Synergistic Effect of Amphotericin B and 1,3-Bis(2-chloroethyl)-1-nitrosourea against a Transplantable AKR Leukemia

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SUMMARY
Long-term survivors of mice bearing a transplanted leukemia have been obtained following treatment with a combination of amphotericin B and 1,3-bis(2-chloroethyl)-1-nitrosourea. The proportion of mice surviving at 28 days depended upon the order and timing of drug administration. Optimal results were obtained when 0.5 mg amphotericin B was given 24 hr before 0.2 mg 1,3-bis(2-chloroethyl)-1-nitrosourea.

Spleen colony assays showed that the survivors harbored viable leukemic cells for a number of days following treatment. Nevertheless, with the most effective regimen, about 75% of the survivors appeared healthy and had no detectable leukemic cells at the end of the 28-day period of observation. The survivors were resistant to further rechallenge with 10^6 leukemic cells, a dose that caused fatal leukemia by Day 6 in all untreated controls. About 25% of the surviving mice became paralyzed and died 4 to 6 weeks after treatment, with leukemic cells assayable in their femoral marrow and widespread leukemic involvement of their central nervous system.

INTRODUCTION
In vitro synergy has been demonstrated for AMB in combination with each of a variety of other agents against cell cultures of human KB and HeLa cells and against mouse L-cells (9, 10). This synergism may be a consequence of an AMB-induced increase in permeability of the cell membrane, which allows more of the 2nd agent to enter the cell (5, 7, 8).

In addition to the potentiation of 2nd agents, AMB has also been shown to modify the resistance of mice to infection with Listeria monocytogenes (13).

This report describes the combined effect of AMB and BCNU against a transplantable AKR leukemia. The treatment regimen rapidly established, at certain times and doses, a fall in colony-forming units which was poorly correlated with prolonged survival (3). Four to 6 weeks after therapy some animals died with extensive central nervous system leukemia.

MATERIALS AND METHODS
Anticancer Agents. AMB in the form of Fungizone was purchased from E. R. Squibb & Sons, Inc., Princeton, N. J. All dosages of AMB refer to amounts of Fungizone. BCNU was obtained from the Drug Development Branch of the National Cancer Institute, Bethesda, Md. Both AMB and BCNU were prepared in 5% glucose in water and administered i.p. in a volume of 0.5 ml.

Mice and Leukemic Cells. Female AKR mice were obtained from The Jackson Laboratories, Bar Harbor, Maine. They were 7 to 9 weeks old and weighed about 22 g. The tumor cell line was a transplantable, widely disseminating, syngeneic, lymphocytic leukemia (1).

Assay for LC FU and NCFU. The assay for LC FU was developed by Bruce and Van der Gaag (3) and involves the quantitation of those leukemic cells capable of proliferation in syngeneic recipient mice sufficient to produce macroscopic spleen colonies. Mice were killed following treatment, their femurs were removed, and a monodispersed suspension of cells in an enriched minimal essential medium (αMEM; Flow Laboratories, Rockville, Md.) was prepared from the marrow (15). Into each of 10 recipient syngeneic AKR mice was injected i.v. of 0.5 ml of appropriate dilutions. Eight days later, the recipient mice were killed, their spleens were placed in Bouin's fixative, and the number of macroscopic colonies was counted. The results provide an estimate of the number of LC FU in the femoral marrow of the donor mice at the time of assay.

The toxicity of the drugs to NCFU was determined by a similar spleen colony assay developed by Till and McCulloch (14). In this assay, nonleukemic donor mice were used; the recipients were supralethally irradiated prior to cell injection, and the spleens were removed 9 days later.
RESULTS

Survival of Leukemia-bearing Mice. In a previous publication (10), we demonstrated that treatment with a single dose of AMB followed 24 hr later by a single dose of BCNU resulted in long-term survival of leukemia-bearing mice. In the 1st part of the study reported here, we confirm our previous results and show that the increased survival is a result of cellular and therapeutic synergism of the AMB and BCNU. Chart 1 shows that, in 3 separate experiments, using 100 mice in the combined treatment group, 44, 40, and 30% of the animals survived for 28 days, whereas 100% of untreated and AMB (0.5 mg)-treated animals were dead on Day 6 (1 and 2 mg of AMB alone did not prolong survival when compared to control animals). Of animals treated with BCNU (0.2 mg), 100% were dead by Day 8; 100% of animals treated with 0.4 mg BCNU were dead by Day 13.

The survival experiments were arbitrarily ended at 28 days. Mice harboring 1 viable leukemic cell die in about 12 days (2).

Timing of BCNU Administration. Mice received 10^6 leukemic cells, and 3 days later groups of 5 mice were given 0.5 mg of AMB and, at different times thereafter, a single dose of 0.2 mg of BCNU. The percentage of animals surviving for 28 days is shown in Chart 2. The combination was successful in generating long-term survivors only when BCNU was given 18 hr or longer after the AMB. The maximal survival was obtained when the interval between the drugs was 24 hr, and by increasing the interval to greater than this the survival decreased rapidly. As in the previous experiments, untreated mice or mice given AMB alone all died within 6 days of the injection of leukemic cells and mice given 0.2 mg BCNU alone died by Day 8.

Kinetics of LCFU Response following the Different Treatments. In our previous publication (10) we noted an apparent discrepancy between the survival data and the LCFU data obtained 24 hr after drug treatment. Although at 24 hr the mice treated with AMB and BCNU had about 9- to 10-fold reduction in LCFU, compared to the mice treated with BCNU alone, the AMB- and BCNU-treated mice still had enough leukemic cells so that their survival should have been prolonged by only 1 to 2 days. Table 1 shows that there is a poor correlation between the number of LCFU per femur at 24 hr and the percentage of animals surviving for 28 days. In this experiment the lowest number of LCFU per femur (7 x 10^5) was seen with the 0.4 mg BCNU treatment, but none of these animals was alive by 28 days. Although the animals receiving the drug combination had fewer LCFU per femur than the animals treated with 0.2 mg of BCNU alone, the levels within the different combination groups were similar; in contrast, the percentages of survival varied greatly.

Chart 3 shows the average LCFU per femur for groups of 5 mice 3 days after the injection of leukemic cells and on subsequent days up to 28 days. Although this chart is made up of 5 different experimental groups (different symbols), a consistent pattern can be discerned. In the untreated controls, there was a constant increase in LCFU per femur from Day 3 through Day 5. All of the untreated mice were dead by Day 6. AMB alone had no effect on the number of LCFU per femur of the leukemic mice. BCNU alone decreased the number of LCFU per femur by a factor of 10, but the LCFU population then increased at approximately the same rate as controls. All of the animals treated with BCNU alone were dead by Day 8. The combination of AMB and BCNU decreased the number of LCFU from controls by a factor of 50 with a subsequent gradual increase through Day 12. There was then a 2nd sharp decline in the measurable LCFU per femur which fell below the limits of our assay (<0.2 LCFU/femur) by about Day 17.

Three weeks after treatment it became apparent that the surviving mice included at least 2 populations. Seventy-five % of the mice appeared completely healthy, and there were no further deaths in this group during the period of observation. The other 25% of the mice that survived for 3 weeks developed paralysis of their hind legs, and some of these animals developed retroorbital and soft tissue tumors.
Table 1
Comparison of LCFU/femur and 28-day survival after different treatment regimens

All mice received $10^6$ leukemic cells i.v. on Day 0. AMB was given i.p. on Day 3, and BCNU was given either on Day 4 or at times noted. Groups of 10 mice were used for each treatment regimen. Five mice in each group were sacrificed, and the femoral marrow content of LCFU was determined. The other 5 mice in each group were followed for survival.

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>LCFU/femur 24 hr after treatment</th>
<th>% survival at 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$6.4 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>0.5 mg AMB</td>
<td>$5.0 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>0.2 mg BCNU</td>
<td>$3.6 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>0.4 mg BCNU</td>
<td>$7.0 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>0.5 mg AMB + 0.2 mg BCNU simultaneously</td>
<td>$1.4 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>0.5 mg AMB + 0.2 mg BCNU 3 hr later</td>
<td>$1.4 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>0.5 mg AMB + 0.2 mg BCNU 6 hr later</td>
<td>$1.2 \times 10^4$</td>
<td>20</td>
</tr>
<tr>
<td>0.5 mg AMB + 0.2 mg BCNU 12 hr later</td>
<td>$2.5 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>0.5 mg AMB + 0.2 mg BCNU 18 hr later</td>
<td>$1.0 \times 10^4$</td>
<td>60</td>
</tr>
<tr>
<td>0.5 mg AMB + 0.2 mg BCNU 24 hr later</td>
<td>$8.0 \times 10^3$</td>
<td>100</td>
</tr>
</tbody>
</table>

DISCUSSION

The administration of AMB followed by the anticancer agent BCNU resulted in long-term survival of mice inoculated with a large number of leukemic cells. Although the percentage of survivors varied greatly in the experiments shown in Charts 1 and 2 and Table 1, they are impressive considering that the animals were quite ill at the time of treatment and received only 1 dose of each drug. Moreover, from the data in Chart 3, there are about $10^5$ LCFU in the femoral marrow of each mouse treated with the drug combination. Because this number represents between 0.1 and 0.01% of the actual number of leukemic cells in the host (2, 3), we can estimate that approximately $10^4$ to $10^5$ leukemic cells are being eliminated from each mouse that subsequently survives for 28 days after treatment with the combination.

The effects of AMB may include the enhancement of cell membrane permeability to BCNU so that it becomes a more effective agent. This is suggested by our in vitro data using fungi and animal cells and data from other laboratories showing AMB potentiation of uptake of a labeled rifamycin derivative into animal cells (5, 9). In vivo, the further decline in LCFU seen 24 hr after treatment with AMB and BCNU when compared to each agent alone further supports this hypothesis (Chart 3; Table 1).

However, the pharmacological effect of AMB on membrane permeability cannot explain our results. For example, 0.4 mg of BCNU decreases LCFU survival further than the drug combination but BCNU alone does not lead to long-term survivors (2), whereas the combination does. Moreover, serial determination of LCFU in the combination-treated animals demonstrates that the long-term survival is not the result of an immediate complete elimination of leukemic cells, but rather a stabilization of cell number for some days followed by an apparent disappearance of leukemic cells.
Synergism of AMB and BCNU

Fig. 1. Photomicrographs of sections of cerebral cortex from AKR mice demonstrating dense infiltration of the subarachnoid space with leukemic cells (A); normal brain is shown for comparison (B). H & E. Original magnification, × 250. The prints have been enlarged 3-fold.

Table 2
Survival of NCFU 24 hr after drug treatment
The experimental procedure is described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of NCFU/femur 24 hr after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.0 × 10^4</td>
</tr>
<tr>
<td>0.5 mg of AMB</td>
<td>2.2 × 10^4</td>
</tr>
<tr>
<td>0.2 mg BCNU</td>
<td>9.5 × 10^3</td>
</tr>
<tr>
<td>0.5 mg AMB + 0.2 mg BCNU</td>
<td>9.6 × 10^3</td>
</tr>
<tr>
<td>given 24 hr later</td>
<td></td>
</tr>
</tbody>
</table>

The apparent stabilization of LCFU and the poor correlation between LCFU and long-term survival suggest that AMB might be affecting host immune response to the leukemic cells. There is considerable evidence that polyenes can boost the immune response. Vitamin A (like Bacillus Calmette-Guérin) has been shown in independent studies to have adjuvant properties (6). In addition, we have earlier reported evidence for a strong effect of AMB on the response of mice to infection with the intracellular pathogen L. monocytogenes: that effect appears to be mediated by modification of host immunity (13). Also consistent with an immune-mediated response in the leukemia model are the observations that the leukemic cells in the treated animals were still lethal to previously unchallenged mice and that the surviving animals were immune to rechallenge with 10^6 leukemic cells.

Other workers have achieved results comparable to ours using another polyene, vitamin A alcohol, in combination...
with BCNU (4) against murine L1210 leukemia; however, nearly curative doses of BCNU had to be used in that system and only survival was measured. The critical dependence on the time between the administration of the drugs in our experiments is similar to the observations in the study with vitamin A and also in other studies using Bacillus Calmette-Guérin in combination with cyclophosphamide (11). This is also consistent with an adjuvant effect of AMB.

Although our experiments do not provide direct proof of the mechanism of action of AMB, we suggest that the observations are potentially important for several reasons. (a) An apparent synergistic relationship between AMB and BCNU is established which is therapeutic for mice with advanced leukemia. The therapeutic effect may be dependent on the membrane-active properties of AMB and perhaps on an increase in the host’s resistance to the tumor. A thorough determination of the mechanism of the AMB effect which leads to prolonged mouse survival may reveal important features about host reaction to tumors. (b) The meningeal leukemia, manifested by paralysis and the pathology shown in Fig. 1, which develops after 3 weeks following the combined treatment, is similar to the central nervous system leukemia that develops in children who have been in prolonged remission of acute lymphoblastic leukemia (12). Our experimental system may provide a means to investigate the mechanism of this form of treatment failure. (c) The experimental design of determining serial LCFU per femur and comparing this with survival data and studies of pathology provides an important technique for investigating the mechanisms of responses in different kinds of cancer therapy.

REFERENCES


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*Cancer Res* 1974;34:974-978.

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