Synergistic Effect of Adriamycin and Ricin on L1210 Leukemic Cells in Mice

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ABSTRACT

The effect of Adriamycin and ricin, singly and together, on the survival of L1210 leukemic cells in mice has been assessed by spleen colony and end-point dilution assays. Adriamycin, as well as ricin, had a stronger effect on leukemic than on normal bone marrow cells. Rapidly proliferating normal cells were as sensitive to Adriamycin as were the leukemic bone marrow cells. In contrast, the preferential effect of ricin was not proliferation dependent.

Combinations of Adriamycin and ricin gave a less than additive toxicity, as measured by the survival of nonleukemic mice. In mice with systemic leukemia, combinations of Adriamycin and ricin exhibited a synergistic effect on the life span of the animals, corresponding to a reduction in the leukemic cell burden by 1 to 2 logs. In the bone marrow, the synergism amounted to a reduction in the fraction of surviving leukemic cells by up to 5 logs, whereas in liver, spleen, and brain the synergism was several orders of magnitude lower. The combinations showed no increased effect on resting normal bone marrow cells, and only an additive effect on rapidly proliferating normal cells.

In mice inoculated with leukemic cells intracerebrally, i.v. administration of either Adriamycin or ricin did not affect the survival, whereas the combination definitely increased the life span of the animals.

In experiments on L1210 cells in culture, only a moderate synergistic effect was found, of a magnitude similar to that found for the liver and spleen of leukemic animals treated in vivo. Similar results were obtained when leukemic bone marrow was exposed in vitro and the effect on the leukemic cells was assayed in vivo.

The results indicate that the observed synergistic effect of Adriamycin and ricin is due to some special property of the leukemic cells and cannot be accounted for by their rapid rate of proliferation. Conceivably, the presence of one agent facilitates the entry of the other one into the cells. The activity of the combination in intracerebral leukemia may involve an effect on the blood-brain barrier. The particular high potentiation observed in the bone marrow remains unexplained.

INTRODUCTION

Recent progress in the chemotherapy of cancer has to a large extent been due to a more rational use of previously established cytostatic agents and, in particular, to the use of combination therapy involving several drugs with different mechanisms of action (23). With the many clinically active cytostatic agents now available, a systematic testing of the numerous possible combinations is only feasible in animal models.

In previous studies on one of the most useful murine tumors, L1210 leukemia, it was found that the toxic lectin, ricin, prolongs the life span of mice inoculated i.p. with the leukemic cells (9). Spleen colony assays showed that ricin had a much greater effect on leukemic than on normal bone marrow cells and that the differential effect was about as strong as that of Adriamycin, a drug widely used in the treatment of various types of human cancer (3). Ricin and the closely related plant toxin, abrin, act by a mechanism different from that of Adriamycin, namely, by specifically inhibiting cellular protein synthesis (for review, see Refs. 16 and 17), and they have only a slight effect on normal myelopoiesis (6). Recently, it has been shown that abrin can potentiate the effect of other chemotherapeutic agents (5, 11). Against this background, we decided to test in L1210 leukemia the effect of ricin and Adriamycin given concurrently. Part of the data have previously appeared in a preliminary report (10).

MATERIALS AND METHODS

Tumors and Animals. L1210 lymphoid leukemic cells were obtained from the NIH, Bethesda, Md., in 1974 and have been serially transplanted as an ascitic tumor in DBA/2 mice. Mice of both sexes, weighing 20 to 25 g, were used. The animals were purchased from the Laboratory Breeding and Research Center, Gml. Bomholt Gaard, Ry, Denmark. The leukemic cells used in the experiments were obtained from the ascitic mice and were diluted to appropriate concentrations.

Chemotherapeutic Agents. Adriamycin was obtained from Farmitalia, Milan, Italy, and ricin was isolated from the seeds of Ricinus communis (15). The 50% lethal dose for Adriamycin was 18 mg/kg, and the minimum lethal dose for ricin was 2.7 μg/kg.

Spleen Colony Assays. The spleen colony assay for leukemic colony-forming units was performed as previously described (9), according to the method of Bruce and van der Gaag (1). Mice were made leukemic by injecting 1 × 10⁶ L1210 cells i.v. on Day 0, and the animals were divided into groups of 2 to 8 mice. One group was used as control, and the other groups were treated on Day 2 with Adriamycin, ricin, or the 2 drugs given concurrently. The doses used are given in the charts and tables. The mice were killed on Day 5, femurs were isolated, and the bone marrow cells were washed out with Medium 199 (Gibco, Glasgow, Scotland). Appropriate dilutions (sufficient to give approximately 10 to 20 colonies/spleen) were injected into the tail veins of recipient mice (groups of 8). These were killed on Day 13, and the spleens were removed and fixed in Bouin's solution. The microscopic colonies were counted, and the results were normalized to the untreated control and expressed as the fraction of surviving colony-forming units per femur.

Normal bone marrow colony-forming units were assayed in the same way as the leukemic colony-forming units, except that the bone marrow cells were injected into irradiated recipient mice.
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Rapid proliferation of normal stem cells was induced by injecting $5 \times 10^6$ normal bone marrow cells into mice irradiated with 660 rads. These mice were treated with the drugs 7 days later, and the assay was then continued as for normal colony-forming units (19).

**End-Point Dilution Assay.** To assay the effect of the treatment on the leukemic cells in different tissues, an end-point dilution assay, essentially as described by Hewitt (13), was used. Mice were given injections of $1 \times 10^6$ L1210 cells on Day 0 and treated on Day 2 with Adriamycin, ricin, or the 2 drugs given concurrently. Three days later, the animals were killed, and cell suspensions were made from bone marrow, liver, spleen, and brain. Appropriate dilutions of cells obtained from treated and untreated animals were injected i.p. into recipient mice, and the survival time was recorded. The fraction of leukemic cells surviving treatment in the different tissues was estimated by determining the titers of the cell suspensions from treated and untreated animals that resulted in the same life span of the recipient mice.

**RESULTS**

**Effect of Adriamycin and Ricin on Bone Marrow Cells.** In normal bone marrow the majority of the stem cells are in a noncycling resting state (20), whereas in mice with systemic L1210 leukemia probably all the leukemic bone marrow cells are rapidly proliferating. To see whether the previously demonstrated preferential effect of Adriamycin and ricin on leukemic cells (9) could be accounted for by their higher rate of proliferation, the sensitivity to Adriamycin and ricin of rapidly proliferating stem cells was compared with that of resting stem cells and leukemic cells.

The results in Chart 1 demonstrate that after treatment of the animals with Adriamycin or ricin, exponential dose-effect curves were obtained in the spleen colony assays and that both Adriamycin and ricin had a much stronger effect on leukemic than on normal cells. It is apparent (Chart 1A) that the proliferating normal cells were as sensitive to Adriamycin

**Chart 1.** Influence of cell proliferation on the sensitivity of bone marrow cells to Adriamycin and ricin. Normal and leukemic mice were treated i.v. with the doses of Adriamycin (A) and ricin (B) indicated, and the number of residual colony-forming cells in the bone marrow was assayed as described in “Materials and Methods.” The proliferation of normal cells was induced by injecting normal bone marrow cells into mice previously irradiated with 660 rads. Each symbol represents the mean for 6 mice; bars, S.E.; O, normal cells; x, proliferating normal cells; A, leukemic cells; CFU, colony-forming units.

as were the leukemic cells. In contrast, the sensitivity of the proliferating cells to ricin (Chart 1B) was much less than that of the leukemic cells. In fact, it was about the same as that of the resting normal cells. The results show that the effect of ricin, in contrast to that of Adriamycin, is not proliferation dependent.

This is consistent with the observation (18) that ricin is equally effective in different phases of the cell cycle.

In Chart 2, the results are shown for combinations containing a constant dose of ricin and increasing doses of Adriamycin on normal and leukemic bone marrow cells. Groups of mice were treated i.v. with combinations of $1 \mu g$ of ricin per kg plus the indicated doses of Adriamycin, and the number of surviving colony-forming bone marrow cells was determined by spleen colony assays, as in Chart 1. The survival of leukemic cells was also measured by end-point dilution, as described in “Materials and Methods.” X, end-point dilution. The other symbols denote spleen colony assays. Each symbol represents the average; bars, S.E.; CFU, colony-forming units.

**Chart 2.** Effect of combinations containing a constant dose of ricin and increasing doses of Adriamycin on normal and leukemic bone marrow cells.

Groups of mice were treated i.v. with combinations of $3.75 \mu g$ of Adriamycin per kg plus the indicated doses of ricin, and the number of surviving colony-forming bone marrow cells was determined by spleen colony assays, as in Chart 1. The survival of leukemic cells was also measured by end-point dilution, as described in “Materials and Methods.” X, end-point dilution. The other symbols denote spleen colony assays. Each symbol represents the average; bars, S.E.; CFU, colony-forming units.

**Chart 3.** Effect of combinations containing a constant dose of Adriamycin and increasing doses of ricin on normal and leukemic bone marrow cells. Groups of mice were treated i.v. with combinations of $3.75 \mu g$ of Adriamycin per kg plus the indicated doses of ricin, and the number of surviving colony-forming bone marrow cells was determined by spleen colony assays, as in Chart 1. The survival of leukemic cells was also measured by end-point dilution, as described in “Materials and Methods.” X, end-point dilution. The other symbols denote spleen colony assays. Each symbol represents the average; bars, S.E.; CFU, colony-forming units.
a fixed dose of ricin together with increasing doses of Adriamycin. Comparison of the results with those in Chart 1A shows that the addition of 1 µg of ricin per kg did not enhance the effect of Adriamycin on normal colony-forming cells. However, this dose of ricin, which had only a slight effect on leukemic cells when given alone (Chart 1B), strongly potentiated the effect of Adriamycin, as already reported (10). The same results were obtained whether the survival of the leukemic cells was assayed by spleen colony formation or by end-point dilution. As expected from the effect of Adriamycin alone (Chart 1A), combinations containing increasing amounts of Adriamycin had a stronger effect on proliferating than on resting stem cells; however, it was much less than that on the leukemic cells. It follows that the rapid proliferation of leukemic cells cannot explain the synergistic effect observed. The exponential dose-effect curve obtained with leukemic cells implies that the cell population was homogeneous with respect to sensitivity.

To see how the synergistic effect depends on the ricin dose, mice were treated with a fixed dose of Adriamycin (3.75 mg/kg) together with increasing doses of ricin. The results in Chart 3 were in principle similar to those shown in Chart 2. Again, the combinations showed a strongly enhanced effect on the leukemic cells (Chart 1B), whereas the effect on normal stem cells was not enhanced. The proliferating bone marrow cells were somewhat more sensitive to the combinations than were the resting cells, as would be expected (Chart 1A).

**Effect of Adriamycin and Ricin on Leukemic Cells in Different Tissues.** The effect of Adriamycin and ricin on the survival of leukemic cells in some other tissues was measured by end-point dilution assay. The results in Table 1 are expressed as the cell kill, i.e., as the percentage of cells per organ unable to proliferate after the treatment. The cell kill was calculated from the fraction of the leukemic cells surviving treatment. The results in Table 1 were obtained with doses of 5 mg of Adriamycin per kg and 1 µg of ricin per kg. These doses were well tolerated by the mice, both when they were given singly and together. It is seen that Adriamycin alone had a rather good effect in spleen and liver, where from 90 to 99.5% of the cells lost their proliferative capacity after treatment. The effect in bone marrow and brain was definitely less pronounced. With the rather low dose of 1 µg of ricin per kg, a modest, but definite cell kill (33%) was seen in the bone marrow (see Chart 1B), whereas this dose had no measurable effect on the leukemic cells in liver, spleen, and brain.

When Adriamycin and ricin were given together, the observed cell kill was much greater than expected, assuming additivity. The synergism was appreciable in liver, spleen, and brain, and, as pointed out above, it was striking in the bone marrow. Moreover, the data in Chart 2 show that by increasing the Adriamycin dose from 5 to 7.5 mg, the fraction of surviving leukemic bone marrow cells was further decreased by approximately 2 logs.

**Effect of Adriamycin and Ricin on the Survival of Leukemic Mice.** Treatment with Adriamycin alone increased considerably the life span of the leukemic animals. For this reason, the mice were treated 3 days after i.v. injection of 1 x 10⁶ cells, i.e., in an advanced state of leukemia. It was found that 1 µg of ricin per kg alone did not enhance the survival, whereas 10 mg of Adriamycin per kg given alone increased the average life span by 134%. Concurrent administration of the 2 drugs gave an increased life span of 198%. It was estimated that the extra increase in life span due to the administration of ricin involved an additional reduction in the leukemic cell burden by about 2 logs. The calculation was based on the findings of Wodinski et al. (22) that the generation time of L1210 cells injected i.v. is about 9.5 hr and that a 10-fold reduction in the number of leukemic cells injected results in an increased life span of 1.3 days.

**Studies of Intracerebral Leukemia.** To see whether the observed effects of the combination of Adriamycin and ricin on leukemic cells in the brain could be accounted for by a systemic effect, 1 x 10⁶ leukemic cells were injected i.c.1 into mice, and the animals were treated i.v. 24 hr later. The results in Table 2 demonstrate that Adriamycin and ricin given separately did not increase the life span of the animals. This was expected, inasmuch as neither Adriamycin (14) nor ricin (7) seems to penetrate the blood-brain barrier. The finding reported above (Table 1), that Adriamycin caused a reduction in the number of leukemic cells in the brain of animals inoculated i.v., must therefore be secondary to a systemic effect.

It is seen from Table 2 that combinations containing 1 µg of ricin per kg and Adriamycin doses in the range of 4.4 to 7.5 mg/kg caused a definite increase in the life span of the animals given the cells i.c. Since, as pointed out above, Adriamycin

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**Table 1**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Adriamycin</th>
<th>Ricin</th>
<th>Expected</th>
<th>Observed</th>
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<tbody>
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<td>33</td>
<td>66.5</td>
<td>99.983</td>
</tr>
<tr>
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<td>0</td>
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</tr>
<tr>
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<td>0</td>
<td>99-99.5</td>
<td>99.7-99.9</td>
</tr>
<tr>
<td>Brain</td>
<td>50-75</td>
<td>0</td>
<td>50-75</td>
<td>95-99</td>
</tr>
</tbody>
</table>

1 The abbreviation used is: i.c., intracerebrally.
alone strongly increased the survival of animals with systemic leukemia but had no effect on the survival of animals inoculated i.c. (Table 2), it follows that these animals die from the cerebral lesions. Hence, it can be concluded that the above effect of the combination of Adriamycin and ricin is exerted directly on the leukemic cells in the brain.

**Toxicity of Adriamycin-Ricin Combinations.** To test the overall toxicity of Adriamycin-ricin combinations, we measured the survival of nonleukemic mice treated with various combinations. The results are given in Table 3. It is seen that in mice given 1 μg of ricin per kg and 17.5 mg of Adriamycin per kg, 3 of 4 animals survived, and when the Adriamycin dose was raised to 20 mg/kg, 2 of 4 animals survived. Since the 50% lethal dose was 18 mg/kg, the data indicate that the toxicity of Adriamycin is not increased by the concurrent administration of 1 μg of ricin per kg. The experiments in which the ricin dose was varied indicate that the addition of 3.75 mg of Adriamycin per kg increased the toxicity of ricin only slightly.

**Effect of Adriamycin and Ricin on Cells in Vitro.** To see whether the synergistic effect of Adriamycin and ricin is exerted directly on the cells, L1210 cells grown in suspension were exposed to Adriamycin and ricin, singly and together, and they were subsequently grown in a soft-agar system, according to the method of Courtenay and Mills (2). It was found that the effect of a combination of Adriamycin and ricin reduced the number of colony-forming leukemic cells by a factor of 2 to 3 compared to that expected on the basis of additive action. This corresponds roughly to the synergistic effect found in the liver and spleen in vivo but is several orders of magnitude lower than that found in leukemic cells in the bone marrow of treated animals. The results show that Adriamycin and ricin have a synergistic effect that is exerted directly on the cells. However, the dramatic effect of the combination observed on leukemic cells in the bone marrow can be explained only in part by a synergistic effect on the cells as such.

In attempts to investigate whether the exceptionally high synergism might be explained by the presence in bone marrow tissue of some enhancing substance, bone marrow obtained from the femur of leukemic mice was exposed in vitro to the drugs for 1 hr, and the number of leukemic cells surviving the treatment was assayed in vivo by either end-point dilution or spleen colony formation. It is seen (Table 4) that the synergism obtained was of the same magnitude as that found in the soft-agar experiments. Similar results were obtained in experiments where the incubation time was increased to 12 hr.

**DISCUSSION**

Murine leukemias offer several advantages as experimental tumor models. By using spleen colony assays, the effect of treatments on leukemic bone marrow cells and on their normal counterparts, the stem cells, can be compared. Moreover, by using end-point dilution, it is possible to examine the effect of drugs on leukemic cells present in the different tissues.

Several interesting conclusions can be drawn from the present study. The finding that the toxic lectin, ricin, did not act more strongly on proliferating than on resting bone marrow cells shows that the preferential effect of ricin on leukemic *versus* normal bone marrow cells cannot be accounted for by cell kinetic differences between the different cell populations but that it must be due to some inherent abnormality in the leukemic cells. This is in contrast to the situation with Adriamycin, where the seemingly preferential effect on leukemic cells could be accounted for by their higher rate of proliferation.

The most interesting finding in the present paper is the dramatic synergistic effect of Adriamycin and ricin on leukemic cells in the bone marrow. Recently, Valeriote et al. found that amphotericin B potentiates the effect of Adriamycin in L1210 leukemia. However, a synergistic effect of the magnitude found here in the bone marrow has not, to our knowledge, been previously reported. The possibility that this effect might be due to an artifact was ruled out by the fact that the same results were obtained with 2 independent methods, namely, spleen colony assays and end-point dilution.

The mechanism underlying the synergistic effect of Adriamycin and ricin on leukemic cells is not clear. Ricin is a lectin which binds to receptors on the cell surface. There is evidence that only a small fraction of the molecules bound are internalized and that the entrance of a single molecule (or its toxic moiety) into the cytosol is sufficient to kill a cell (4). Moreover, there is good evidence that transport into tumor cells may be critical for the action of Adriamycin and other anthracyclins (12). One obvious possibility is, therefore, that when Adriamycin and ricin are administered together, one of the agents may somehow facilitate the cellular uptake of the other one.

In the experiments in which the leukemic cells were exposed to Adriamycin and ricin *in vitro*, the synergistic effect was of the same order of magnitude as that found in the liver and spleen after treatment *in vivo*. This potentiation by a factor of 2 to 5 seems to reflect an interaction at the cellular level. It is clear that the greater effect observed on leukemic cells in the brain, and in particular the striking effect in the bone marrow, must be due to some additional factor. No evidence was found that the particularly great effect in the bone marrow could be due to the presence in this tissue of a special substance or factor. Thus, when whole leukemic bone marrow tissue was exposed to the substances *in vitro* and the survival of the leukemic cells was assayed *in vivo*, the observed synergism

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2 K. M. Tveit, unpublished data.

3 F. A. Valeriote, G. Medoff, and J. Dieckman, personal communication.
was no greater than that observed in liver and spleen in the in vivo experiments.

Since the effect of Adriamycin alone on leukemic cells in the bone marrow was comparatively low (Table 1), an alternative possibility to be considered is that the concurrent administration of ricin will somehow facilitate the access of Adriamycin to the leukemic cells in the bone marrow. Using labeled Adriamycin and ricin, we now attempt to study whether either one of these agents will alter the concentration of the other one in the bone marrow. The finding that the combination of Adriamycin and ricin increased the life span of animals given leukemic cells i.e. indicates that the combination acted directly on the leukemic cells in the brain. This is remarkable, as either agent was totally ineffective when administered individually. One possibility is that the administration of the one agent somehow facilitates the penetration of the other one through the blood-brain barrier.

The present results raise a number of questions and, obviously, further experiments are needed to elucidate the mechanism underlying the synergistic effects here observed. Experiments are now in progress to study whether a similar synergism is found in other forms of leukemia and with substances related to ricin and Adriamycin.

The synergistic effect of Adriamycin and ricin was achieved with combinations that had little effect on normal bone marrow cells and that were well tolerated by the mice. In human leukemias, the bone marrow is assumed to be the tissue of origin, and in the treatment the toxic effect on normal bone marrow cells is frequently the dose-limiting factor. Moreover, even though effective combinations are now available for the treatment of acute leukemias, the manifestations in the central nervous system still constitute a difficulty. Against this background, the observed effect of Adriamycin-ricin combinations may have interesting clinical implications.

ACKNOWLEDGMENTS

The valuable assistance of Bente Mikkelsen and Roy Kroka is gratefully acknowledged.

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