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Control of Permeation of Bleomycin A_2 by Polyene Antibiotics in Cultured Chinese Hamster Cells[1]

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ABSTRACT

Control of permeation of bleomycin A_2, a well-known antitumor antibiotic, in combination with various polyene macrolide antibiotics was analyzed in cultured Chinese hamster cells *in vitro*. Three polyene antibiotics, filipin, pentamycin, and pimaricin, were found to enhance the action of bleomycin A_2 remarkably, while amphotericin B or nystatin could not. Although DNA synthesis and colony-forming activity of polyene-sensitive Chinese hamster V79 cells were synergistically inhibited by the combination of filipin and bleomycin A_2, in a polyene-resistant subline (AMB^R-1) derived from V79, they were only slightly affected in the presence of both drugs. The cellular uptake of [^{14}C]bleomycin A_2 by V79 was enhanced 2- to 4-fold in the presence of increasing doses of filipin or pentamycin, but not in the presence of amphotericin B. The treatment of V79 cells with filipin for 20 to 30 min was enough to block DNA synthesis almost completely when combined with 20 μg bleomycin A_2 per ml. The pretreatment of the hamster cells with 6 μg filipin per ml for 60 min continued to enhance the inhibitory action by bleomycin A_2 of DNA synthesis up to 5 hr after the removal of filipin from the cultured medium.

INTRODUCTION

Polyene macrolide antibiotics are known to enhance the permeation of combined second agents through interaction with sterols of cell membranes (1, 4, 9, 17), and the synergistic combination with polyenes is found to be a useful therapeutic method not only against fungi-infectious diseases (10) but also against tumors (15, 18). A polyene antibiotic, pentamycin, could potentiate bleomycin A_2, a potent antitumor agent (8, 19, 22), but not fusidic acid, an inhibitor of protein synthesis (7). In contrast, another polyene, amphotericin B, could potentiate fusidic acid (8, 16). In this report, we examine whether the cytocidal action of bleomycin A_2 *in vitro* is enhanced by other polyene antibiotics besides pentamycin, and we discuss diversity among polyene-induced membranous alterations.

MATERIALS AND METHODS

Cell Culture and Cell Lines. Chinese hamster cell line V79 and its amphotericin B-resistant subline (AMB^R-1) were obtained and maintained as described previously (7). The cells were routinely grown in monolayers in glass Petri dishes in minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 1 mg bactopeptone per ml, 0.292 mg L-glutamine per ml, 10% fetal bovine serum (Microbiological Associates, Bethesda, Md.), and penicillin (100 units/ml). These cells could grow with a doubling time of 9 to 10 hr.

Chemicals. [^{3}H]Thymidine (20 Ci/mmol) (New England Nuclear, Boston, Mass.) was used. Radioactive [^{14}C]bleomycin A_2 (10 μCi/400 μg; Nippon Kayaku Co., Tokyo, Japan) was prepared and highly purified when bleomycin A_2 was methylated with ^{14}C-containing methyl iodide at 42° in methanol, as described previously (5). Amphotericin B and nystatin were given to us by Sankyo Co., Tokyo, Japan, and pentamycin was given by Nikken Chemical Co., Tokyo, Japan. Filipin was kindly given by The Upjohn Co., Kalamazoo, Mich. Pimaricin was given by Torii Pharmaceutical Co., Tokyo, Japan. Nonradioactive bleomycin A_2 which does not contain other bleomycin molecules was kindly donated by Nippon Kayaku Co., and bleomycin A_2 was used as bleomycin throughout this study. Aquasol was purchased from New England Nuclear.

Synergistic Study *in Vitro* by [^{3}H]Thymidine Incorporation. V79 and AMB^R-1 (1 to 2 × 10^5 cells/ml) cultured in 3 ml medium in Petri dishes were exposed to various doses of bleomycin with or without each of the polyene antibiotics for 10 or 18 hr and were then exposed to [^{3}H]thymidine (1 μCi/ml) for another 4 hr in the presence of drugs. After exposure to [^{3}H]thymidine, DNA synthesis was measured by counting the radioactivity of 10% trichloroacetic acid-insoluble fraction retained on glass filter paper as described previously (13). Polyene antibiotics used in this study, including amphotericin B, pentamycin, pimaricin, filipin, and nystatin, 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.

Synergistic Study by Colony Formation. Cell survival of V79 or AMB^R-1 cells under synergistic conditions was also measured by colony formation. Cells (250 each) were plated in duplicate 60-mm dishes in the absence of any drug and incubated for 18 hr. Then the cells were exposed to bleomycin A_2 with or without filipin and incubated at 37° for 8 days. Colony number was counted when stained with Giemsa as described previously (7, 8).

Cellular Uptake of Radioactive Bleomycin. Cells were treated with each polyene antibiotic for 2 hr and then exposed to [^{14}C]bleomycin A_2 (30,000 cpm/dish) for another 4 hr in the presence of the polyene. The cells in each dish were harvested and washed 3 times with phosphate-buffered saline containing 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and 1 mM bleomycin A_2 by repeated centrifugation. Finally, the cellular pellets were suspended in 1 ml of H_2O and mixed thoroughly with 10 ml of Aquasol. Then radioactivities were counted. Protein concentrations were determined by the

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method of Lowry *et al.* (16), with bovine serum albumin as a standard.

RESULTS

Enhancement of the Action of Bleomycin by Filipin, Pentamycin, and Pimaricin, but not by Amphotericin B and Nystatin. The Chinese hamster V79 cell is not highly sensitive to bleomycin (8), which is possibly due to difficulty in membrane permeability by bleomycin. The cytocidal activity of bleomycin has been shown to be enhanced remarkably when combined with pentamycin in Chinese hamster V79 cells (8). To test if any other polyene antibiotics can potentiate bleomycin, we examined the effect of bleomycin in combination with 5 polyene antibiotics such as amphotericin B, filipin, nystatin, pentamycin, and pimaricin. We used 2 different concentrations of each polyene antibiotic with respect to inhibitory extent of DNA synthesis, low concentration of polyene (0 to 20% inhibition of DNA synthesis), and high concentration of polyene (40 to 60% inhibition). Cellular DNA synthesis was synergistically inhibited when 10 μg bleomycin A_2 per ml was combined with polyene antibiotics such as pentamycin, filipin, and pimaricin, but not when combined with amphotericin B or nystatin (Table 1). Bleomycin A_2 could not be potentiated even when combined with higher doses of amphotericin B or nystatin.

Synergistic Effect of Bleomycin and Filipin upon DNA Synthesis, as well as Colony Formation of Amphotericin B-sensitive and -resistant Cell Lines. Three polyene antibiotics, filipin, pentamycin, and pimaricin, effectively potentiate bleomycin in Chinese hamster cells, and the effect of pentamycin is in good agreement with our previous results (8, 19). To study an effective combination of bleomycin and filipin in more detail, we compared the synergistic effect between V79 and an amphotericin B-resistant (AMB^R-1) cell line derived from V79 (7). Dose response of V79 and AMB^R-1 cells to bleomycin was compared when combined with or without 3 or 6 μg filipin per ml (Chart 1). In the absence of filipin, both V79 and AMB^R-1 show almost identical dose-response curves to bleomycin, while addition of filipin was found to efficiently enhance the inhibition of DNA synthesis of V79, but not of AMR^R-1. DNA synthesis of V79 which is amphotericin B sensitive, was blocked by 70 to 75% when 5 to 10 μg bleomycin A_2 per ml was combined with 6 μg filipin per ml (Chart 1*a*). Almost the same synergistic effect of bleomycin and filipin as in DNA synthesis was observed when assayed by colony formation of V79 and AMB^R. As shown in Chart 2, bleomycin A_2 alone, below the concentration of 0.8 μg/ml, inhibited colony formation of both V79 and AMB^R cells by about 40 to 50% of the control activity. Colony-forming activity of V79, however, was decreased to below 10^{-2} of the control value by 0.3 μg bleomycin A_2 per ml, in combination with 3 μg filipin per ml (Chart 2*a*). In contrast, colony formation of AMB^R cells was blocked by 75% only when 0.8 μg bleomycin A_2 per ml was combined with 3 μg filipin per ml (Chart 2*b*). In addition, filipin (4 μg/ml) decreased the survival fraction of V79 to 7×10^{-3} of the control value when combined with 0.1 μg bleomycin A_2 per ml, while the same dose of the polyene antibiotic decreased that of AMB^R to 10^{-2} when combined with 0.8 μg bleomycin A_2 per ml. Thus, 2 independent assays (DNA synthesis and colony formation) reinforced an argument that filipin potentiated bleomycin A_2 and also that, to obtain the same degree of synergistic effect, AMB^R cells required about an 8-fold higher dose of bleomycin A_2 than did the V79 cells.

The differential effect of filipin and bleomycin A_2 upon V79 and AMB^R-1 cells was also confirmed by examining the dose response to filipin with or without bleomycin A_2. More than 70% of DNA synthesis activity in V79 cells was inhibited by 10 μg bleomycin A_2 per ml in combination with 5 μg filipin per ml (Chart 3*a*), whereas DNA synthesis of AMB^R cells was inhibited only by 25% of the control when bleomycin A_2 (10 μg/ml) was combined with 20 μg filipin per ml (Chart 3*b*). The finding that AMB^R-1 cells are apparently resistant to filipin is consistent with our previous report (8). Since resistant phenotype of AMB^R to polyene antibiotics is due to the altered cholesterol content (7),[3] it is suggested that the extent of synergistic effect of bleomycin and filipin is mediated through the level of cholesterol.

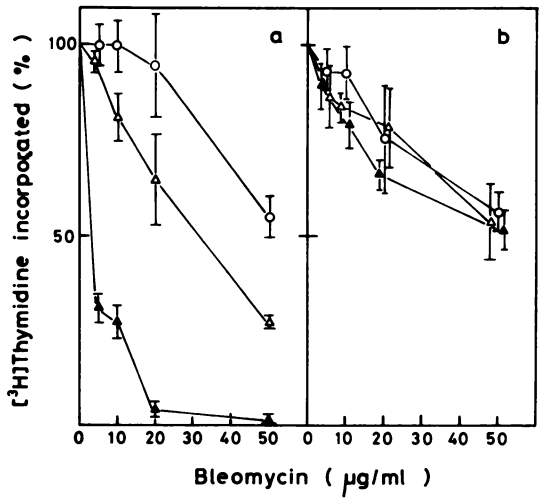

Chart 1. Dose response to bleomycin in the absence (○) or presence (△, 3 μg/ml; ▲, 6 μg/ml) of filipin of V79 (*a*) and AMB^R-1 (*b*). Both V79 and AMB^R-1 cells were exposed to various doses of bleomycin-A2 with or without filipin for 18 hr. Cellular DNA synthesis was determined as described in "Materials and Methods." Normalized activity of DNA synthesis from triplicate experiments is shown when 100% corresponds to 6741 cpm (V79) and 5454 cpm (AMB^R-1) in the absence of filipin, 6717 cpm (V79) and 5489 cpm (AMB^R-1) in the presence of filipin (3 μg/ml), and 5900 cpm (V79) and 4067 cpm (AMB^R-1) in the presence of filipin (6 μg/ml). *Bars*, S.E.

Table 1

Synergistic effect of bleomycin and various polyene antibiotics upon DNA synthesis of V79 cells

Polyene antibiotics	Concentration (μg/ml)	cpm at bleomycin concentration of 0 μg/ml		cpm at bleomycin concentration of 10 μg/ml	
None	0	2489 ± 120[a]	(100)[b]	2385 ± 122	(100)
Amphotericin B	20	2344 ± 140	(94)	2367 ± 153	(99)
	60	1115 ± 58	(45)	1125 ± 46	(47)
Nystatin	20	1921 ± 61	(77)	2125 ± 86	(89)
	60	1083 ± 59	(44)	982 ± 68	(41)
Filipin	6	2215 ± 160	(89)	546 ± 90	(23)
	15	1492 ± 54	(60)	28 ± 10	(1)
Pentamycin	1	2248 ± 293	(90)	897 ± 88	(38)
	2	1555 ± 64	(64)	29 ± 7	(1)
Pimaricin	50	2403 ± 196	(97)	120 ± 32	(5)
	75	1218 ± 45	(49)	119 ± 36	(5)

[a] Mean ± SE of triplicate experiments.
[b] Numbers in parentheses, DNA synthesis activity of polyenes alone, or of each combination of polyene and 10 μg bleomycin A_2 per ml when 100% corresponds to the activity in the absence (2489 cpm) and the presence (2385 cpm) of 10 μg bleomycin A_2 per ml, respectively.

[3] K. Hidaka, S. Akiyama, and M. Kuwano, submitted for publication.

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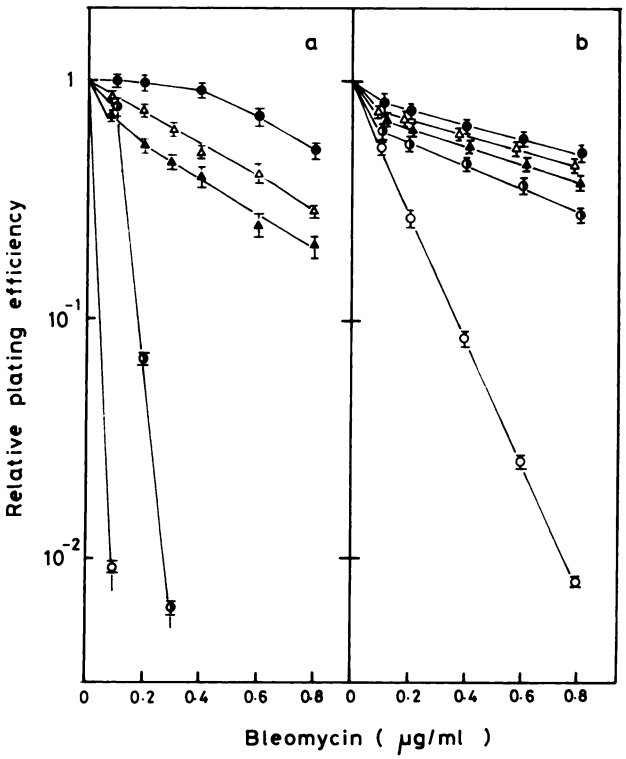

Chart 2. Dose response of the parental cell V79 (*a*) and AMB^{R}-1 (*b*) to bleomycin A_2 in the absence or presence of filipin, assayed by colony-forming ability. Cells (250 each) of V79 and AMB^{R}-1 were plated and exposed to various concentrations of bleomycin A_2 alone (●) or in combination with filipin [1 (△), 2 (▲), 3 (◑), or 4 (○) μg/ml] for 8 days. The *curves* drawn for each combination show the survival values obtained from triplicate experiments. *Bars*, S.E. Number of colonies in the absence of bleomycin A_2 with or without 1 to 4 μg filipin per ml was 150 to 216 in V79 (*a*) and 139 to 193 in AMB^{R}-1 (*b*).

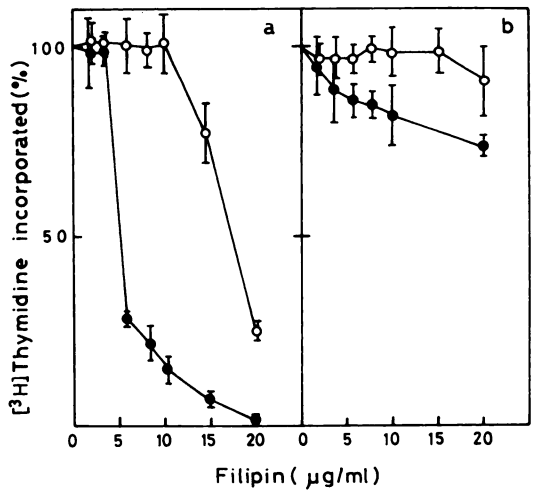

Chart 3. Dose response of V79 (*a*) and AMB^{R}-1 (*b*) to filipin in the absence or presence of bleomycin A_2. After cells were exposed to various concentrations of filipin alone (○) or in combination with bleomycin A_2 (10 μg/ml) (●) for 18 hr, [^{3}H]thymidine incorporation into acid-insoluble fraction was measured. The normalized activity of DNA synthesis is presented from triplicate experiments when 100% corresponds to 3825 cpm (V79) and 4253 cpm (AMB^{R}-1) in the absence of bleomycin A_2 and 3901 (V79) and 3785 cpm (AMB^{R}-1) in the presence of bleomycin A_2. *Bars*, S.E.

Duration of the Effect of Filipin to Enhance the Action of Bleomycin-A2 in Chinese Hamster Cells. To examine when the synergistic effect of bleomycin A_2 and filipin appears after treatment of cells with polyene antibiotics, V79 cells were exposed to 6 μg filipin per ml for various times, and then the culture medium was changed to remove filipin. At indicated times after the treatment, Bleomycin A_2 (20 μg/ml) was added immediately and was followed by incubation for another 10 hr. As shown in Chart 4, short time (20 to 30 min) exposure of V79 cells to filipin was enough to inhibit more than 80% of DNA synthesis when combined with bleomycin A_2. There appeared to be no inhibition of DNA synthesis in the absence of filipin.

We then examined how long the effect of filipin to potentiate the antitumor antibiotic continued after the removal of polyene antibiotics. The cells which were pretreated with filipin for 60 min were incubated in fresh medium and then 20 μg bleomycin A_2 per ml were added immediately (at Time 0) or at 1, 3, 5, 7, and 24 hr. It was found that the DNA synthesis of V79 cells was markedly inhibited by bleomycin A_2 even at 5 hr after the removal of filipin (Chart 5). The inhibition of DNA synthesis, however, could not be observed when V79 cells were cultured in the absence of the polyene for 7 hr or longer. It is concluded that the effect of filipin to enhance the action of bleomycin A_2 appears when V79 cells are exposed to the polyene briefly for 20 to 30 min and also that the cells treated with filipin could be susceptible to bleomycin A_2 during 5 hr after the removal of filipin.

Cellular Uptake of [^{14}C]Bleomycin A_2 in the Presence of Polyene Antibiotics. Our previous study showed that a synergistic effect of bleomycin A_2 and polymyxin B, which is not a polyene antibiotic, could be partly due to enhancement of permeation of bleomycin A_2 by polymyxin B in cultured rat cells (14). In this work, we found that pentamycin, pimaricin, and filipin could potentiate bleomycin (Table 1). To test whether or not the synergistic effect is due to altered permeation, we assayed cellular uptake of bleomycin in the absence or presence of polyene antibiotics. Autoradiographic study with radioactive bleomycin *in vitro* showed that the radioactivity was observed near the nuclear membrane after exposure of the cultured cell for 4 hr (6). We therefore assayed the cellular

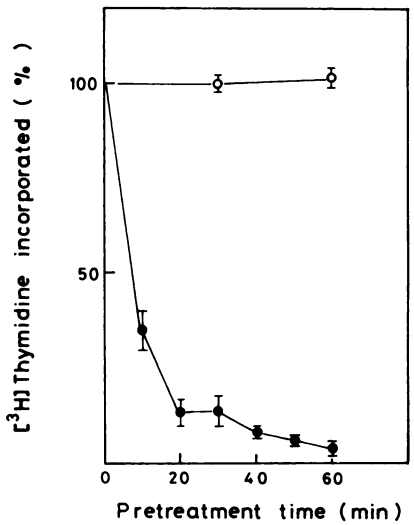

Chart 4. Exposure time to filipin and synergistic effect. V79 cells were exposed to filipin (6 μg/ml) for various times, and filipin was removed by changing medium at indicated time (●). Immediately after the removal of filipin, 20 μg bleomycin A_2 per ml were added, followed by incubation for 10 hr. Cellular DNA synthesis was determined from triplicate samples, as described in "Materials and Methods." *Bars*, S.E. As control (○), cells were cultured in the absence of filipin, but bleomycin A_2 alone was added at indicated times. Normalized activity of DNA synthesis is shown when 100% corresponds to 2063 cpm at time 0.

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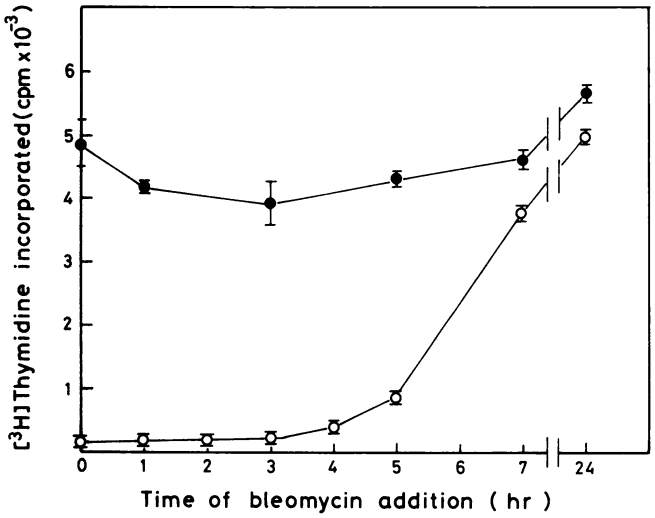

Chart 5. Duration of the effect of filipin to potentiate bleomycin A_2. V79 cells were exposed to 6 μg filipin per ml for 60 min (○), followed by incubation in fresh medium without filipin, and then 20 μg bleomycin A_2 per ml were added at indicated times. Cellular DNA synthesis was assayed by labeling with [^{3}H]thymidine for 4 hr after the cells were incubated with bleomycin A_2 for 10 hr. As control (●), V79 cells were not treated with filipin, but 20 μg bleomycin A_2 per ml were added. Values are the mean of 3 samples. *Bars*, S.E.

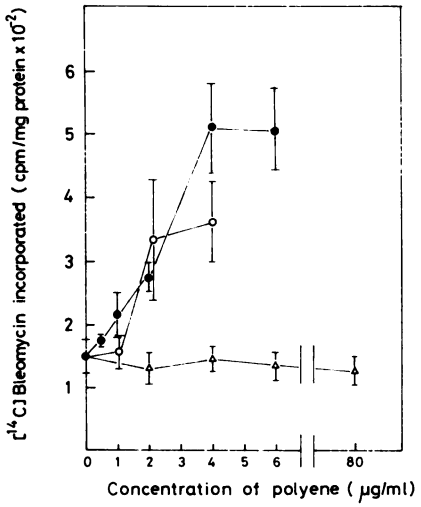

Chart 6. Incorporation of [^{14}C]bleomycin A_2 into V79 cells in the absence or presence of polyenes. Cells were treated with various doses of pentamycin (○), filipin (●), or amphotericin B (△). Incorporation of [^{14}C]bleomycin A_2 was determined from triplicate samples, as described in "Materials and Methods." *Bars*, S.E.

uptake of bleomycin A_2 when V79 cells were incubated with [^{14}C]bleomycin A_2 for 4 hr. As shown in Chart 6, the cellular uptake of [^{14}C]bleomycin A_2 was stimulated 2- to 4-fold reproducibly in the presence of pentamycin or filipin. The percentage of uptake of bleomycin A_2 ranged from 0.42 to 0.59% of input count in the presence of 4 μg pentaene polyenes per ml, while the uptake was 0.17% in the absence of the polyene.

DISCUSSION

Among 5 polyene antibiotics (filipin, amphotericin B, nystatin, pentamycin, and pimaricin) examined, filipin, pentamycin, and pimaricin could remarkably potentiate an anticancer agent, bleomycin A_2 (23). In accordance with synergistic effects, cellular uptake of radioactive bleomycin A_2 was enhanced by filipin or pentamycin, but not by amphotericin B. Since bleomycin A_2 was shown to induce breakdown of DNA chains *in vitro* (3, 20), enhanced permeation of the antitumor agent into the cells might cause reduction in cell survival. Bleomycin A_2, the structure of which was recently determined (21), may not be able to permeate the pore being formed by amphotericin B or nystatin (1, 4). One may assume that the membranous alteration induced by pentamycin or pimaricin is akin to that indicated by filipin, because these polyenes, filipin, pentamycin, and pimaricin, enhance the action of bleomycin A_2 remarkably. Recently, polyene antibiotics were classified into 2 groups: Group 1, antibiotics that caused K^+ leakage and cell death (or hemolysis) at the same doses of added polyenes; and Group 2, antibiotics that caused K^+ leakage at low doses and cell death (or hemolysis) at high doses (11). They found that Group 1 drugs included pimaricin (tetraene) or filipin (pentaene), while Group 2 included amphotericin B (heptaene) or nystatin (degenerated heptaene). Our work thus far appears to indicate that bleomycin A_2 is potentiated by Group 1 polyenes, but not by Group 2 antibiotics. In addition, amphotericin B or nystatin could make macrophages tumoricidal, but filipin and pimaricin, which are not ionophoretic, could not stimulate the tumoricidal activity of macrophages (2). It is interesting to note that the immunological study by Chapman and Hibbs (2) and our synergistic study thus far are consistent with the classification of polyene antibiotics, according to chemical structure and biological effects proposed by Kotler-Brajtbrug *et al.* (11).

Continuous incubation of animal cells with amphotericin B is necessary to enhance the cellular uptake of gigantic *Escherichia coli* DNA (12). We also found that the cells preexposed to amphotericin B lost their ability to potentiate the combined agents immediately after washing the cells once to remove the polyene.[4] By contrast, the cells treated briefly with filipin could continue to enhance the effect of bleomycin A_2 during 5 to 6 hr, even in the absence of the polyene antibiotic (Chart 5). This result may be due to higher affinity of filipin than of amphotericin B to membranous sterols. Alternatively, some membranous damage caused by the pentaene antibiotic may not be repaired rapidly, presumably until new synthesis of membrane structure resumes.

On the other hand, bleomycin A_2 was well known to show differential response to various tumors in humans, and it was suggested that cellular level of inactivation enzyme of bleomycin A_2 was correlated with the sensitivity of tumors to the anticancer agent (23). However, differences in permeation of the antibiotic may be involved in the different responses of tumors to bleomycin A_2, as discussed previously in the bacterial system (24). Further study will be necessary to verify the latter possibility with mammalian cells or tumors.

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