cell growth, provided the drug is present for a short time only. The primary cause seems to be EB binding to DNA, either virus or mitochondrial (Richert & Hare, 1972).

However, not only DNA, but also RNA can bind EB by intercalation (Bittman, 1969). The less rigid structure of RNA offers fewer binding sites for the dye than DNA and the consequences of this binding are in any case overshadowed by the effect on DNA. In the cell, transcription is primarily inhibited, the inhibition of translation being a secondary effect.

The direct effect of EB binding to RNA should be demonstrable in a system where transcription is mediated by RNA as when RNA viruses multiply. We have shown previously that pyronine, a xanthene related to acridines and capable of intercalation, does act...
in such a way (Semmel & Huppert, 1970). The effect of EB binding would be particularly strong if the virus template RNA were circular, as has been recently claimed for mouse encephalomyocarditis (EMC) virus (Algol et al. 1970). We have investigated the effect of EB on mouse fibroblasts (L cells), either normal or infected with EMC virus, on EMC virus multiplication and on cellular and virus RNA synthesis.

METHODS

Reagents and solutions. EB was a gift from Boots Company (Nottingham). The dye was dissolved in 0.14 M-NaCl at a concentration of 3 mg/ml, sterilized by autoclaving and stored at 4 °C in the dark. Actinomycin D was a gift from Merck, Sharpe and Dohme. [5-3H]-uridine (specific activity 17 Ci/m-μmol) from C. E. A. Saclay was used. DEAE-dextran came from Pharmacia. Other reagents were Merck purest grade products. Extinction coefficients were measured with a Zeiss spectrophotometer, model M4QIII. Radioactivity was measured with a Beckman scintillation counter.

Virus and cells. L cells were grown in Roux glass bottles, Falcon plastic bottles or dishes, in Eagle’s medium supplemented with 10% calf serum and 10% tryptose phosphate broth. If the cells were to be labelled with [5-3H]-uridine, the medium was not supplemented with tryptose phosphate broth. For infection, stock EMC virus with a titre of 10⁹ p.f.u./ml was diluted in phosphate-buffered saline (Dulbecco & Vogt, 1954) to an input multiplicity of 3 p.f.u./cell and left in contact with the washed monolayer for 1 h at 4 °C in order to synchronize virus absorption. The monolayers were then incubated in Eagle’s medium at 37 °C. Samples of either whole cell suspensions or supernatant fluids were stored in sealed ampoules at −70 °C. Plating of virus was done by incubating a monolayer with virus suspension for 45 min at 37 °C, after which time the medium was replaced by an agar overlay. Infectivity of the RNA preparations was determined in cells pretreated with DEAE-dextran (Pagano & Vaheri, 1965). Monolayers were incubated with appropriate dilutions of RNA for 15 min at 37 °C, then the medium was replaced by an agar-overlay.

For determination of cell growth, 5 ml of a cell suspension containing 10⁵ cells/ml were seeded in plastic dishes and incubated at 37 °C. Every 24 h, the cells of two duplicate dishes were trypsinized and counted.

Labelling, RNA extraction and sucrose gradients. Incorporation of [5-3H]-uridine into RNA was determined after precipitation of whole cells with perchloric acid (Blade & Harel, 1968). For qualitative analysis, the RNA was extracted from cells, disrupted by freezing and thawing, lysed with sodium dodecyl sulphate and deproteinized with cold phenol (Montagnier & Sanders, 1963). Single-stranded RNA was separated from double-stranded RNA by precipitation with MgCl₂ (Harel et al. 1964), and dialysed against two changes of NaCl, 10⁻¹ M, tris, 10⁻² M, EDTA, 10⁻³ M, pH 7.2 (NTE). MgCl₂ precipitates ribosomal and virus single-stranded RNA, while transfer and virus double-stranded RNA remain in the supernatant fluid. Sucrose gradients (5 to 20% sucrose in NTE) were centrifuged in a Spinco ultracentrifuge (model L50 or model L4) in either a SW39 or a SW65 rotor. The maximum vol. of RNA samples layered over the gradient was 0.2 ml. At the end of the run, fractions were collected by piercing the bottom of the tube. Fractions were dried on Whatman glass fibre filters and counted in scintillation liquid. In order to distinguish double-stranded and single-stranded virus RNA after sedimentation, total RNA was layered on the gradient, fractions collected as above and diluted in SSC, 1.5 M. Each fraction was separated into two equal parts. One part was precipitated with 5% trichloracetic acid in the presence of 100 μg yeast RNA, the second part was treated with 10 μg RNase/ml for
Fig. 1. Effect of EB and/or EMC virus on the growth of L cells. L cells were grown on Petri dishes as described in Methods. Cells were harvested and counted on four plates each day. A: 30 μg EB/ml present for 4 h. B: 30 μg EB added 1.5 h after infection and permanently present in the medium. ○—○, controls; ●—●, EB treated; △—△, EMC virus and EB; ▲—▲, EMC virus.

15 min at 37 °C and then precipitated. The precipitates were collected on Whatman glass fibre filters which were then washed, dried and counted in scintillation liquid in a Beckman scintillation counter.

Chemical determinations. Protein was determined by the method of Lowry (Lowry et al. 1951).

RNA content was determined by optical density measurements. One μg RNA/ml was equated with an $E_{260}$ of 0.025. All RNA extracted showed ratios of $E_{260}/E_{280}$ and $E_{280}/E_{230}$ between 1.9 and 2.2.

RESULTS

Growth of L cells in the presence of EB

When L cells were incubated with 30 μg EB/ml for 6 h, only 1 to 2 μg EB/ml were bound by the cells: plastic dishes containing either monolayers ($10^6$ cells/ml) or no cells were incubated at 37 °C. When the dishes contained cells, the $E_{480}$ of the medium after incubation
Fig. 2. Morphology of L cells after 21 h incubation with 30 μg EB/ml and/or EMC virus. L cells were grown in Petri dishes, EB was added 1.5 h after infection. Photographs were taken of the dish under a phase contrast microscope (magnification × 750). A, controls; B, treated; C, infected with EMC virus; D, infected with EMC virus and treated with EB.
Ethidium bromide, L-cells and EMC virus

Fig. 3. Effect of EB concentration on EMC virus multiplication. EB was added 1.5 h after infection, supernatant was harvested 6 h after infection and p.f.u. assayed as described in Methods.

Fig. 4. Multiplication of EMC virus in the presence of 30 μg EB/ml added at different times of the multiplication cycle. Supernatant was harvested after 7 h incubation and p.f.u. assayed as described in Methods.

was 0.421 ± 0.006 and that of medium from empty dishes was 0.441 ± 0.005 (8 duplicates). The difference, i.e. $E_{480} = 0.020$, corresponds to 1.36 μg EB/ml.

When L cells were incubated for 4 h with 30 μg EB/ml and the medium was changed after this time, the cell number remained stationary for 24 h; afterwards, the cells resumed growth and attained almost the number of control cells (Fig. 1 A). When infected cells were treated for 4 h with 30 μg EB/ml, and the medium was changed after this time, the cells were found to be destroyed 24 h later. When the cells were permanently left in contact with
EB, their number declined (Fig. 1B). Morphologically, cells treated for 24 h with EB resemble control cells (Fig. 2). L cells infected with EMC virus for 24 h are destroyed; most cells were detached from the plastic and the remaining cells were round (Fig. 2C). When the infected cells were treated with EB 90 min after infection, and examined, they resembled, after 24 h, control cells and their number was half that of non-infected EB-treated cells (Fig. 2D). At later times, the number of EB-treated infected cells decreased faster than that of EB-treated uninfected cells.

**Effect of EB on EMC virus multiplication**

When infected cultures were incubated with EB the release of infectious EMC virus as measured by the p.f.u. in the supernatant fluid 6 h after infection decreased proportionately to the amount of EB in the medium (Fig. 3).

EB had no direct effect on EMC virus. When virus was incubated for 6 h at 37 °C with 30 µg EB/ml, no decrease of infectivity was observed.

The effect of EB on virus multiplication depended on the time relative to infection at which the dye was added. Fig. 4 illustrates this point: the decrease of virus production was greatest when 30 µg EB/ml was added 1 to 2 h after infection, that is, before virus RNA synthesis was maximal. At later stages of virus replication, the effect of adding the drug was less and after 5 h nil. In the presence of EB, no new virus is produced during the first 6 h of infection and only a very small amount at later times (Fig. 5). Figs. 4 and 5 show that neither virus absorption nor virus release are primarily influenced by EB, but that the replication of the virus is affected.
Ethidium bromide, L-cells and EMC virus

Table 1. Incorporation of [5-^H]-uridine into L cells treated with EB and/or EMC virus

<table>
<thead>
<tr>
<th>Time of addition of [H]-uridine</th>
<th>Treatment</th>
<th>Ct/min/mg protein (± 5%)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>None</td>
<td>18 866</td>
<td>100</td>
</tr>
<tr>
<td>10 μCi[5H]-uridine/ml</td>
<td>+ 30 μg EB/ml</td>
<td>3 940</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>+ EMC virus</td>
<td>14 975</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>+ EB + EMC</td>
<td>3 563</td>
<td>18</td>
</tr>
<tr>
<td>21 h</td>
<td>None</td>
<td>12 639</td>
<td>100</td>
</tr>
<tr>
<td>10 μCi[5H]-uridine/ml</td>
<td>+ 30 μg EB/ml</td>
<td>5 901</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>+ EMC virus</td>
<td>4</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>+ EB + EMC</td>
<td>5 065</td>
<td>40</td>
</tr>
<tr>
<td>3 h</td>
<td>None</td>
<td>2 064</td>
<td>100</td>
</tr>
<tr>
<td>1 μCi[5H]-uridine/ml</td>
<td>+ 5 μg AMD*/ml + EB</td>
<td>20</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>+ 5 μg AMD/ml + EMC</td>
<td>120</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>+ 5 μg AMD/ml + EMC + EB</td>
<td>15</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* AMD = actinomycin D.

RNA synthesis in cells in the presence of EB and/or EMC virus RNA

RNA synthesis decreased in cells treated with EB and/or infected with EMC virus. In EB-treated cells, whether normal or infected, some RNA synthesis continued for 24 h, whereas in infected and untreated cells RNA synthesis had ceased 21 h after infection.

Actinomycin D (AMD) was used to distinguish cellular from virus RNA synthesis. Five μg AMD/ml did not affect EMC virus multiplication, but suppressed cellular RNA synthesis (Reich et al. 1962). When infected cells were grown in the presence of AMD, RNA was produced. When EB was added, this synthesis decreased, indicating that EB inhibits virus RNA synthesis (Table 1).

Fig. 6 shows the sedimentation pattern of cellular and virus RNA extracted from cells treated with EB and/or EMC virus. Infected cells were treated with AMD.

In Fig. 6A RNA extracted from control cells is compared with RNA extracted from cells treated with EB for 7.5 h: a shift towards the slowly sedimenting RNA species is taking place. When EB was present for 24 h, the label was essentially in the slowly sedimenting fractions (Fig. 6B). The effect of EB is reversible. When EB was removed after 3 h, synthesis of the major RNA species was resumed (Fig. 6B).

EB severely inhibited synthesis of fast sedimenting single-stranded virus RNA: most of the newly synthesized RNA sedimented slowly (Fig. 6C). None of the RNA from EB-treated cells is infectious (Table 2). When EB was removed from the medium, synthesis of infectious single-stranded virus RNA was resumed (Fig. 6C). Double-stranded virus RNA synthesis was inhibited by EB though to a lesser degree as some of the infectivity of the RNA remained (Table 2). Most of this RNA does not sediment in a peak at 18s (Fig. 6D). When virus RNA extracted from EB treated cells was assayed for RNase-resistance, indicating the proportion of ds RNA, this RNA was found to be present in all fractions, indicating that it had remained in the replicative intermediate form (Figs. 6E, F). Precipitation of replicative intermediate form by MgCl₂ depends on the lengths of the single strands attached to the double strands. If there are few or only short pieces of single strands, the intermediate form remains in the supernatant fraction.
Fig. 6A–E. For legend see facing page.
Ethidium bromide, L-cells and EMC virus

Fig. 6. Sedimentation patterns of cellular and virus RNAs synthesized in the presence of EB. In order to obtain comparable patterns at equal concentrations of RNA, specific radioactivity was adjusted by diluting highly radioactive samples (controls) with unlabelled carrier RNA.

A. Total RNA extracted from cells labelled with 10 µCi/ml [3H]-uridine for 7.5 h. ———, control; ———, cells treated with 30 µg EB/ml 1.5 h prior to and during labelling.

B. Total RNA extracted from cells labelled with 10 µCi [3H]-uridine/ml for 21 h. ———, cells treated for 3 h with 30 µg EB/ml, labelled after removal of EB; ———, cells treated with 30 µg EB/ml for 3 h prior to and during labelling.

C. MgCl₂-precipitable RNA extracted from cells infected with EMC virus and treated with 5 µg actinomycin D/ml for 4 h. ———–, cells labelled with 10 µCi [3H]-uridine/ml from 3 to 5 h after infection; ———, cells treated with 30 µg EB/ml from 1.5 h prior to and during labelling. Labelled with 10 µCi [3H]-uridine/ml from 3 to 5 h after infection; ———, cells treated with 30 µg EB/ml from 1.5 to 3 h after infection. EB was then removed, and the cells labelled with 10 µCi [3H]-uridine from 3 to 6.5 h after infection.

D. MgCl₂-soluble RNA extracted from cells infected with EMC virus and treated with 5 µg actinomycin D/ml. ———–, cells labelled with 10 µCi [3H]-uridine from 3 to 5 h after infection (left scale); ———, cells treated with 30 µg EB/ml from 1.5 h prior to and during labelling. Labelled with 10 µCi [3H]-uridine/ml from 3 to 5 h after infection (left scale); ————, Cells treated with 30 µg EB/ml from 1.5 to 3 h after infection EB removed. Labelled with 10 µCi [3H]-uridine from 3 to 6.5 h after infection (left scale).

E. RNA extracted from cells infected with EMC virus and treated with 5 µg actinomycin D/ml, labelled with 10 µCi [3H]-uridine from 3 to 5 h after infection. ++++, total RNA after TCA precipitation (left scale); Δ—Δ, RNA after RNAase treatment and TCA-precipitation (right scale).

F. RNA extracted from cells infected with EMC virus, treated with 30 µg EB/ml. Treated with 30 µg EB/ml from 1.5 to 5 h after infection. Labelled with 10 µCi [3H]-uridine/ml from 3 to 5 h after infection. ++, total RNA after TCA-precipitation (left scale); Δ—Δ, RNA after RNAase treatment and TCA-precipitation (right scale).

Synthesis of ds RNA was resumed when EB was removed from the medium (Fig. 6D). Transfer RNA synthesis continues in controls and EB-treated cells.

DISCUSSION

Inhibition of cell growth by EB has been shown to be preceded by alterations of nucleic acid metabolism (Smith et al. 1972). When the drug is removed, the cells recover. When EB concentration is low, recovery of cells can take place after longer exposure to the drug than when it is high (Nass, 1970).

In this report, we have shown that at a concentration of 30 µg EB/ml, 1 to 2 µg/ml were bound by the cells during the first 6 h. During this time, the cells were capable of synthesizing...
Table 2. Infectivity and synthesis of single and double stranded RNA

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Ct/min/100 µg RNA</th>
<th>%</th>
<th>p.f.u./100 µg RNA</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl2-precipitable RNA (ribosomal and virus single-stranded RNA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (5 h incubated, labelled from 3 to 5 h). (344 µg RNA/5 × 10⁷ cells)</td>
<td>880 650</td>
<td>100</td>
<td>964</td>
<td>100</td>
</tr>
<tr>
<td>EB-treated (5 h incubated, labelled from 3 to 5 h). (480 µg RNA/5 × 10⁷ cells)</td>
<td>34 650</td>
<td>3'9</td>
<td>0</td>
<td>&lt;0'2*</td>
</tr>
<tr>
<td>EB-treated for 3 h (EB removed, labelled from 3 to 6'5 h) (344 µg RNA/5 × 10⁷ cells)</td>
<td>329 100</td>
<td>37</td>
<td>1 000</td>
<td>103</td>
</tr>
<tr>
<td>MgCl2-soluble RNA (transfer and virus double-stranded RNA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (5 h incubated, labelled from 3 to 5 h). (44 µg RNA/5 × 10⁷ cells)</td>
<td>1 079 000</td>
<td>100</td>
<td>3 700</td>
<td>100</td>
</tr>
<tr>
<td>EB-treated (5 h incubated, labelled from 3 to 5 h). (32 µg RNA/5 × 10⁷ cells)</td>
<td>1 070 000</td>
<td>99</td>
<td>560</td>
<td>15</td>
</tr>
<tr>
<td>EB-treated for 3 h (EB removed, labelled from 3 to 6'5 h). (30 µg RNA/5 × 10⁷ cells)</td>
<td>3 725 700</td>
<td>345</td>
<td>6 800</td>
<td>183</td>
</tr>
</tbody>
</table>

* less than 2 p.f.u./100 µg RNA could not be detected.

the major RNA species, though in reduced amounts and in abnormal proportions, with a shift towards slowly sedimenting components. RNA synthesis and cellular growth were resumed if EB was removed after this time. When EB was present for 24 h, at least 50% of the cells remained attached to the culture dish and could synthesize RNA, though this RNA was abnormal. Under the same conditions, EMC virus multiplication was completely suppressed as was synthesis of infectious single-stranded virus RNA. However, some infectious double-stranded virus RNA was produced in the presence of EB. EB-treated infected cells retained an apparently normal aspect, whereas without EB practically all cells were destroyed by the virus. After removal of EB from the medium, synthesis of virus RNA and virus multiplication were resumed.

It appears from our results that virus replication is blocked mainly at the level of transcription of ss RNA from ds RNA and is not only a consequence of the effect of EB on the host cell. The synthesis of infectious ds RNA decreases by 85%, that of infectious ss RNA by at least 99-8% (Table 2). 80% of host cell RNA synthesis, as evaluated by the incorporation of [3H]-uridine, is inhibited (Table 1), and the major RNA species are produced, though in abnormal proportions (Fig. 6A). If EB affected the synthesis of all cellular and virus RNA species in the same way, one would expect the synthesis of at least some infectious virus RNA.

Our results may be interpreted in the following way: part of the EB present in the medium penetrates into the cells. There it binds to mitochondrial DNA, virus replicative ds RNA and nuclear DNA. When bound it causes alterations of the tertiary structure of the nucleic acids, which affect the template efficiency of the nucleic acids. If the nucleic acid is circular, for instances mitochondrial DNA (Zylber et al. 1969), the alterations will have more severe biological consequences than when the drug is bound by linear nucleic acids.

The sensitivity of EMC virus multiplication to EB would be in favour of a circular form of its nucleic acid (Algol et al. 1970).

However, EB could be intercalated and bound if the replicative form of the virus RNA
Ethidium bromide, L-cells and EMC virus

were a double-stranded structure maintained by hydrogen bonds as postulated by Montagnier and Sanders (Montagnier & Sanders, 1963). This would seem less likely if EMC virus replication followed the model proposed by Borst and Weissmann (Borst & Weissmann, 1965) for MS2 phage, that is the apposition of the plus and minus strand virus RNA maintained by proteins.

Both the structure and the composition of the nucleic acid are probably important: less EB is needed to inhibit mitochondrial RNA synthesis mediated by circular DNA than EMC virus ss RNA synthesis. Though the main cause of inhibition seems to be binding of EB by nucleic acids, it is possible that secondary effects arise simultaneously through EB binding to different cellular components and by binding modes other than intercalation.

This work has been supported by a grant from the D.G.R.S.T. ‘Action Concertée Biologie Moléculaire’ Contrat No. 6600292 and ‘Ligue Française contre le Cancer’.

REFERENCES


(Received 24 September 1972)