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Differential Control of Synergistic Effect with Polyene Macrolide Antibiotics upon Chinese Hamster Cells *in Vitro*[1]

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ABSTRACT

An amphotericin B-resistant cell (AMB^R-1), which was isolated from aneuploid Chinese hamster cells (V79), was found to show much higher resistance than its parent V79 cells to other polyene antibiotics, such as pentamycin and filipin. To obtain the 50 to 60% inhibition of the control protein synthesis activity by a synergistic combination of fusidic acid and amphotericin B, 50 μg fusidic acid per ml were combined with 10 μg amphotericin B in V79 cells, whereas in AMB^R cells 50 μg fusidic acid per ml were combined with 100 μg polyene antibiotic per ml. Bleomycin (10 μg/ml), which alone did not affect cellular DNA synthesis, inhibited DNA synthesis of V79 cells by more than 90% of the control activity when combined with only 1 μg pentamycin per ml, whereas a similar extent of inhibition in AMB^R cells was observed by combination with more than 5 μg pentamycin per ml.

INTRODUCTION

Specific control of membrane permeability to anticancer agents could be a final goal in cancer chemotherapy. Membrane-active antibiotics that perturb the cell membrane to permit the penetration of a second agent include polyene macrolide antibiotics (9), such as amphotericin B or pentamycin (8, 12-14, 17, 18, 20, 21, 28). These polyene antibiotics interact specifically with sterols in the membranes. Synergism of anticancer agents with amphotericin B has been also shown to be effective against various tumors *in vivo* (16, 19). Although cellular sensitivity to polyene antibiotics differs among cell lines, especially between transformed and untransformed cells *in vitro* (1, 14, 18), the molecular mechanism underlying the difference in sensitivity remains to be studied. Lago *et al.* (15) recently indicated that fibroblastic cells transformed with SV40 virus had lower affinity for radioactive amphotericin B. Recent genetic studies (11, 24) have also indicated a close correlation between sensitivity to polyene antibiotics and the cellular level of cholesterol content. Thus, to understand the basic mechanism involved in the synergistic effect caused by the polyene antibiotics, we compare the synergistic effects *in vitro* of an amphotericin B-resistant clone (11) and its parental V79 cells (Chinese hamster cells). In this report we also discuss the cellular specificity in the potentiation of antibiotic action by polyene antibiotics.

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MATERIALS AND METHODS

Cell Culture and Cell Lines. An aneuploid Chinese hamster cell line V79 and its amphotericin B-resistant subline (AMB^R-1) were used as described previously (11). The cells were routinely grown in monolayer in glass Petri dishes in minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan) containing 0.1% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 10% fetal bovine serum (Microbiological Associates, Inc., Bethesda, Md.), kanamycin (100 μg/ml), and penicillin (200 units/ml). These cells grow with a doubling time of 9 to 10 hr.

Chemicals. [^{3}H]Leucine (60 Ci/mmol) and [^{3}H]thymidine (20 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Fusidic acid and amphotericin B (Fungizone) were given to us by Sankyo Co., Ltd., Tokyo, Japan, and pentamycin was given by Nikken Chemicals Co., Ltd., Tokyo, Japan. Filipin was kindly given to us by Dr. W. Friis (The Upjohn Co., Kalamazoo, Mich.). Bleomycin A_2 was obtained from Nippon Kayaku Co., Ltd., Tokyo, Japan.

Cell Survival by Colony Formation. The dose-response curves of V79 cells and their amphotericin B-resistant subline (AMB^R-1) for polyene antibiotics were determined by plating 250 cells in duplicate 60-mm dishes with various doses. After incubation at 37° for 7 days, the dishes were stained with Giemsa and the colonies were counted. Plating efficiencies of V79 cells and AMB^R cells ranged from 75 to 85%.

Synergism Study *in Vitro*. One to 2×10^5 V79 or AMB^R-1 cells/ml cultured in 3 ml medium in Petri dishes were exposed to various doses of fusidic acid or bleomycin with or without polyene antibiotics for 18 hr and then were exposed to [^{3}H]leucine (2 μCi/ml) (protein synthesis) or [^{3}H]thymidine (0.2 μCi/ml) (DNA synthesis) for 4 hr. After exposure to radioisotopic compounds, DNA synthesis was measured by counting the radioactivity in a 10% trichloroacetic acid-insoluble fraction retained onto glass-filter paper, and protein synthesis was measured by counting the radioactivity of a hot acid-insoluble fraction as described previously (12, 21). Polyene antibiotics used in this study were prepared by dissolving in dimethyl sulfoxide before each experiment, and all control experiments were done by adding the same amount of dimethyl sulfoxide alone.

RESULTS

Comparison of Colony Formation of V79 and AMB^R Cells in the Presence of Polyene Antibiotics. We have isolated independently 3 amphotericin B-resistant sublines from Chinese hamster V79 cells after nitrosoguanidine mutagenesis (11). To compare the dose response of the colony-

forming ability of amphotericin B-resistant (AMB^R) cells with that of V79 cells, we used a representative culture (AMB^R-1) the resistant phenotype of which was maintained stably for 2 months in the absence of amphotericin B (11), and in the experiment reported here, we used AMB^R-1 cells that had been cultured continuously in the absence of the drug for 5 months. A clear difference in the dose response of the colony-forming ability appeared between V79 and AMB^R-1 cells. Amphotericin B (75 μg/ml), pentamycin (5 μg/ml), or filipin (7 μg/ml) decreased the survival fraction of V79 cells to below 10^{-2} of the control value in the absence of the drugs, whereas the respective concentrations of the above polyene antibiotics inhibited only slightly, if at all, the growth of AMB^R cells (Chart 1). Thus, the results clearly showed that AMB^R was resistant to general polyene antibiotics and that the resistant phenotype was very stably maintained.

Comparative Study on the Synergistic Effect with Combinations of Polyene Antibiotics between V79 and AMB^R Cells. With 2 different but effective synergistic combinations, fusidic acid with amphotericin B (12, 14) and bleomycin with pentamycin (21, 28), we examined the possible correlation between the extent of synergistic effect and the difference in the sensitivity of both V79 and AMB^R cells to polyene antibiotics, which overcome the membrane barrier to increase the permeability to chemical drugs. Since fusidic acid is an inhibitor of protein synthesis in mammalian cells as previously reported (12, 14), the action of fusidic acid was assayed by measuring cellular protein synthesis. Amphotericin B alone, below the concentration of 30 μg/ml, inhibited protein synthesis of V79 cells by about 30% of the control activity, whereas even the higher dose of amphotericin B (100 μg/ml) did not inhibit protein synthesis of AMB^R cells (Chart 2). However, the inhibition of protein synthesis in V79 cells by fusidic acid was remarkably enhanced in the presence of amphotericin B, and the extent of inhibition increased with the increasing dose of amphotericin B (Chart 2*A*). Protein synthesis activity was blocked almost completely by 50 μg fusidic acid per ml in combination with 20 to 30 μg amphotericin B per ml. In contrast, protein synthesis of AMB^R cells was blocked by 50% only when 50 or 75 μg fusidic acid per ml was combined with such higher doses as 100 μg amphotericin B per ml (Chart 2*B*). It was also found that V79 and AMB^R showed almost identical response to fusidic acid (data not shown). Thus, to obtain the same extent of synergistic effect, AMB^R cells required about a 10-fold-higher dose of the polyene antibiotic than did the V79 cells.

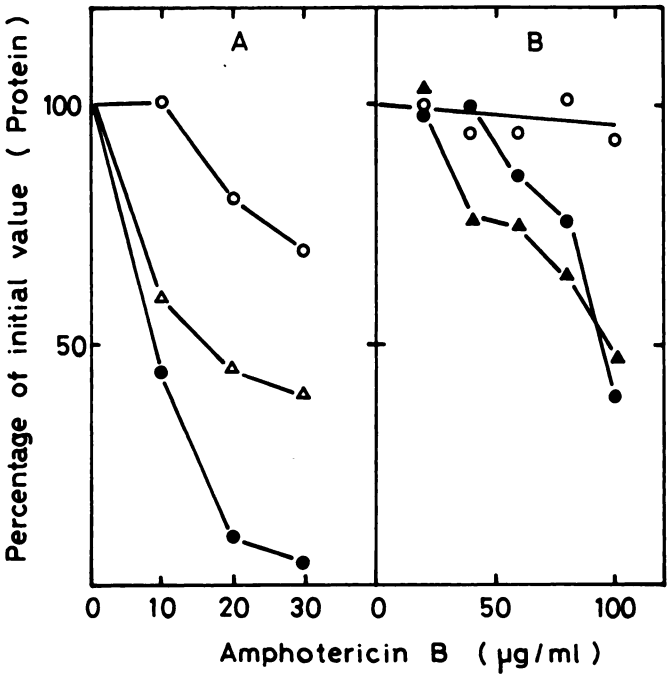

Chart 2. Dose response of V79 (*A*) and AMB^R-1 (*B*) to amphotericin B in the absence or presence of fusidic acid on protein synthesis. After cells were exposed to various concentrations of amphotericin B alone (○) or in combination with fusidic acid [25 (△), 50 (●), or 75 μg/ml (▲)] for 18 hr, [^{3}H]leucine incorporation into hot acid-insoluble fraction was measured. The normalized activity is presented when 100% corresponds to 1291 (none), 1433 (25 μg fusidic acid per ml), and 751 cpm (50 μg fusidic acid per ml) in *A* and to 1407 (none), 920 (50 μg fusidic acid per ml), and 701 cpm (75 μg fusidic acid per ml) in *B*, respectively.

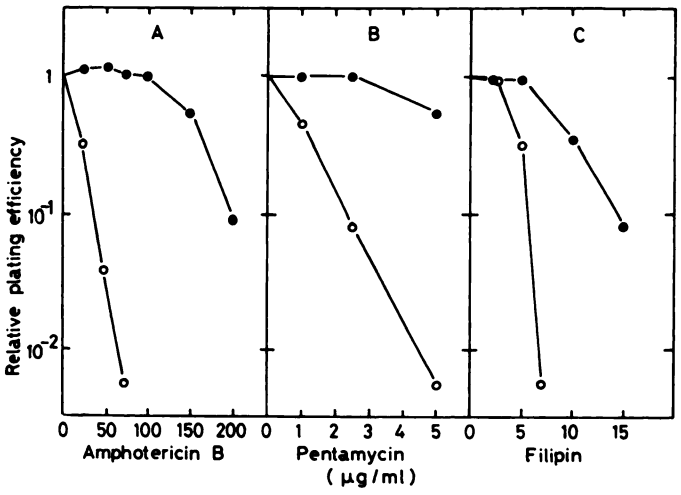

Chart 1. Dose response of the parental cell V79 and AMB^R-1 to polyene antibiotics, assayed by colony-forming ability. Cells (250 each) of V79 (○) and AMB^R-1 (●) were plated and incubated in the presence of various doses of amphotericin B (*A*), pentamycin (*B*), and filipin (*C*) for 7 days. The *curves* drawn for each cell line show the survival values obtained from duplicate experiments. The plating efficiency for V79 was 85%, and that for AMB^R-1 was 75%.

We also examined whether differential sensitivities of the synergistic effect of bleomycin, a potent antitumor antibiotic (29), were observed between V79 and AMB^R cells when combined with pentamycin. Pentamycin is a pentaene polyene that enhances the cytotoxic action of bleomycin (21, 28). This polyene antibiotic alone did not inhibit DNA synthesis of V79 cells below the concentration of 2 μg/ml, whereas at 5 μg/ml the pentamycin inhibited 80% of DNA synthesis activity (Chart 3A). This pentaene polyene at concentrations of 0.5 to 1 μg/ml could enhance greatly the action of bleomycin against V79 cells, whereas pentamycin could not enhance the action of fusidic acid (Chart 3*A*). On the other hand, less than 10% of DNA synthesis activity in AMB^R cells was blocked with the polyene (5 μg/ml) alone (Chart 3*B*). Significantly enhanced action of bleomycin (10 μg/ml) appeared against AMB^R cells when a high dose (5 μg/ml) of pentamycin was combined (Chart 3*B*). As seen in Chart 4, the dose response of bleomycin in the absence or presence of pentamycin was compared between V79 and

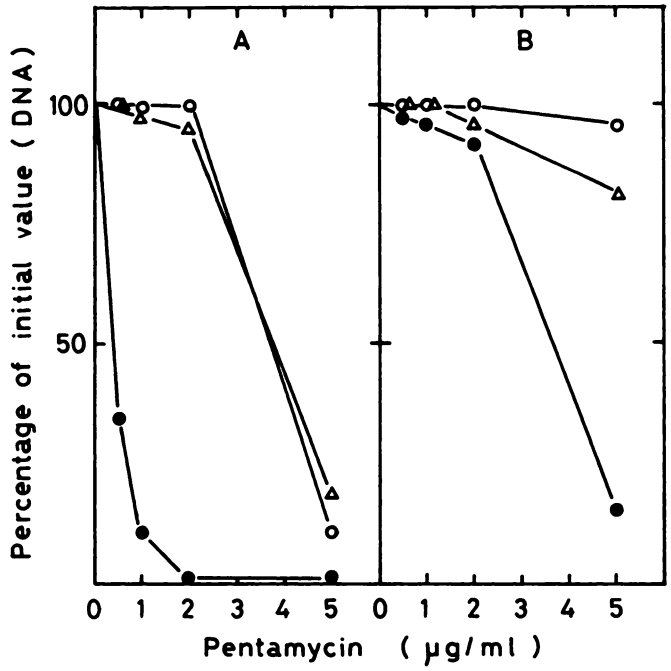

Chart 3. Dose response of V79 (*A*) and AMB^R-1 (*B*) to pentamycin in the absence or presence of fusidic acid or bleomycin on DNA synthesis. V79 and AMB^R cells were exposed to various doses of pentamycin in the absence (○) or presence of bleomycin (10 μg/ml) (●) or fusidic acid (50 μg/ml) (△) for 18 hr. Normalized activity of DNA synthesis is shown when 100% corresponds to 9536 (in the absence of pentamycin), 9312 (bleomycin alone), and 6575 cpm (fusidic acid alone) in *A* and to 10776 (none), 9617 (bleomycin alone), and 7326 cpm (fusidic acid alone) in *B*.

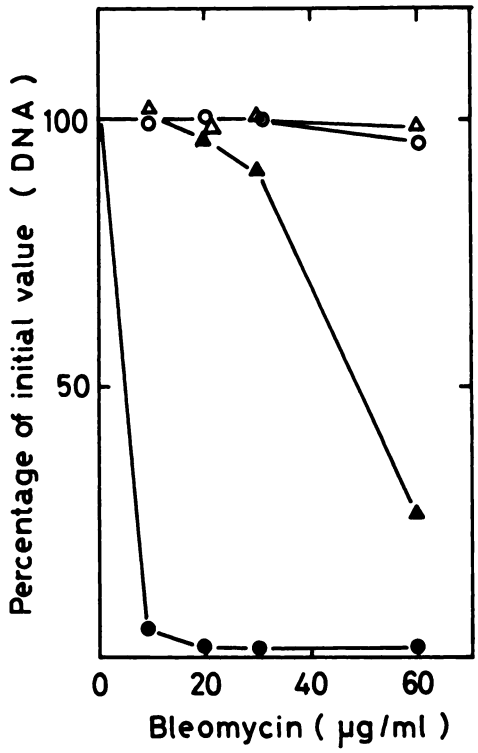

Chart 4. Dose response to bleomycin in the absence or presence of pentamycin of V79 (○ and ●) and AMB^R-1 (△ and ▲). Both V79 and AMB^R-1 cells were exposed to various doses of bleomycin without (○ and △) or with pentamycin (2 μg/ml) (● and ▲) for 18 hr. Normalized activity of DNA synthesis is shown when 100% corresponds to 6634 (V79) and 8773 cpm (AMB^R-1) in the absence of pentamycin and to 7634 (V79) and 10017 cpm (AMB^R-1) in the presence of the drug.

AMB^R cells. The extent of inhibition of DNA synthesis increased in the presence of 2 μg pentamycin per ml with an increasing dose of bleomycin, but bleomycin alone did not affect DNA synthesis of both V79 and AMB^R cells within the concentration used. Pentamycin (2 μg/ml) could apparently magnify the inhibitory action by bleomycin (10 μg/ml) on DNA synthesis in V79, whereas the same dose of pentamycin inhibited DNA synthesis in AMB^R-1 cells to the same extent as in V79 cells only when more than bleomycin (60 μg/ml) was combined (Chart 4). The data in Charts 3 and 4 therefore suggest an apparent difference by a factor of 5 to 10 in the sensitivity between V79 and its amphotericin B-resistant cell line to the synergistic effects with polyene antibiotics.

DISCUSSION

In this report a remarkable difference in the sensitivity to polyene antibiotics was observed between V79 and its amphotericin B-resistant clone. Our study showed that the cholesterol content or the synthetic rate of cholesterol in AMB^R cells was found to be about one-half of those of V79 cells (11) and that the reduced level of cholesterol in the amphotericin B-resistant cell line was closely correlated with the lowered sensitivity to other polyene antibiotics, such as filipin, pentamycin, or nystatin. Thus, the resistant phenotype to amphotericin B is thought to be mediated through the level of cholesterol, which is known to be a target site for polyene antibiotics (5, 6, 22, 26). An independent study (25) has shown that the increased sterol content in mouse leukemia L1210 cells incubated with ergosterol-containing liposomes makes the cells highly susceptible to amphotericin B. We also observed that AMB^R-1 cells treated with cholesterol-containing liposomes showed much reduced resistance to polyene antibiotics (S. Akiyama, K. Hidaka, and M. Kuwano, unpublished data).

Amphotericin B and its related compounds powerfully enhance the action of second agents in the *in vitro* tissue culture system (see "Introduction"). It is expected that, through the holes in the membrane being formed by polyene antibiotics (2, 4), second agents efficiently permeate the cells. In fact, the cellular uptake of radioactive actinomycin D was enhanced by amphotericin B (20).

The polyene antibiotic also remarkably potentiates the antitumor activity of nitrosourea agents against animals bearing leukemia or ependymoblastoma (16, 19). However, it remains unclear whether the effective synergistic combination *in vivo* is due to enhanced permeation or other mechanisms caused by the polyene antibiotic. Biosynthesis of sterol is balanced in normal cells with the level of external cholesterol, whereas the exogenous cholesterol fails to suppress the sterol synthesis in neoplastic cells (10, 27, 31). The sterol contents of various tumor cells, such as hepatoma or leukemia, show higher levels of cholesterol than do the contents of normal tissue or cells (3, 7, 23, 30, 31). Thus, one could expect selective control of antitumor activity by a polyene antibiotic-mediated synergism against tumors with higher levels of cholesterol. However, Medoff *et al.* (19) argue that the antitumor activity enhanced by polyene is not solely attributed to altered permeability but

also is attributed to enhanced immune response. Further study is necessary to elucidate the mode of action *in vivo* as well as *in vitro* by polyene antibiotics.

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