EFFECT OF POLYENE MACROLIDE ANTIBIOTICS ON INVERTEBRATE TISSUE CULTURE CELLS

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The effect of amphotericin B (AB) and amphotericin B methyl ester (AME) on viability and DNA synthesis in three insect cell lines, *Trichoplusia ni* (Tn), *Carpocapsa pomonella* 169 (Cp) and *Aedes aegypti* (Aa), has been evaluated. In all cases AME was less toxic than AB and inhibited DNA synthesis to a lesser degree than AB. However, the three cell lines differed in their response to the two polyene macrolide antibiotic preparations. Tn and Aa cell responded in a similar manner when exposed to either AME or AB, whereas Cp cells were more sensitive to both antibiotics than Tn or Aa cells.

Polyene macrolide antibiotics are routinely used as antifungal agents in tissue culture.[1~3] Mode of action studies indicate that these antibiotics bind to membrane sterols and can damage eukaryotic cells either by causing permeability changes or by fragmenting the cell surface membrane.[4~9] In addition, polyene macrolides have been used as membrane probes to study structural relationships between sterols and other membrane constituents.[6~9]

The use of amphotericin B (AB) as an antifungal agent in tissue cultures is restricted because of its marked toxicity toward diverse cell types.[3,10~12] Recently, FISHER *et al.*[10~12] demonstrated that amphotericin B methyl ester (AME), a structurally modified derivative of AB[13,14], was less toxic to tissue culture cells than AB or the commercially available desoxycholate complex of AB, Fungizone^R (E. R. Squibb & Sons). This observation was important since AB alone or as Fungizone is the most widely used antifungal agent in tissue culture, and concentrations of this antibiotic required both to prevent and to eliminate contamination are destructive to a large proportion of the cell population.[3,10~12]

Insect cells have become important as potential vehicles for studying the effects of invertebrate viruses useful in pest control.[15] In addition, insect cells do not synthesize endogenous cholesterol but require an exogenous source of this compound in the medium.[16,17] This sterol requirement might result in unique membrane properties for which the polyene macrolides could be used as probes. We have, therefore, investigated the effects of AB and AME on insect cells in culture.

Materials and Methods

Cell Lines and Media:

Trichoplusia ni (Tn) and *Carpocapsa pomonella* 169 (Cp) cells were grown in a modified mammalian culture medium[18] at 27°C. *Aedes aegypti* (PELEG) (Aa) cells were grown in mosquito culture medium (Grand Island Biological Co.) at 27°C. Both types of media were supplemented with 10% fetal bovine serum (Microbiological Associates). All three cell lines were kindly provided by Dr. A. H. MCINTOSH,

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Polyene Macrolide Antibiotics:

Amphotericin B (AB) was supplied by E. R. Squibb & Sons, New Brunswick, New Jersey. Amphotericin B methyl ester (AME) was synthesized and kindly supplied by Dr. W. MECHLINSKI, Waksman Institute of Microbiology, Rutgers University, New Brunswick, New Jersey. Both antibiotics were dissolved in dimethyl sulfoxide (DMSO) immediately prior to use.

Cell Viability Studies:

Growth studies on the three cell lines in the presence or absence of various concentrations of AB or AME were performed over a 48-hour test period.[3] Equal numbers of cells ($2\sim3\times10^5$) in one ml of growth media were added to replicate Leighton tubes followed by 10 μl of antibiotic dissolved in DMSO; yielding a final concentration of 1~20 μg/ml of AB or 10~100 μg/ml of AME. Control tubes received either no treatment or 10 μl of DMSO. The final concentration of DMSO was 1%. The tubes were then incubated for 48 hours at 27°C, and viable cell counts were performed using the trypan blue dye exclusion technique.[3,10~12] Since insect cells adhere poorly to culture vessels, floating as well as attached cells were counted. Each replicate study was performed a minimum of two times. Viability was expressed as percent of control. The concentration of polyene macrolide antibiotic resulting in approximately a 50% reduction in cell number, in comparison with control cultures, after 48 hours was expressed as TCD_{50}.

(^{3}H) Thymidine Incorporation into DNA:

Four hundred thousand Tn, Cp or Aa cells in 2 ml of growth media were placed in 5 ml Falcon tissue culture tubes. Cultures were treated with 20 μl of polyene macrolide antibiotic to yield a final concentration of 1~100 μg/ml of AB or AME dissolved in DMSO. Control cultures received no treatment or 20 μl of DMSO. After 1-hour incubation (with shaking every 15 minutes) at 27°C, 5 μCi of (^{3}H) thymidine (sp. act. 15 Ci/mM) was added to the polyene macrolide treated and control cultures. Incubation of cultures was continued for 1 additional hour at 27°C. Two 0.5-ml aliquots were removed from each culture, diluted with 3.5 ml of HANK's balanced salt solution, and the cells were pelleted by centrifugation at $1,000\times g$ for 15 minutes at 4°C. To the pellet 0.5 ml of 0.2 N sodium hydroxide was added, and the cells were vortexed and kept on ice for 5 minutes. Two ml of cold 20% trichloroacetic acid (TCA) was added to each tube, and the samples were kept for 15 minutes at 4°C. Acid-insoluble DNA was collected on scintillation pads (Whatman 3MM, Arthur H. Thomas Co.) by vacuum filtration, washed 5 times with 5% TCA, dried at 70°C for 1 hour and counted in a Packard Tricarb liquid scintillation spectrophotometer (Model 3003, Packard Instrument Co.) using Aquasol as the cocktail.

Results

The toxicity of AME and its parent compound AB toward three insect tissue culture cell lines, Tn, Cp and Aa, was evaluated using cell viability and (^{3}H) thymidine incorporation into DNA as indicators of drug toxicity. In all cases, AME was found to be less toxic than AB.

The concentration of AME required to reduce 48-hour cell viability of Tn and Aa cells to approximately 50% of control value (TCD_{50}) was 75 μg/ml, whereas a similar reduction in viability resulted after a single exposure to 20 μg/ml of AB. The high inate resistance of Tn and Aa cells to AB is 4 to 20 fold higher than found in vertebrate cells[10~12]. In contrast, Cp cells were more sensitive to both AME and AB, with a TCD_{50} of 50 and 5 μg/ml respectively.

The results of experiments dealing with the effects of AB and AME on (^{3}H) thymidine incorporation into DNA (Figs. 1~3) were comparable to cell viability studies. As can be seen in Fig. 1, the effect of AME on Tn cells remains constant over the concentration range of 1~100 μg/ml. However, 1~20 μg/ml of AB resulted in an initial drop in DNA synthesis, whereas a plateau effect was evident with higher concentrations of AB. At the highest concentration of AB tested (100 μg/ml), DNA synthesis proceeded at approximately 60% of control values. Similar results were found

Fig. 1. DNA synthesis in *Trichoplusia ni* (Tn) cells in the presence of AME and AB.

(^{3}H) Thymidine was added to Tn cell cultures in the presence of 1~100 μg/ml of AME or AB. Incorporation of the radioactivity into TCA precipitable DNA after 2 hours was determined. Results are expressed in relation to untreated or DMSO treated controls (100%).

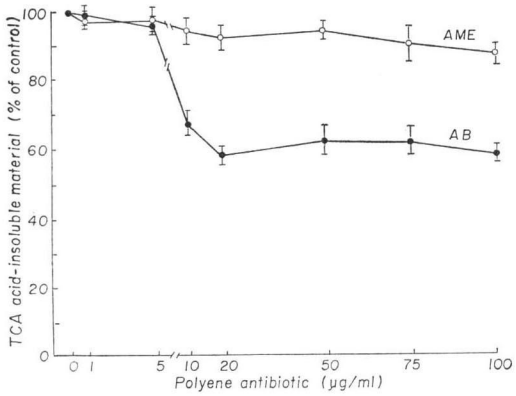


Fig. 2. DNA synthesis in *Aedes aegypti* (PELEG) (Aa) cells in the presence of AME and AB.

Experimental details the same as in Fig. 1 and "Materials and Methods".

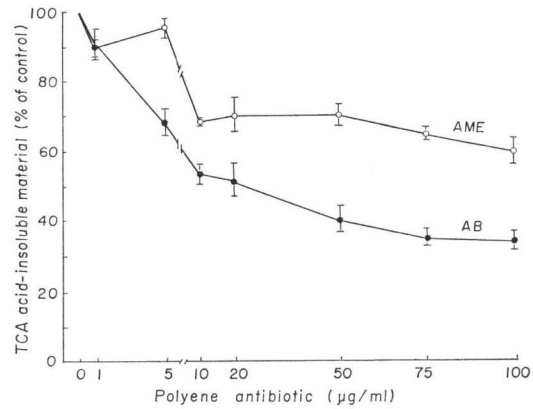
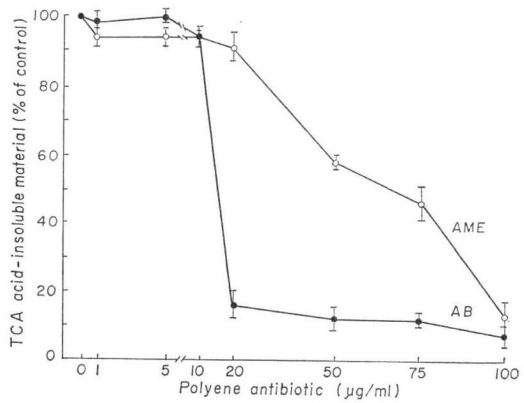


Fig. 3. DNA synthesis in *Carpocapsa pomonella* 169 (Cp) cells in the presence of AME and AB.

Experimental details the same as Fig. 1 and "Materials and Methods".



with Aa cells (Fig. 2). The effect of AME and AB on Cp cells was unusual (Fig. 3). Between 20 to 100 μg/ml of AME there was a steady decline in (^{3}H) thymidine incorporation, with DNA synthesis proceeding at 15% of control values after a single exposure to 100 μg/ml of AME. In contrast, studies using various vertebrate cell cultures indicated no significant alterations in DNA synthesis after treatment with 1~100 μg/ml of AME for 2 hours (unpublished data). AB inhibited DNA synthesis to a greater extent than AME in Cp cells. There was a steep decline in DNA synthesis between 10 and 20 μg/ml of AB to a rate approximately 15% of control cultures.

Discussion

AB alone or as Fungizone^R is a widely used antifungal agent in tissue culture systems.[1~3] However, this antibiotic has been found to impair cellular growth[3,10~12,19] and change the permeability of the plasma membrane[12] at concentrations required to rid a culture of fungi.

In recent years we have been investigating the effect of AME, a structurally modified derivative of AB,[13,14] on diverse tissue culture cells.[10~12] AME has similar antifungal activity as AB,[20,21] but is less toxic to vertebrate cells in culture.[10,12] In addition, AME is more effective against various mycoplasmal strains than AB.[22]

The reason for the lowered toxicity of AME in comparison with AB may depend on the physicochemical properties of these two polyene macrolide antibiotic preparations. AME is more soluble in

aqueous solution than AB; in fact, AME more closely approaches molecular dispersion, whereas AB forms large micelles in water. At similar concentrations, the larger micelle size of AB compared to AME could result in a local saturation of AB at the cell surface membrane and subsequently produce membrane alterations with permeability changes and cellular death.

Another major consideration in evaluating the susceptibility of cells to polyene macrolide antibiotics is sterol-polyene macrolide interactions. For this reason, insect cells represent a unique experimental system because they cannot synthesize cholesterol and require an exogenous source of sterols.[16,17] The results of our experiments indicated that the three insect cell lines were generally more sensitive to AB than AME, but varied in their response to the same antibiotic preparation. Tn and Aa cells were more resistant to both AME and AB induced toxicity than Cp cells as indicated by cell viability after 48 hours. Differential sensitivity between the three cell types was also found when monitoring (^{3}H) thymidine incorporation into DNA (Figs. 1~3). Low levels of AME or AB resulted in a sharp decline in DNA synthesis in Cp cells, whereas DNA synthesis in Tn and Aa cells was not inhibited to the same extent after a single exposure to the same or higher levels of AME or AB. Alterations in the type, amount or molecular orientation of specific sterol(s) in the membranes[12] of these insect cells may be the reason for their differential sensitivity to the same polyene macrolide antibiotic. We are presently investigating the relationship between membrane structure and polyene macrolide toxicity.

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References

1) PERLMAN, D. & S. A. BRINDLE: Antibiotic control of contamination in tissue culture. Antimicr. Agents & Chemoth. 3: 458~461, 1963
2) HEMPHILL, J. J.; Y. F. HERMAN & Y. M. YOUNG: Comparative antifungal activity of nystatin and amphotericin B in tissue culture for virus propagation. Antibiotics Annual. 1956/1957: 690~696, 1957
3) BONNER, D. P.; P. B. FISHER, N. I. GOLDSTEIN, W. MECHLINSKI, V. BRYSON & C. P. SCHAFFNER: Effect of fetal bovine serum on polyene antibiotic cytotoxicity and antifungal activity. In Vitro 12: 399~404, 1976
4) CASS, A.; A. FINKELSTEIN & V. KRESPI: The ion permeability in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. J. Gen. Physiol. 56: 100~124, 1970
5) DENNIS, V. W.; N. W. STEAD & T. E. ANDREOLI: Molecular aspects of polyene- and sterol-dependent pore formation in thin lipid membranes. J. Gen. Physiol. 55: 375~400, 1970
6) KINSKY, S. C.: Antibiotic interaction with model membranes. Ann. Rev. Pharmacol. 10: 119~142, 1970
7) DE KRUIJFF, B.; W. J. GERRITSEN, A. OERLEMANS, R. A. DEMEL & L. L. M. VAN DEENEN: Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. 1. Specificity of the membrane permeability changes induced by the polyene antibiotics. Biochim. Biophys. Acta 339: 30~43, 1974
8) DE KRUIJFF, B.; W. J. GERRITSEN, A OERLEMANS, P. W. M. VAN DIJCK, R. A. DEMEL & L. L. M. VAN DEENEN: Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. II. Temperature dependence of the polyene antibiotic-sterol complex formation. Biochim. Biophys. Acta 339: 44~56, 1974
9) DE KRUIJFF, B. & R. A. DEMEL: Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. III. Molecular structure of the polyene antibiotic-cholesterol complexes. Biochim. Biophys. Acta 339: 57~70, 1974
10) FISHER, P. B.; N. I. GOLDSTEIN, D. P. BONNER, W. MECHLINSKI, V. BRYSON & C. P. SCHAFFNER: Toxicity of amphotericin B and its methyl ester toward normal and tumor cell lines. Cancer Res. 35: 1996~1999,

1975

11) FISHER, P. B.; N. I. GOLDSTEIN, D. P. BONNER, W. MECHLINSKI, V. BRYSON & C. P. SCHAFFNER: Stimulatory effect of amphotericin B methyl ester on the growth of L-M and Vero cells. J. Antibiotics 28: 896~902, 1975
12) FISHER, P. B.; N. I. GOLDSTEIN, V. BRYSON & C. P. SCHAFFNER: Reduced toxicity of amphotericin B methyl ester (AME), *vs.* amphotericin B and Fungizone in tissue culture. In Vitro 12: 133~140, 1976
13) MECHLINSKI, W. & C. P. SCHAFFNER: Polyene macrolide derivatives. I. N-Acylation and esterification reactions with amphotericin B. J. Antibiotics 25: 256~258, 1972
14) SCHAFFNER, C. P. & W. MECHLINSKI: Polyene macrolide derivatives. II. Physical-chemical properties of polyene macrolide esters and their water soluble salts. J. Antibiotics 25: 259~260, 1972
15) SUMMERS, M. D.: Biophysical and biochemical properties of Baculoviruses. *In* "Baculoviruses for Pest Control: Safety Considerations". M. D. SUMMERS, R. ENGLER & L. A. FALCON, *Eds.*, ASM, Washington, 1975
16) VAUGHN, J. L.; S. J. LOULOUDES & K. DOUGHERTY: The uptake of free and serum-bound sterols by insect cells *in vitro*. *In* "Arthropod cell cultures and their application to the study of viruses". E. WEISS, *Ed.*, Springer-Verlag, New York 1971
17) VAUGHN, J. L.: Insect cell nutrition: Emphasis on sterols and fatty acids. In Vitro 9: 122~128, 1973
18) MCINTOSH, A. H.; K. MARAMOROSCH & C. RECHTORIS: Adaptation of an insect cell line (*Agallia constricta*) in a mammalian cell culture medium. In Vitro 8: 375~378, 1973
19) DOLBERG, D. & M. J. BISSELL: Side effect of amphotericin B-deoxycholate (Fungizone) and nystatin in chick cells in culture. In Vitro 10: 26~29, 1974
20) BONNER, D. P.; W. MECHLINSKI & C. P. SCHAFFNER: Polyene macrolide derivatives. III. Biological properties of polyene macrolide ester salts. J. Antibiotics 25: 261~262, 1972
21) HOWARTH, W. R.; R. P. TEWARI & M. SOLOTROVSKY: Comparative *in vitro* antifungal activity of amphotericin B and amphotericin B methyl ester. Antimicr. Agents & Chemoth. 7: 58~63, 1975
22) GOLDSTEIN, N. I.; A. H. MCINTOSH, P. B. FISHER, K. MARAMOROSCH & C. P. SCHAFFNER: *In vitro* antimycoplasmal activity of amphotericin B methyl ester. J. Antibiotics 29: 656~661, 1976