Enhancement of Infectivity of Encephalomyocarditis Virus RNA by Amphotericin B Methyl Ester

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SUMMARY

The methyl ester of amphotericin B (AmBME), a macrolide polyene antibiotic, enhanced the infectivity of encephalomyocarditis (EMC) virus RNA for L929 cells. AmBME alone (100 µg/ml) resulted in increases in EMC virus RNA infectivity of 10- to 100-fold. Addition of DEAE dextran at concentrations (5 µg/ml), which alone slightly suppressed EMC virus RNA infectivity, further augmented the effects of AmBME (augmentation in infectivity up to 750-fold). AmBME did not inhibit RNase, did not enhance EMC virus infectivity and increased infectivity of EMC virus RNA which was already cell-associated. The polyenes are probably acting by increasing intracellular penetration of polyribonucleotides.

INTRODUCTION

Macrolide polyene antibiotics, such as amphotericin B and its methyl ester, nystatin, and filipin, interact with sterols in eukaryotic cells (Kinsky, 1970; Andreoli, 1973; Hamilton-Miller, 1974). Morphological pits (pores) develop in fungal and vertebrate cell membranes, liposomes and even virus envelopes after polyene treatment (Kinsky, 1970; Finkelstein & Holz, 1973; Dekruijff & Demel, 1974; Hamilton-Miller, 1974; Majuk et al. 1977). Substantial alterations in membrane permeability, which favour the movement of anionic compounds, also result. These permeability changes, which are probably the primary basis for antifungal action (Hamilton-Miller, 1974), also occur in mammalian cells (Morton & Lardy, 1967; Kinsky, 1970; Andreoli, 1973; Finkelstein & Holz, 1973). Augmentation of effects of antitumour agents and inhibitors of macromolecular synthesis has resulted from concomitant treatment of cells with polyenes; these changes have been attributed to enhanced intracellular penetration of the metabolic inhibitors (Medoff et al. 1973a, b, c). We have reported enhancement of the biological effects of polyriboinosinic-polyribocytidyllic acid [poly(rI).poly(rC)], including interferon production and cell cytotoxicity, from treatment of L929 cells with polyenes (Borden & Leonhardt, 1976; Borden, 1978). Mechanistic studies suggested that the polyenes enhanced cell penetration by the macromolecular, synthetic polynucleotide (Borden et al. 1977); increased availability of a specific membrane binding site for interferon induction by poly(rI).poly(rC) could not, however, be eliminated. To clarify these observations further and to determine whether polyenes increase the bio-

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logical effects of a natural polynucleotide, which must penetrate the cell to become active, the effect of amphotericin B methyl ester (AmBME) on the infectivity of encephalomyocarditis (EMC) virus RNA was investigated.

METHODS

Virus RNA. EMC virus (obtained from Roland R. Rueckert) was propagated in L929 mouse fibroblasts (ATCC-CCL1), which were grown in roller bottles in minimal essential medium (MEM; Gibco Biologicals, Grand Island, New York), supplemented with 10% foetal calf serum, 2 mM-glutamine and 50 μg/ml gentamicin (Schering Diagnostics, Port Reading, New Jersey). Cells were infected with EMC virus at an approximate multiplicity of 5. Growth medium was added at 1 h and cells were harvested by trypsinization at 7 h. Cell-associated virus was released by repeated freeze-thawing, followed by centrifugation at 10000 g for 10 min and passage through a 0.45 μm filter. RNA was extracted from these virus preparations using a cold phenol technique (Bachrach, 1960). The virus was diluted into 0.02 M-phosphate buffer (pH 7.6), which contained 0.1% sodium dodecylsulphate and 0.10% disodium ethylenediamine tetra-acetic acid (EDTA). This material was extracted with an equal volume of phenol saturated with aqueous 0.10% EDTA. Phenol was removed from the aqueous phase by extracting five times with peroxide free diethyl ether. Residual ether was removed by vacuum evaporation at 4°C and samples of RNA solution dispensed into siliconized glass ampoules for storage in liquid nitrogen. Infectivity of the RNA preparation was markedly sensitive to pancreatic RNase (PL Biochemicals, Milwaukee, Wisconsin). RNase digestion was performed at 37°C for 10 min in Dulbecco’s phosphate buffered saline without calcium or magnesium (pH 7.3). The ratio of absorbance of the preparation at 260 nm to 280 nm in a Beckman model 25 scanning spectrophotometer was 2.2 to 1.

Effects of AmBME. AmBME was a gift, kindly provided by E. R. Squibb and Sons, Princeton, New Jersey. It was partially solubilized by grinding the powder in a minimal amount of Earle’s balanced salt solution (EBSS) and diluted to the desired concentration. Confluent L929 cell monolayers, grown in 60 × 15 mm dishes (Linbro Chemical Co., New Haven, Connecticut), were washed three times with EBSS and inoculated with equivolume mixtures of appropriate concentrations of AmBME in EBSS and EMC virus or its RNA in Dulbecco’s phosphate buffered saline without calcium or magnesium (DPBS). DEAE dextran (Sigma) when added, was diluted into DPBS. Virus RNA and drugs were all kept at 4°C before being added to cells. After 1 h at 37°C, 4 ml of MEM was added to each well and the cells incubated for another hour. After washing again, 2 × MEM with 2% FCS and agarose were added. Following incubation for 30 to 36 h, a neutral red liquid overlay was added and plaques counted. Each experimental point was assayed in triplicate or quadruplicate and mean plaque counts determined. All results represent findings from several experiments.

RESULTS

AmBME potentiated the infectivity of EMC virus RNA for L929 cells both in the presence and absence of DEAE dextran (Table 1). AmBME (100 μg/ml) alone enhanced infectivity 25-fold. Addition of DEAE dextran further augmented the effects of AmBME (750-fold increase in infectivity) (Table 1). The degree of enhancement of EMC virus RNA infectivity by AmBME (100 μg/ml) in the presence of DEAE dextran was related to the concentration of DEAE dextran (Fig. 1). AmBME augmented infectivity at all concentrations of DEAE dextran from 0.2 μg/ml to more than 1000 μg/ml. However, the greatest
Table 1. Effect of amphotericin B methyl ester on infectivity of EMC virus and its RNA

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Infectivity (p.f.u./ml)</th>
<th>AmBME (μg/ml)</th>
<th>DEAE dextran (μg/ml)</th>
<th>EMC virus</th>
<th>EMC virus RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>1.5 × 10⁹</td>
<td>4.0 × 10³</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>0</td>
<td>5</td>
<td>1.3 × 10⁹</td>
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<tr>
<td>100</td>
<td></td>
<td>5</td>
<td>5</td>
<td>1.4 × 10⁹</td>
<td>4.0 × 10⁵</td>
</tr>
</tbody>
</table>

* Cells were treated concomitantly with EMC virus or its RNA. After 60 min, cells were washed three times, overlaid with agarose and plaques enumerated at 36 h.

Augmentation in infectivity by AmBME occurred at DEAE dextran concentrations of less than 5 μg/ml. At maximal DEAE dextran concentrations only a small augmentation in infectivity was observed. DEAE dextran alone at low concentrations slightly decreased (2 to 5 times) infectivity of the EMC virus RNA; AmBME completely overcame this inhibition in infectivity (Fig. 1). In contrast to its effects on the infectivity of EMC virus RNA, AmBME, either alone or with DEAE dextran, had no effects on the infectivity of intact EMC virus (Table 1). At a DEAE dextran concentration of 50 μg/ml, EMC virus RNA infectivity increased with increasing concentrations of AmBME (Fig. 2). Enhancement in infectivity was noted at AmBME concentrations as low as 20 μg/ml. Maximal augmentation of infectivity occurred at approx. 100 μg/ml of AmBME. Amphotericin B also potentiated EMC virus RNA infectivity but not as effectively as AmBME, perhaps because of lower solubility or greater cytotoxicity.

Further experiments were undertaken to elucidate the possible mechanisms for the enhancement in virus RNA infectivity by AmBME. AmBME and EMC virus RNA did not need to be present simultaneously to enhance virus RNA infectivity. After 30 min absorp-
Fig. 2. Effect of varying AmBME on infectivity of EMC virus RNA (p.f.u.). Confluent L929 cell monolayers were washed with EBSS and inoculated with a 1:1 mixture of appropriate concentrations of AmBME and EMC virus RNA containing DEAE dextran (50 μg/ml). After 1 h at 37 °C, cells were again washed, an agarose overlay added, and virus plaques enumerated at 36 h.

Table 2. Effect of separate addition of AmBME on EMC virus RNA infectivity

<table>
<thead>
<tr>
<th>Time of treatment (min) with *</th>
<th>AmBME (100 μg/ml)</th>
<th>DEAE dextran (50 μg/ml)</th>
<th>P.f.u./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-30</td>
<td>7.5 × 10³</td>
<td></td>
<td>7.5 × 10³</td>
</tr>
<tr>
<td>30-60</td>
<td>9.5 × 10³</td>
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<td>9.5 × 10³</td>
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<tr>
<td>0-30</td>
<td>5.5 × 10⁴</td>
<td></td>
<td>5.5 × 10⁴</td>
</tr>
<tr>
<td>30-60</td>
<td>8.5 × 10⁴</td>
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<td>8.5 × 10⁴</td>
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<tr>
<td>0-30</td>
<td>1.3 × 10⁵</td>
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</tr>
<tr>
<td>30-60</td>
<td>3.9 × 10⁶</td>
<td></td>
<td>3.9 × 10⁶</td>
</tr>
</tbody>
</table>

* L929 cells infected with EMC virus RNA from 0 to 30 min (37 °C) and other additions at indicated times.
Table 3. Effect of amphotericin B methyl ester on RNase function*

<table>
<thead>
<tr>
<th>RNase (µg/ml)</th>
<th>AmBME (µg/ml)</th>
<th>DEAE dextran (µg/ml)</th>
<th>EMC virus (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8 × 10^4</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>3.5 × 10^5</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>500</td>
<td>9.9 × 10^4</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>500</td>
<td>8.1 × 10^4</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
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<td>2.7 × 10^5</td>
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<tr>
<td>2</td>
<td>100</td>
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<td>2.5 × 10^5</td>
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<tr>
<td>10</td>
<td>0</td>
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</tr>
<tr>
<td>10</td>
<td>100</td>
<td>500</td>
<td>&lt;5 × 10^5</td>
</tr>
</tbody>
</table>

* Varying dilutions of EMC virus RNA were treated with chromatographically purified bovine pancreatic RNase (PL Biochemicals, Milwaukee, WI) in Dulbecco’s PBS (pH 7.3; without Ca^2+ or Mg^2+) at 37 °C for 10 min both in the presence and absence of AmBME. An equal volume of DEAE dextran (1 mg/ml in EBSS, 4 °C) was then added and EMC virus RNA was added to washed L929 cell monolayers.

Membrane permeability alterations, induced by the macrolide polyenes, probably result from the binding of polyenes to membrane sterols (Kinsky, 1970; Andreoli, 1973; Finkelstein & Holz, 1973). Conceptual models for the membrane interaction of polyenes, based on their amphipathic nature, have been proposed (Andreoli, 1973; DeKruijff & Demel, 1974). These permeability alterations may be the basis for the synergistic effects of amphotericin B and actinomycin D, tetracycline and rifamycin derivatives on RNA and protein synthesis which resulted in L929, transformed 3T3 and HeLa cells (Medoff et al. 1973a, b, c). These effects occurred at concentrations of amphotericin B which by themselves were not cytotoxic.

The polyene macrolides enhanced the biological activity of both natural (EMC virus RNA) and synthetic [poly(rI).poly(rC)] polyribonucleotides in L929 cells (Table 1; Borden & Leonhardt, 1976; Borden et al. 1977; Borden, 1978). Polyamines, calcium and polycations such as DEAE dextran have also been used to enhance the infectivity of virus nucleic acids and the production of interferon by poly(rI).poly(rC) (Dubes & Klinger, 1961; Pagano et al. 1967; Dianzani et al. 1968; McCutchan & Pagano, 1968; Lampson et al. 1969; Billiau et al. 1970; Graham & Van der Eb, 1973; Booth & Borden, 1978). The mechanism by which these polybasic substances potentiate the action of nucleic acids remains uncertain; increased cell absorption and protection from nucleases may both contribute. Since interferon production was maximal when an electroneutral complex was formed by the anion, poly(rI).poly(rC) and the cation, DEAE dextran (Pitha & Carter, 1971), it may also be that DEAE dextran enhances the biological effects of polynucleotides by increasing transport through the hydrophobic membrane interior. The enhancement in biological effects of polynucleotides by polyenes occurred in the absence of any added DEAE dextran but augmented the effect of DEAE dextran when present.

Our previous observations of the mechanism of enhancement of the biological effects of
poly(rI).poly(rC) (Borden et al. 1977) and the results reported here strongly suggest that polyenes augment the biological effects of polyribonucleotides by increasing their intracellular penetration. Since neither virus-induced interferon production nor the infectivity of EMC virus was enhanced, the effects of polyenes on biologically active polynucleotide function is probably at a step prior to virus uncoating. No direct interaction of the polyenes with RNA could be demonstrated. Polyenes did not increase cell-binding of radiolabelled poly(rI).poly(rC) and augmented the activity of both poly(rI).poly(rC) and EMC virus RNA which were already cell associated. They did not inhibit pancreatic RNase activity. Thus, alterations in cell membrane penetration seem the most likely basis for our findings. Whether the changes in membrane permeability result from development of defined pores, increased endocytosis, or another mechanism remains to be determined. Membrane penetration may prove a limiting step for interferon production by poly(rI).poly(rC) and for genetic information transfer between cells by nucleic acids.

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Polyenes and EMC Virus RNA


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