Amphotericin B Potentiation of the Cytotoxicity of Anticancer Agents Against Both Normal Hematopoietic and Leukemia Cells in Mice[1,2]

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ABSTRACT—Dose-response curves in AKR or $CD2F_1$ mice were obtained for AKR leukemia, L1210 leukemia, and hematopoietic stem cells following in vivo administration of one of the following anticancer agents alone or in combination with amphotericin B (AmB): doxorubicin, lomustine, or melphalan. AmB potentiated drug cytotoxicity of all agents against both leukemias. Potentiation of all three agents was greatest with the AKR leukemia demonstrating dose-modifying factor (DMF) values of 1.8, 3.0, and 2.7, respectively, for lomustine, melphalan, and doxorubicin. L1210 leukemia was somewhat less sensitive to drug potentiation by AmB with respective DMF values of 1.8, 1.8, and 1.3. AmB did not potentiate the action of any agent against hematopoietic stem cells. The increased life-span assays generally confirmed the clonogenic assays supporting the validity of the latter results. The specificity of AmB potentiation of anticancer agent cytotoxicity for tumor cells indicates that AmB may be a useful addition to antitumor drug combinations.—JNCI 1984; 73:475-482.

We previously demonstrated that the polyene antibiotic AmB can potentiate the cytotoxicity of a variety of anticancer agents against AKR leukemia (*1*). Two important questions result from these studies: 1) Is the level of potentiation of individual anticancer agents by AmB the same for all tumors? 2) Is the potentiation specific for tumor cells compared to normal cells? Answers to both of these questions are necessary not only to develop drug combinations that are likely to work clinically but also to judge whether results from experimental animal models can be used as a basis for planning human studies. We show in this study that AmB can potentiate the action of several different anticancer agents against both the AKR and L1210 leukemias; in addition, AmB does not potentiate the toxicity of the agents that we tested against normal CFU-S.

MATERIALS AND METHODS

Mice.—Studies with the AKR leukemia were done in AKR mice; studies with the L1210 leukemia were done in $CD2F_1$ [(DBA/2♂ × BALB/c♀)F_1] mice; studies on CFU-S were done in AKR mice. All mice were bred in our specific-pathogen-free animal facilities from foundation breeders provided by The Jackson Laboratory, Bar Harbor, Maine. They were housed 8 per cage and had free access to food and water.

For the in vivo studies on proliferating CFU-S, AKR mice were lethally irradiated (1,000 rad, ^{137}Cs γ-rays), and then immediately each was given an iv injection of 5×10^6 bone marrow cells harvested from syngeneic donors. Seven days later, when the CFU-S were in a rapidly proliferating state (*2*), the studies determining drug sensitivity were done.

Leukemia cells.—Both the AKR and L1210 leukemias are transplantable, widely disseminating lines. Their method of passage and the preparation of monodispersed leukemia cells have been discussed (*3, 4*). AKR mice received 10^6 leukemia cells iv, and $CD2F_1$ mice received 5×10^5 L1210 cells.

Drugs.—AmB, in the form of Fungizone, was purchased from E. R. Squibb & Sons, Inc., Princeton, N.J., and was dispersed in 5% dextrose in water immediately before use. The dose of AmB refers to dry weight of the commercial preparation (AmB + deoxycholate + salts). CCNU, Adria, and L-PAM were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, Md. CCNU was dissolved in a 1:1 mixture of polyethoxylated oil (Protameen Chemical Co., Totowa, N.J.) and absolute ethanol. Adria was dissolved in deionized water. L-PAM was first dissolved in 1 ml acidified ethanol (pH 1). All agents were diluted further in 0.9% NaCl and administered ip in a volume of 0.5 ml. In all of the experiments AmB was administered as 4 daily doses of 0.5 mg/mouse starting 1 day after tumor cell injection; the anticancer agent was administered immediately after the fourth AmB dose.

LCFU assay.—This assay is a spleen colony assay previously described (*4, 5*). Groups of 3 leukemic donor mice were killed 24 hours after drug treatment, and a monodispersed cell suspension of the femoral marrow

ABBREVIATIONS USED: Adria=doxorubicin (adriamycin); AmB=amphotericin B; CCNU=lomustine [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea]; CFU-S=colony-forming units of hematopoietic stem cells; DMF=dose-modifying factor(s); ILS=increased life-span; LCFU=leukemia colony-forming units; NCFU=normal hematopoietic colony-forming units; L-PAM=melphalan (phenylalanine mustard).

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was prepared. Fractions of this suspension were injected in 0.5-ml volumes into the tail veins of 8 recipient mice. The mice were killed 8 days later, their spleens were removed and placed in Bouin's fixative, and the macroscopic colonies were counted. The number of LCFU in the original donor femur was determined and expressed as fractional survival of LCFU relative to that of an untreated control.

Assay for NCFU.—NCFU were assayed in the femoral marrow of normal, treated mice by a procedure similar to that outlined above and previously described (*4*). Fractions of the cell suspension were injected into groups of 8 supralethally irradiated mice (1,000 rad, ^{137}Cs γ-rays), and the spleen colonies were counted 9 days later. In each experiment the endogenous control value was subtracted for each experimental result. Results are expressed as fractional survival of an untreated control.

Dose-survival relationships.—Dose-survival relationships of normal and leukemia cells were obtained after administration of different doses of anticancer agents alone or in combination with AmB. All data were fit by the least-squares methods to a simple exponential line. The extent of potentiation was expressed in terms of the DMF, which is the ratio of the slopes of the dose-survival curve for the drug alone compared to the curve for the AmB-drug combination. The slope was defined in terms of the $D_{½}$ value, i.e., the dose of agent required to reduce the surviving fraction by 50% on the exponential portion of the dose-survival curve.

ILS studies.—Groups of 10 AKR or CD2F$_1$ mice received 10^6 AKR or L1210 leukemia cells, respectively; 4 days later they were treated with either the anticancer agent alone or the AmB-drug combination. Survival was followed for 30 days. Mean survival time was determined, and the ILS was indicated as an absolute increase in survival time compared to the survival time in an untreated control. The control group for both leukemias had median survival times of 5-5.5 days. The data were all fit by eye.

RESULTS

Dose-Survival Relationships

Dose-survival curves of AKR and L1210 leukemia cells and CFU-S after exposure in vivo to CCNU, L-PAM, or Adria alone or in combination with AmB are presented in text-figures 1-3, respectively. In all cases exponential dose-survival curves were obtained. The $D_{½}$ values for the slopes are presented in table 1. Table 2 shows the DMF values. No cytotoxic effect was noted for AmB alone at the doses used in this study either against AKR leukemia (*6*) or against L1210 leukemia (Valeriote F: Unpublished results).

The results for CCNU (text-fig. 1; tables 1, 2) show that L1210 leukemia was more sensitive than either AKR leukemia or CFU-S to CCNU alone. While the dose-survival curves for AKR leukemia were exponential throughout the dose range studied, biphasic curves were noted for L1210 leukemia. In the calculation of the $D_{½}$ and DMF values for L1210 leukemia, the terminal slope (between 0.02 and 0.08 mg/mouse) was used. Both leukemias yielded a similar DMF value of 1.8. In marked contrast to the effect on leukemia cells, AmB did not significantly potentiate the cytotoxicity of CCNU against normal CFU-S (DMF of 1.2).

The results for L-PAM (text-fig. 2; tables 1, 2) showed a similar pattern to that noted above for CCNU. L1210 leukemia was more sensitive to L-PAM alone than either AKR leukemia or CFU-S. However, AmB potentiation of L-PAM cytotoxicity was greatest against AKR leukemia with a DMF of 2.3; for L1210 leukemia the DMF was 1.8. AmB did not potentiate the cytotoxicity of L-PAM against CFU-S (DMF of 1.1).

The results for Adria (text-fig. 3; tables 1, 2) were different from those for the other two anticancer agents. Again, L1210 leukemia was more sensitive than AKR leukemia to Adria alone; however, there was little potentiation of Adria cytotoxicity by AmB against L1210 leukemia (DMF=1.3). In contrast, there was a high level of potentiation against the AKR leukemia (DMF=2.7). As with the other agents, AmB did not potentiate Adria cytotoxicity against CFU-S (DMF=1.0).

Therefore, there was a marked difference between the AmB effects on leukemia and normal cells. AmB potentiated cytotoxicity of all of the agents tested against the former but had no effect on the latter. The extent of this difference was most striking at the highest dose levels of the anticancer agents. For example, at the highest L-PAM dose studied, AmB potentiation of cytotoxicity against AKR leukemia was greater than 1,000-fold.

Since the preparation of AmB used in this study (Fungizone) contains deoxycholate, we examined here whether it might potentiate the cytotoxicity of these anticancer agents. Accordingly, mice bearing AKR leukemia received 4 daily doses of 0.19 mg deoxycholate (the amount in 0.5 mg Fungizone) followed by single doses of CCNU (2 mg/mouse), L-PAM (0.2 mg/mouse),

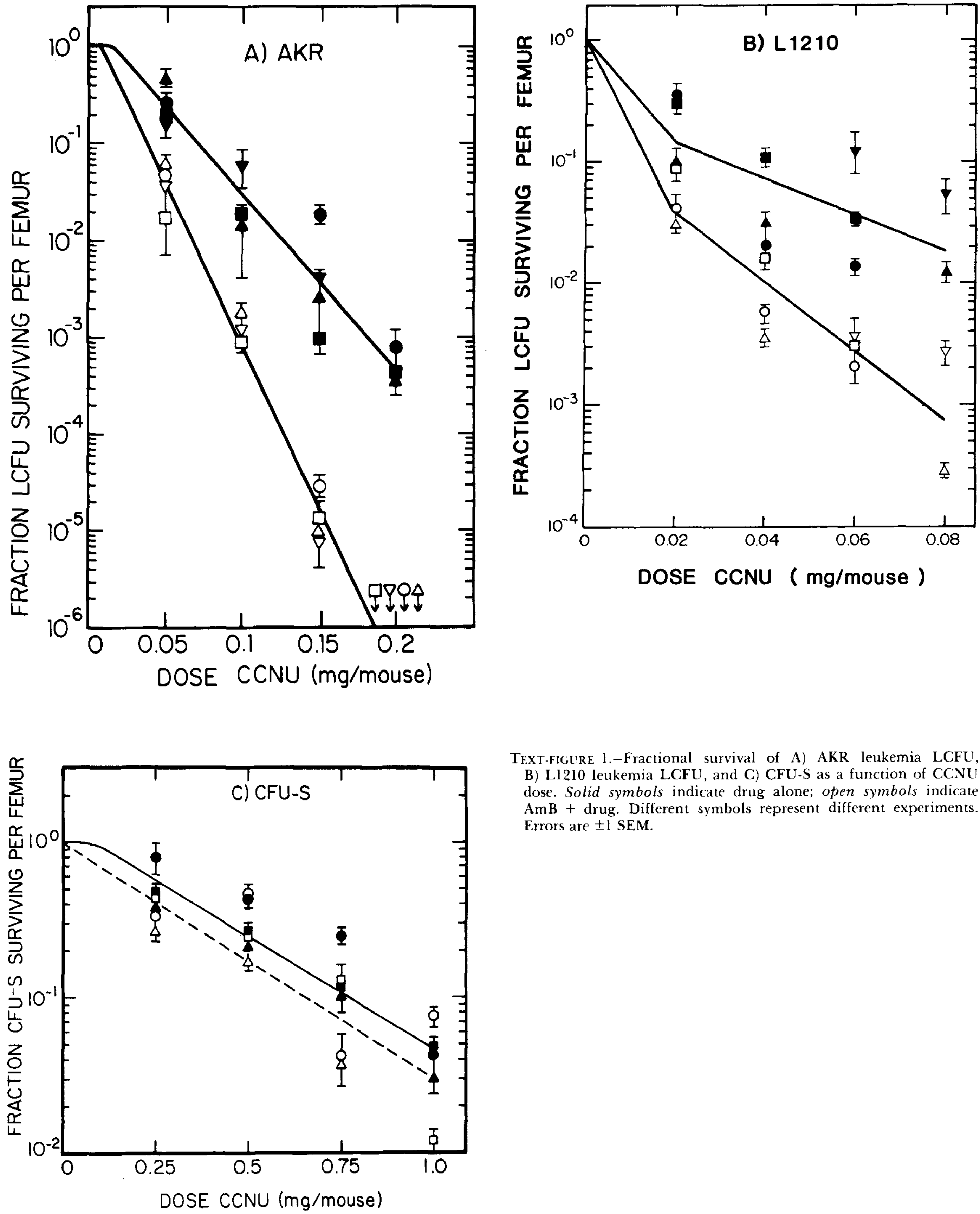
TABLE 1.—*Sensitivity of malignant and normal cells to anticancer agents alone or in combination with AmB*[a]

Agent	AKR leukemia	L1210 leukemia	CFU-S	Proliferating CFU-S
CCNU alone	0.0165	0.020	0.23	0.080
AmB + CCNU	0.0090	0.011	0.20	0.070
L-PAM alone	0.025	0.0018	0.080	
AmB + L-PAM	0.011	0.0010	0.070	
Adria alone	0.046	0.040	0.010	
AmB + Adria	0.017	0.033	0.010	

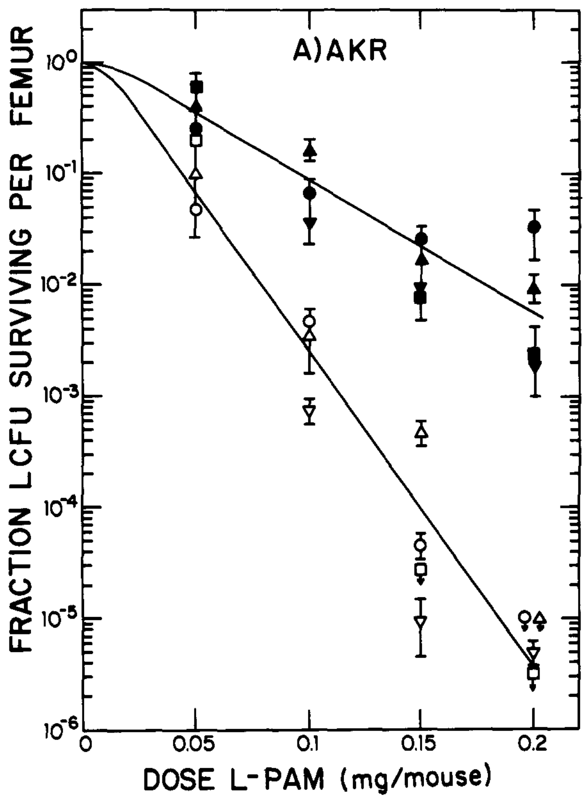
[a] $D_{½}$ values in mg/mouse.

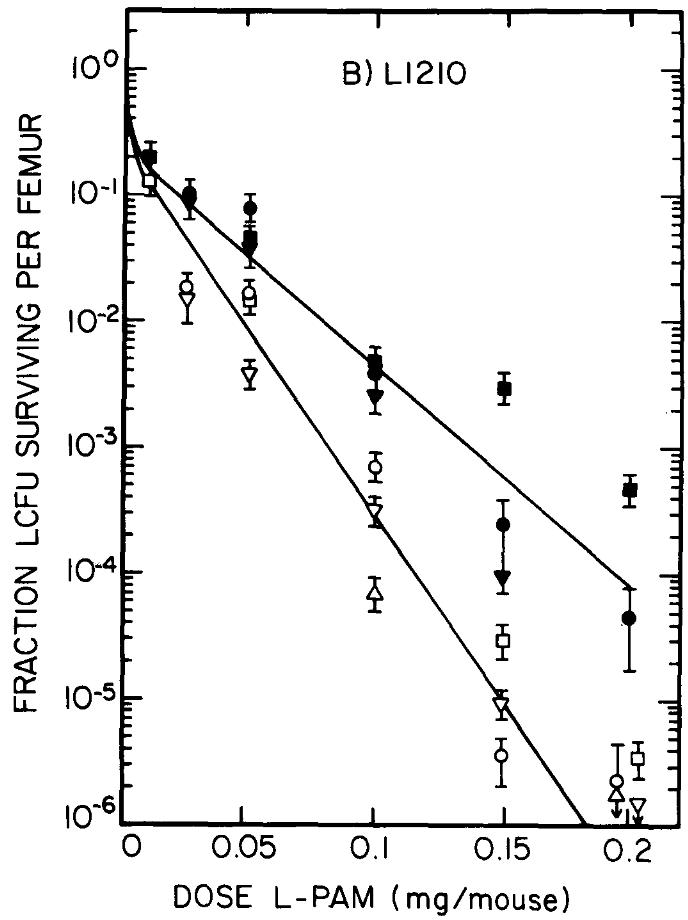
TABLE 2.—*DMF for AmB*

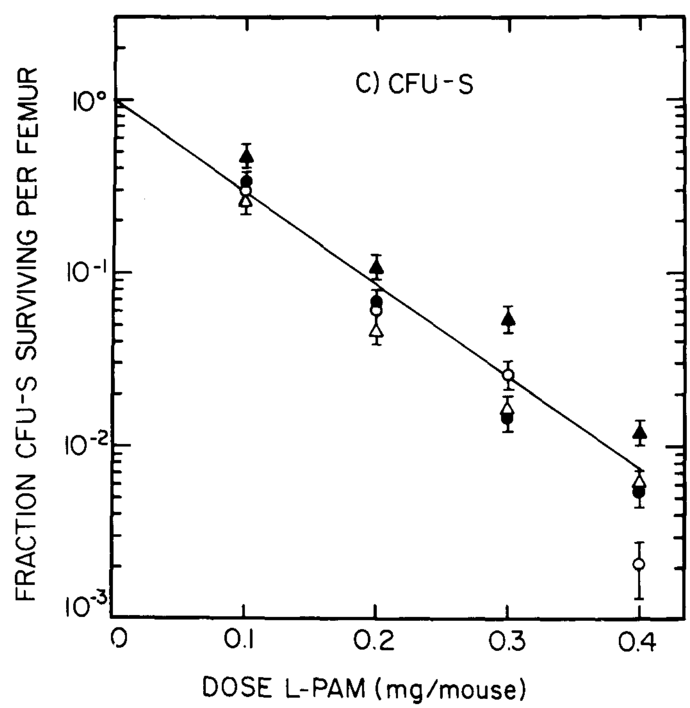
Agent	AKR leukemia	L1210 leukemia	CFU-S	Proliferating CFU-S
CCNU	1.8	1.8	1.2	1.1
L-PAM	2.3	1.8	1.1	
Adria	2.7	1.2	1.0	



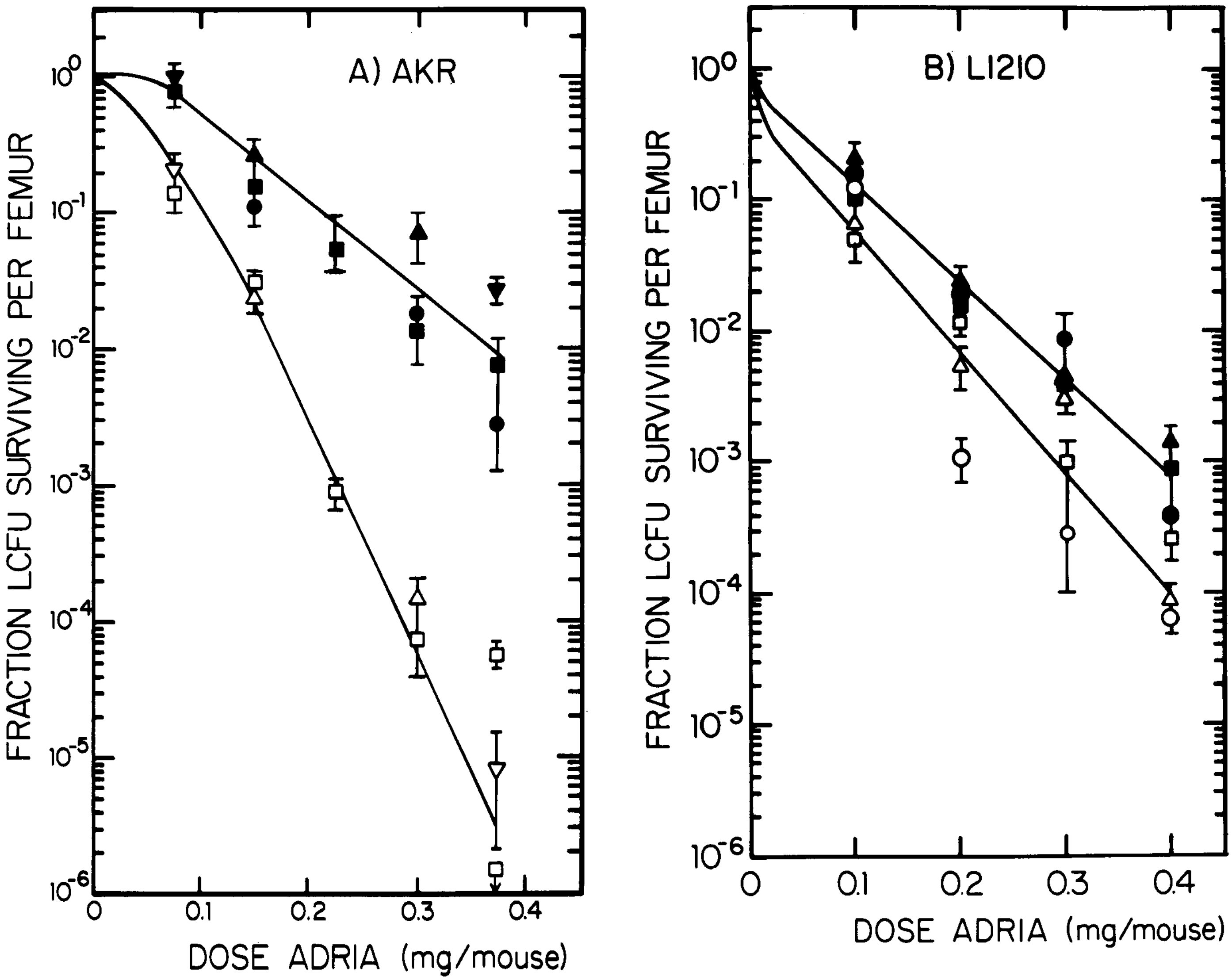
TEXT-FIGURE 1.—Fractional survival of A) AKR leukemia LCFU, B) L1210 leukemia LCFU, and C) CFU-S as a function of CCNU dose. *Solid symbols* indicate drug alone; *open symbols* indicate AmB + drug. Different symbols represent different experiments. Errors are ±1 SEM.

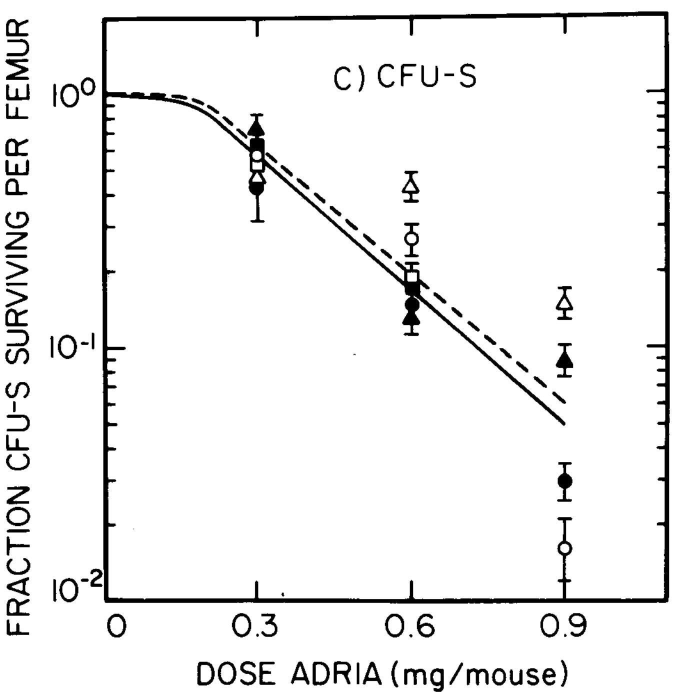




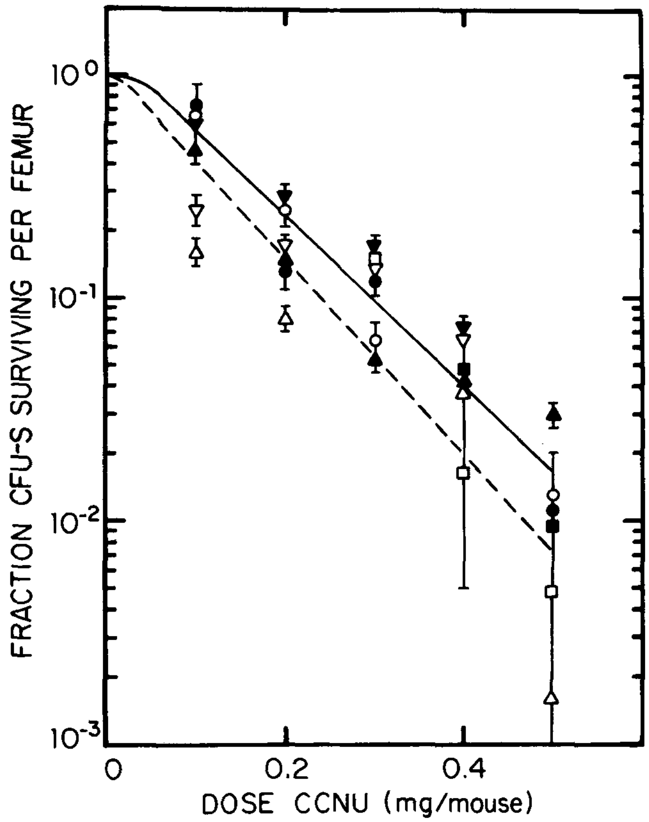


TEXT-FIGURE 2.—Fractional survival of A) AKR leukemia LCFU, B) L1210 leukemia LCFU, and C) CFU-S as a function of L-PAM dose. *Solid symbols* indicate drug alone; *open symbols* indicate AmB + drug. Different symbols represent different experiments. Errors are ±1 SEM.





TEXT-FIGURE 3.—Fractional survival of A) AKR leukemia LCFU, B) L1210 leukemia LCFU, and C) CFU-S as a function of Adria dose. *Solid symbols* indicate drug alone; *open symbols* indicate AmB + drug. Different symbols represent different experiments. Errors are ±1 SEM.



TEXT-FIGURE 4.—Fractional survival of proliferating CFU-S as a function of CCNU dose. *Solid symbols* indicate drug alone; *open symbols* indicate AmB + drug. Different symbols represent different experiments. Errors are ±1 SEM.

or Adria (0.3 mg/mouse). In all cases the surviving fraction of LCFU for the combination was within 20% of the value noted for anticancer agent alone.

One possible reason for the difference in AmB potentiation of anticancer agents between the normal and leukemia cells is the difference in the proliferation states of the two cell populations: The leukemia cells are all in cycle, whereas a large fraction of CFU-S is out of cycle (in a G_0 state). To examine this possibility, we tested the effect of CCNU alone and in combination with AmB on proliferating CFU-S. These results are presented in text-figure 4 and table 1. The proliferating CFU-S were more sensitive to CCNU than normal CFU-S (by a factor of 3.6), but again there was no demonstrable potentiation of CCNU cytotoxicity by AmB as evidenced by the parallel dose-response curves. AmB alone was not cytotoxic to proliferating CFU-S.

Effects of Anticancer Agents Alone and in Combination With AmB on ILS

ILS assays were done on mice bearing AKR or L1210 leukemia. The results for CCNU, L-PAM, and Adria are presented in text-figures 5 and 6.

The two assays were compared with the use of the following standard equation (7): Log_{10} cell kill=ILS value in days/(3.32) (*Td*), where *Td* is the tumor-doubling time and corresponds to about 0.4 days for both leukemias studied here (*8*). Therefore, a survival fraction of 10^{-1} corresponds to an ILS of 1.3 days.

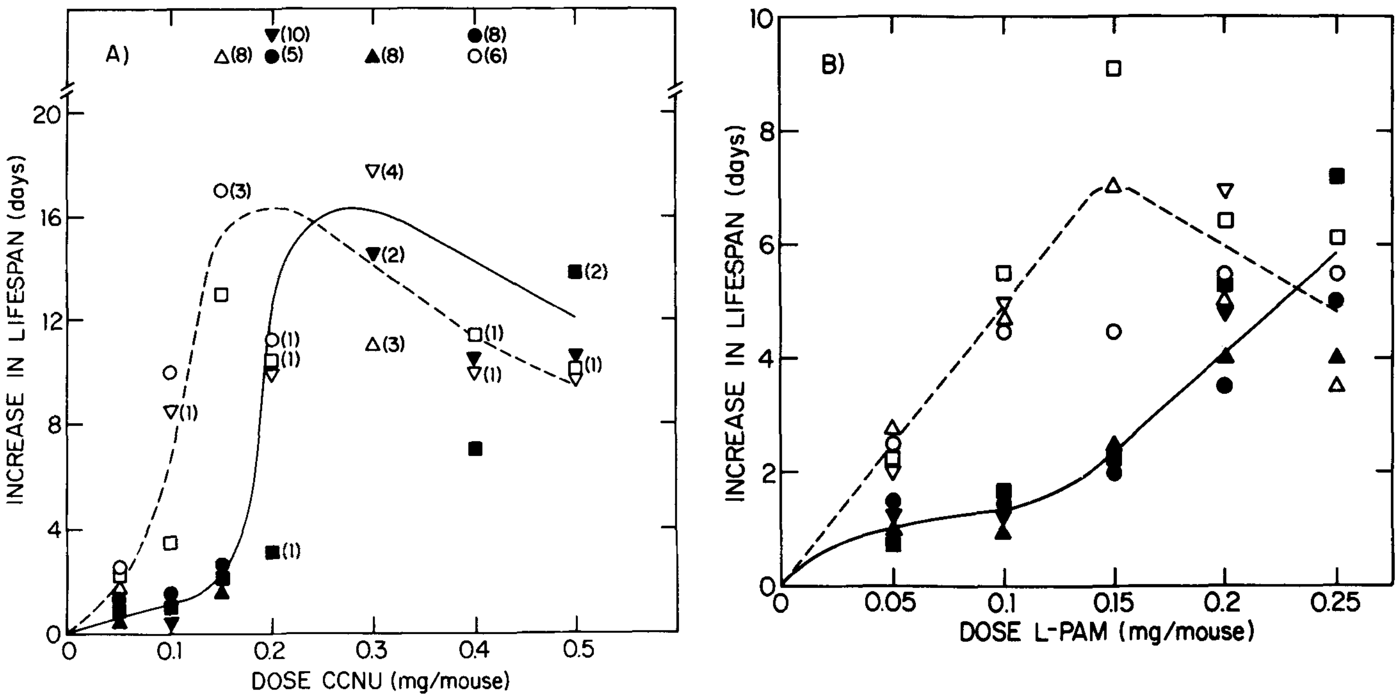
For AKR leukemia, CCNU alone at a dose of 0.15 mg/mouse caused an ILS of 2 days, a result consistent with the clonogenic assay (text-fig. 1, A). However, at 0.2 mg/mouse the life-span increased abruptly, and a significant fraction of mice was cured. When AmB was added to CCNU, both life-span and number of cures increased. The marked increase in life-span was greater than we expected from the approximate 10^3-fold potentiation of the cytotoxicity of 0.15 mg CCNU/mouse by AmB noted in the clonogenic assay (text-fig. 1, A). We believe that the disproportionate increase in life-span and the demonstration of cures for both CCNU alone at doses greater than 0.15 mg/mouse and for the combination with AmB at CCNU doses greater than 0.1 mg/mouse resulted from both the direct cytotoxicity of the combination on the tumor cells and the stimulation of the host's immune response to the tumor. We previously noted this "immunochemotherapeutic" effect in the treatment of AKR leukemia with AmB and another nitrosourea, carmustine (*5, 8*).

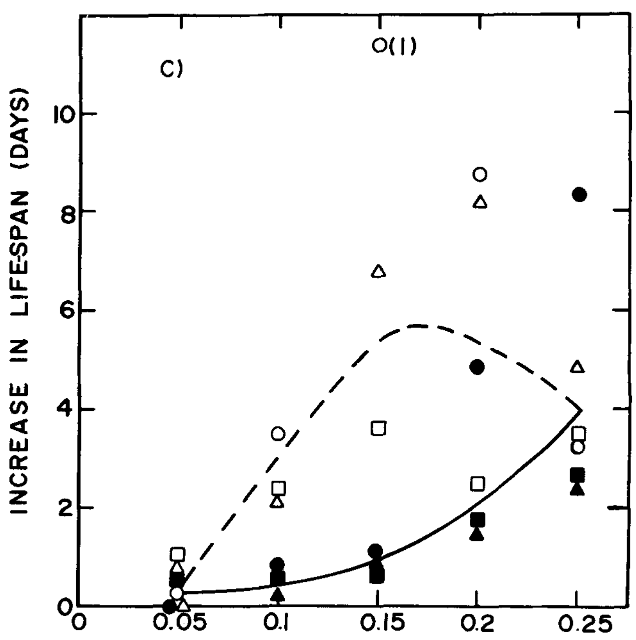
A 4-day ILS was observed with 0.2 mg L-PAM/mouse, and this ILS corresponded to the 10^{-2} to 10^{-3} leukemia cell survival noted at this dose (text-fig. 2, A). The significant potentiation of L-PAM cytotoxicity by AmB noted in the clonogenic studies was also evident in the ILS response for doses below 0.2 mg/mouse. Finally, the 2-day ILS found with 0.2 mg Adria/mouse corresponded to the 10^{-1} leukemia cell survival noted at this dose in the clonogenic assay (text-fig. 3, A). In addition, the further approximate 3-day ILS noted for AmB pretreatment in this dose was in accord with the hundredfold potentiation of Adria cytotoxicity shown by the clonogenic assay.

For L1210 leukemia, the ILS after CCNU was given alone was about 3 days for the group given 0.1 mg/mouse (text-fig. 6) and correlated with the extrapolated cell survival at this dose of 10^{-2} (text-fig. 1, B). The effect of AmB pretreatment was to increase the ILS noted for CCNU alone by about 2 days, again consistent with the corresponding clonogenic assay studied over the dose range to 0.08 mg/mouse. This combination of AmB and CCNU for L1210 leukemia resulted in a significant number of cures when doses greater than 0.1 mg/mouse were used, an observation similar to that noted above for AKR leukemia. No cures were obtained at any dose of CCNU alone.

DISCUSSION

Our data demonstrate that the potentiation of the cytotoxicity of anticancer agents by AmB was not unique to the AKR leukemia. This potentiation was also present when the drug-AmB combinations were tested against L1210 leukemia. With the exception of CCNU for the AKR leukemia, data for the drug alone or the drug-AmB combinations for both leukemias



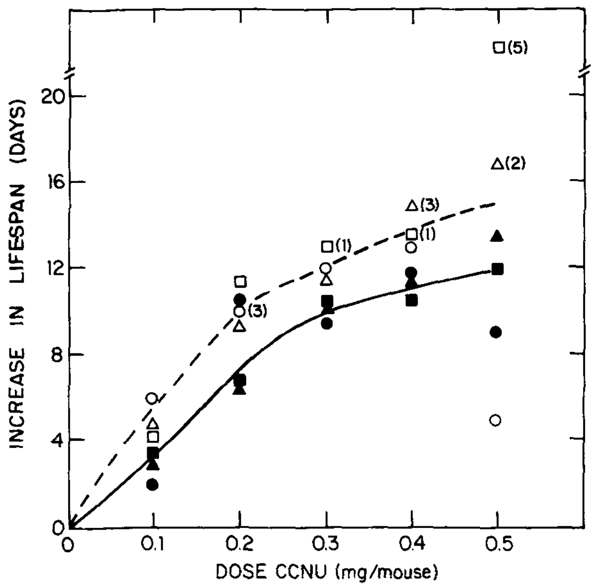


TEXT-FIGURE 5.—ILS of AKR-bearing mice following different doses of A) CCNU, B) L-PAM, and C) Adria. *Solid symbols* indicate drug alone; *open symbols* indicate AmB + drug. Different symbols represent different experiments.

demonstrated an excellent correlation between the ILS and the clonogenic assays. The discrepancy in the assays in the case of CCNU likely relates to the effect of the host immune system interacting with residual tumor (*6*).

Interestingly, the extent of potentiation of anticancer agent cytotoxicity by AmB was not identical in the two leukemias. It was similar for CCNU and L-PAM, even though L1210 leukemia was more sensitive than AKR leukemia to the agents. In contrast, the potentiation of Adria was much less in L1210 than AKR leukemia despite increased sensitivity of L1210 leukemia to Adria alone. Therefore, the level of AmB potentiation is not consistent in all tumor lines, and innate sensitivity to the anticancer agent is not predictive of potentiation by AmB.

A recent study by Ozols et al. (*9*) demonstrated no potentiation of survival for mice bearing L1210 leukemia for the combination of AmB-melphalan compared to the result observed with melphalan alone. The difference between their study and our study most likely relates to the large difference in the extent of



TEXT-FIGURE 6.—ILS of L1210 leukemia-bearing mice following different CCNU doses. *Solid symbols* indicate drug alone; *open symbols* indicate AmB + drug. Different symbols represent different experiments.

pretreatment of the mice with AmB. We chose the dose and schedule of AmB from previous studies that optimized the immunochemotherapy effect of AmB plus anticancer agents (*5*) and was not related to potentiation of the cytotoxicity of anticancer agents. An indepth study of dose and schedule of AmB for potentiation of cytotoxicity is being done.

We demonstrated here that the detergent in Fungizone, deoxycholate, was without effect on potentiation of drug cytotoxicity. Indeed, this finding was expected from our studies with a variety of polyenes (*10*) in which a water-soluble form of AmB, its methyl ester, which does not contain a detergent, was as potent, if not more potent, than AmB with deoxycholate.

AmB did not potentiate the cytotoxicity of the anticancer agents against CFU-S. Although the mechanism underlying this difference in response of normal and leukemia cells is not known, it has obvious therapeutic significance because it implies that increased tumor cell cytotoxicity can be obtained without increased host toxicity. This prediction is in agreement with the ILS assays presented here in which we showed that AmB in combination with the anticancer agents could prolong the life of tumor-bearing mice. The prediction also agrees with the phase I toxicity studies done by Presant et al. (*11*), which indicated little or no increased toxicity when AmB was added to a combination of anticancer agents.

The difference in response between the tumor and normal cells did not seem to be a consequence of a difference in their proliferative state. Although proliferating CFU-S were much more sensitive than the normal, resting population to CCNU, AmB did not potentiate cytotoxicity of CCNU against proliferating CFU-S. The mechanism of the specificity of AmB for the leukemia cells compared to that for normal cells is unknown, but on the basis of the known membrane effects of AmB, we presume that it relates to a difference in membrane constituents.

In summary, we have demonstrated that AmB can potentiate the cytotoxicity of CCNU, L-PAM, and Adria against AKR and L1210 leukemia cells by both clonogenic and ILS assays. There was no potentiation against normal cells. The potentiation was predictive neither from factors such as innate sensitivity of the tumor cell to the anticancer agents nor from the proliferative state of the cells. Therefore, potentiation of a given agent for a specific tumor likely will have to be determined before the combination can be effectively used clinically.

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