STIMULATORY EFFECT OF AMPHOTERICIN B METHYL ESTER ON THE GROWTH OF L-M AND VERO CELLS

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The chemically modified polyene macrolide antibiotic, amphotericin B methyl ester (AME), exhibited a concentration-dependent growth stimulatory effect on established lines of mouse (L-M) and monkey (Vero) cells. Stimulation was indicated by increases in growth rate, and in the enhanced synthesis of DNA and RNA. In contrast, the parental antibiotic amphotericin B and the desoxycholate complex of amphotericin B, Fungizone^{R}, did not elicit a similar proliferative response in L-M or Vero cells. AME was not growth-promoting toward low passage strains of mouse (PMK 6) and monkey cells (GMK 8).

Amphotericin B methyl ester (AME) has been shown to retain the antifungal properties of its parent compound amphotericin B (AB).[1,2] In addition, studies using mice[1,3] and dogs[3] have demonstrated that AME is substantially less toxic than AB when administered intravenously. Tissue culture studies, involving six cell lines of mammalian origin have shown that AME is less toxic than either AB or the desoxycholate complex of AB, Fungizone^{R} (E. R. Squibb & Sons)[4]. In the present experiments, we report a concentration-dependent stimulatory effect of AME on the growth of mouse (L-M) and monkey (Vero) cells.

Materials and Methods

Cell Viability Studies:

L-M cells, derived from normal mouse areolar and adipose tissue, were grown at 37°C in complete growth medium which consisted of DULBECCO's modified EAGLE's minimum essential medium (DMEM) supplemented with 10% uninactivated fetal calf serum (FCS), non-essential amino acids (NEAA), L-glutamine and 50μg/ml of gentamicin (Microbiological Associates). The L-M cell line was kindly supplied by Dr. ROBERT J. KUCHLER, Microbiology Dept., Rutgers University, New Brunswick, N.J. Vero cells, derived from the kidney of an African Green monkey, were grown at 37°C in the same medium as L-M cells. This cell line was a gift from Dr. CLAIRE G. ENGLE, Ciba-Geigy Corporation, Summit, N. J.

Primary mouse kidney cells (PMK) obtained from Swiss mice (approximately 3 weeks old) and primary monkey kidney cells (GMK), derived from a 2~3 kg African Green monkey, were purchased from Flow Laboratories, Rockville, Md. PMK and GMK cells were grown in the same medium and at the same temperature as L-M cells and were studied after passage 6 (PMK-6) or passage 8 (GMK-8).

Fixed volumes of cells, $1\sim5\times10^{5}$ cells, in 1 ml of medium were inoculated into Leighton tubes or 35×10 mm tissue culture plates, followed by a single administration of a 10μl aliquot

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of each polyene macrolide antibiotic, yielding final concentrations of 1~10μg/ml AB, 1~20 μg/ml Fungizone^{R} (FZ), the desoxycholate complex of AB, or 1~400μg/ml AME. Studies were performed in duplicate, a minimum of three times. AB and FZ were kindly supplied by E.R. Squibb & Sons, New Brunswick, N.J. AME was synthesized in our laboratory[5]. FZ was dissolved in sterile distilled water, while AB and AME were dissolved in dimethyl sulfoxide (DMSO). Control cultures received no drug treatment or 10μl DMSO. Viable cell counts were performed on days 0, 1, 3 and 5 using the trypan blue dye exclusion technique and a hemocytometer. Relative TCD_{50}'s (the concentration of the polyene macrolide resulting in approximately 50% reduction in viable cell number after a 5-day test period, as opposed to control cultures after the same period) were determined for each of the three polyene macrolide antibiotic preparations. Residual polyene macrolide levels were monitored by spectrophotometric measurements.

Determination of DNA Synthesis in the Presence of AME:

Monolayer cells in the logarithmic stage of growth were removed by trypsin and resuspended in complete growth medium containing 0.5μCi/ml of [^{3}H] thymidine (specific activity, 20 Ci/mM, New England Nuclear). One ml aliquots of the final cell suspension, 2.5~3.0$\times 10^{5}$ cells/ml, were dispensed into tissue cultures plates (35$\times$10 mm, Microbiological Associates). Two sets of triplicate controls were established with one receiving no additions and the other receiving 10μl DMSO. Triplicate samples received 40, 80, 120, 160 or 200μg/ml of AME (final concentration/ml) in 10μl of DMSO. All cultures were then incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. Media was removed and the cell sheets were washed twice with cold (4°C) DULBECCO's phosphate buffered saline (PBS, Microbiological Associates). Acid-insoluble material was precipitated by the addition of 0.5 ml of ice-cold (4°C) 10% trichloroacetic acid (TCA). The cell sheets were then manually scraped with a rubber policeman and collected on scintillation pads (Whatman 3MM, Arthur H. Thomas Co.) by vacuum filtration. The tissue culture plates and scintillation pads were washed 5 times with ice-cold 5% TCA. Precipitates were dried at 70°C for 1 hour and counted in BBOT cocktail in Toluene : Triton$\times$100 (3:1, volume/volume) on a Packard Tricarb liquid scintillation spectrophotometer (Model 3003, Packard Instrument Co.).

Determination of RNA Synthesis in the Presence of AME:

Cells in the logarithmic stage of growth were removed with trypsin, resuspended in complete growth medium and dispensed in 1 ml aliquots (2.5~3.0$\times 10^{5}$ cells/ml) into 35$\times$10 mm plastic tissue culture dishes. Cells were incubated for 21 hours at 37°C at which time the 1 ml of medium was replaced with an equivalent amount of complete growth medium containing 0.5μCi/ml of [^{3}H] uridine (25 Ci/mM specific activity, New England Nuclear). Triplicate untreated and DMSO treated (10μl DMSO) controls were set up as described for DNA synthesis. Triplicate cultures received 40, 80, 120, 160 or 200μg/ml of AME (final concentration of AME/ml) in 10μl of DMSO. After 3 hours incubation at 37°C in a 5% CO_2 atmosphere the media was removed and the cell sheets were washed twice with cold PBS. Further processing was as for the DNA synthesis experiments.

Assay for Mycoplasmal Contamination:

L-M, Vero, PMK-6 and GMK-8 cells—used for viability, DNA and RNA synthesis experiments—were assayed for the presence of mycoplasmal contamination. A 0.1 ml sample of growth medium from 3 day old semiconfluent cell cultures was added to ATCC plant mycoplasma broth (ATCC 27503) which consisted of 21 g Difco PPLO broth, 1 g fructose, 1 g glucose, 1 g sucrose, 1 g tryptone, 50 g sorbitol in 700 ml sterile distilled water, 200 ml horse serum and 100 ml of 25% fresh yeast extract. The tubes were incubated for 1~3 weeks at 27°C and checked for growth. As a further check 0.1 ml of three week old tube cultures was plated on agar plates composed of 34% Difco PPLO agar in ATCC 27503 medium. Plates were incubated for 1~3 weeks and examined microscopically (20$\times$magnification) for the presence of typical "fried egg" appearing mycoplasmal colonies.

Results

AME was found to be far less toxic than either FZ or AB to all four cell types (Table 1). AME was 12~20 fold less toxic than FZ and 36~250 fold less toxic than AB as monitored by 5 day cell viability in comparison with 1% DMSO control or untreated control cultures. Growth stimulation was not observed when treating the different cell types with FZ or AB at any of the concentrations tested.

AME, however, had a marked stimulatory effect on the growth of L-M and Vero cells. At levels of 40~120 μg/ml both cell lines were stimulated in comparison with control cells. Stimulation was indicated by increases in viable cell number, growth rate, and incorporation of [^{3}H] thymidine and [^{3}H] uridine into

Table 1. Comparative toxicity of amphotericin B (AB), Fungizone (FZ) and amphotericin B methyl ester (AME).

Cell type	TCD_{50} (μg/ml)*		
	AB	FZ	AME
L-M**	1	3	180
Vero	1.5	7	250
PMK-6	4	10	200
GMK-8	5	15	180

* TCD_{50} indicates the concentration of polyene antibiotic, in μg/ml, which results in approximately a 50% reduction in 5-day viable cell number in comparison with control cultures.

** The effect of AB, FZ and AME on 72-hour viability has been previously reported (FISHER *et al.*[4]).

Table 2. Effect of amphotericin B methyl ester (AME) on the average population doubling time* of L-M and Vero cells.

Antibiotic concentration (μg/ml)	Average population doubling time (hrs)	
	L-M	Vero
Control	21.5±1.2	20.4±1.6
40	16.9±0.5	17.7±1.0
80	16.9±0.7	15.5±1.1
120	19.5±1.0	16.0±1.0

* Population doubling times were determined by replicate studies and represent averages of Day 1~5.

Fig. 1. Effect of amphotericin B methyl ester (AME) on the growth of L-M cells.

Approximately 4.2×10^5 L-M cells, in replicate Leighton tubes, were either untreated or treated with 40 μg/ml of AME, 80 μg/ml of AME, or 120 μg/ml of AME at the initial time of cell plating (Day 0).

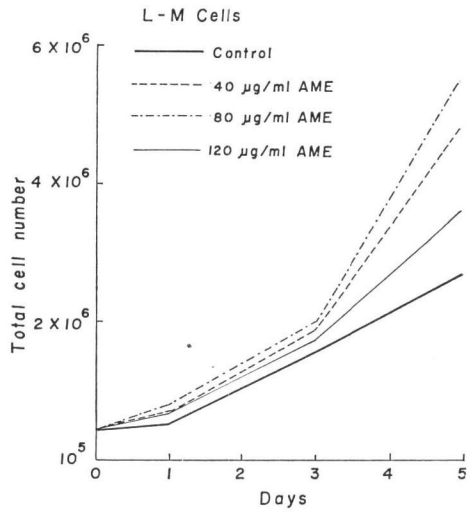
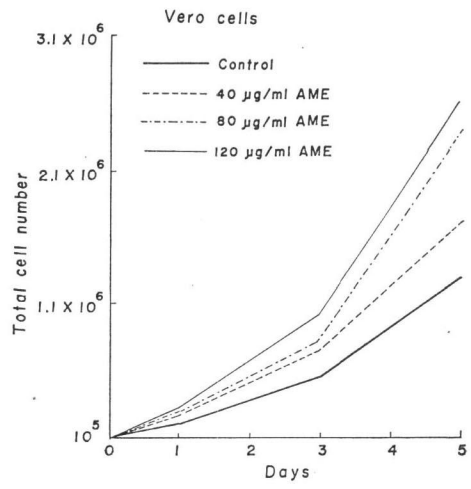


Fig. 2. Effect of amphotericin B methyl ester (AME) on the growth of Vero cells.

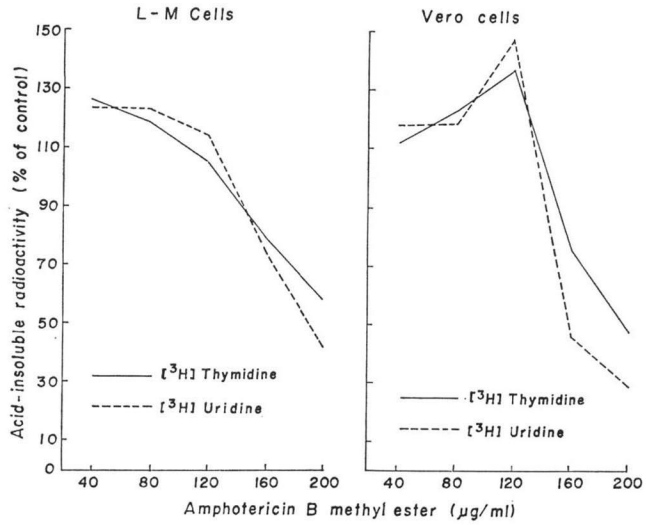
Approximately 1.1×10^5 Vero cells, in replicate Leighton tubes, were either untreated or treated with 40 μg/ml of AME, 80 μg/ml of AME, or 120 μg/ml of AME at the initial time of cell plating (Day 0).



DNA and RNA respectively. A maximum increase in L-M viable cell number, 2.1 times control, resulted after 5 day test period using 80 μg/ml AME (Fig. 1), while a twofold increase in Vero cells resulted following treatment with 120μg/ml AME during the same time period (Fig. 2). As seen in Table 2 the average population doubling for control (untreated or DMSO treated) L-M cells was 21.5$\pm$1.2 hours, whereas treatment with 40μg/ml of AME lowered this to 16.9$\pm$0.7 hours. Similarily, treatment of Vero cells with 80μg/ml of AME resulted in a 16.0$\pm$1.0 hour doubling time. Control (untreated or DMSO treated) Vero cells had a 20.4$\pm$1.6 hour doubling time. In contrast, 1$\sim$400 μg/ml of AME did not exert a stimulatory effect on the growth of PMK-6 or GMK-8 cells.

The effect of AME on DNA and RNA synthesis in L-M and Vero cells was stimulatory between the levels of 40$\sim$120μg/ml (Fig. 3). For both the L-M and Vero cells the concentration-dependent increase in DNA synthesis was paralleled by a concentration-dependent increase in RNA synthesis. For Vero cells the peak increase in DNA and RNA synthesis

Fig. 3. DNA and RNA synthesis in L-M and Vero cells in the presence of AME.
[^{3}H] thymidine or [^{3}H] uridine were added to L-M and Vero cell cultures in the presence of 40, 80, 120, 160 or 200 μg/ml of AME. Incorporation of the radioactivity into TCA precipitable material (DNA or RNA) after 24 hours (DNA) or 3 hours (RNA) was determined.



followed after treatment with 120 μg/ml AME, the same optimum concentration for increases in growth rate and viable cell number. For L-M cells the optimum level of AME for an increase in DNA-RNA syntheses was 40$\sim$80 μg/ml, the range of concentrations also found to best stimulate growth rate and total viable cell number. Levels of 1$\sim$400μg/ml of AME did not stimulate macromolecular synthesis (DNA or RNA) in PMK-6 or GMK-8 cells.

More than 50 % of the initial concentration of the polyene macrolide antibiotics was present after the five day test period in experiments determining drug effects on growth rate and cell number. In general, reductions in AME, FZ and AB concentrations were found to parallel each other, although, somewhat lower levels of AME remained in cell supernatants at the end of the experiments.

Analysis of tube cultures (1$\sim$3 weeks) and agar plates (1$\sim$3 weeks) failed to show the

presence of any mycoplasmal contamination.

Discussion

The results of this study indicate that AME: (1) exerts a concentration-dependent growth stimulatory effect on L-M and Vero cells, but does not stimulate PMK-6 or GMK-8 cell growth; (2) is less toxic than FZ or AB toward L-M, Vero, PMK-6 and GMK-8 cells; and (3) has good stability at 37°C in the tissue culture medium employed.

The possibility that elimination of low level fungal contamination results in increased growth after treatment with AME seems unlikely since AB and AME have essentially the same activity against this group of microorganisms[1,2]. Recent studies by GOLDSTEIN *et al.*[6] have demonstrated that AME is more active than AB against the *in vitro* growth of *Acholeplasma laidlawii* and *Spiroplasma citri*. Since the present study revealed no mycoplasmal contamination in L-M or Vero cultures using tube and agar assays it may be concluded that AME facilitated growth is not the consequence of elimination of such contaminants.

Studies on the mode of action of various growth stimulatory agents such as serum[7~9], LX-conditioned media[10], DNA transforming viruses[8,11], RNA transforming viruses[12,13], and other mitogenic agents[14,15] often indicate a correlation between an increase in uptake of low molecular weight nutrients present in culture media and stimulation of cell growth. Also, DULBECCO and ELKINGTON[16] have shown that the multiplication of fibroblastic cells is limited by the availability of medium factors, as opposed to contact between cells, and is even favored by short intercellular distances (distance-dependent helper effect).

The mechanism involved in the stimulatory effect of AME on L-M and Vero cells, which have a fibroblastic morphology, is not presently known but may involve permeability alterations resulting from polyene-cholesterol interactions. The polyene macrolide antibiotics have been shown to form complexes with sterols in the plasma membrane[17,18] resulting in morphological[19,20] and permeability[20,21] alterations in eucaryotic cells. It is believed that the permeability alterations induced by amphotericin B are a consequence of pore formation within the membrane. Such permeability changes as a result of AME interaction may facilitate the uptake and release of small molecular weight nutrients, such as glucose or essential and nonessential amino acids, thereby stimulating growth. The toxic effect of AME at higher concentrations may be due to excessive membrane modifications preventing cells functioning. The absence of any growth promoting effect as well as the increased toxicity of AB (alone or as FZ) in comparison with AME may be due to structural differences between these compounds with resultant changes in solubility, sterol binding ability, membrane interaction potential or other properties[4]. AMATI and LAGO[22] have reported a positive correlation between resistance of cell lines to AB and the number of cells obtainable from these lines at the time of density-dependent inhibition. Resistant lines yielded higher titers. Whether AME influences density-dependent inhibition, and thus the yield of cells at saturated growth, is not yet known; our studies were not pursued to the point of growth completion.

Investigations on growth stimulation of cells show that the promoting substances characteristically exhibit a high degree of specificity, acting on some types or strains of cells and not on others[23]. In the present study AME did not demonstrate a growth promoting effect on early passage mouse (PMK-6) or African Green monkey (GMK-8) cells. Similarly, previous experiments have indicated that AME does not stimulate the growth of certain mouse (RAG) or human (embryonic lung, WISH, KB or HeLa) cell types.[4] The inability of AME to stimulate these cell types suggests that L-M and Vero cells may be comparatively transport deficient for certain materials needed for more efficient growth. An analogous situation has been postulated for the effect of phytohemagglutinin (PHA), a membrane active agent, on lymphocytes in culture.[15] The uptake of uridine into lymphocytes is restricted. The addition of PHA to lymphocyte cultures facilitates passage into the cell of uridine and certain other substances with a resultant metabolic stimulation. If AME dependent growth stimulation is found to be

restricted to those cell types defective in one or more modifiable routes of membrane-mediated transport, this novel polyene macrolide would be a useful probe for detecting such abnormalities in cultured cells.

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