Synergistic Effect of Ricin in Combination with Daunorubicin, cis-Dichlorodiammineplatinum(II) and Vincristine in Systemic L1210 Leukemia

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ABSTRACT

The antitumor effect of combinations of the toxic proteins ricin and abrin with other drugs was studied in mice with systemic L1210 leukemia. Ricin was tested in combination with daunorubicin, cis-dichlorodiammineplatinum(II), or vincristine, and abrin was tested in combination with Adriamycin. The antileukemic effect of the regimens was gauged by assay of the life span of tumor-bearing animals and by measuring the survival of leukemic cells in bone marrow, spleen, and brain by end point dilution assay. The effect of the drugs on leukemic cells was compared with that on resting and proliferating normal bone marrow cells in spleen colony assays.

Administration of a fixed dose of ricin (1 ¿g/kg) enhanced considerably the antileukemic effects of the conventional drugs given concurrently without increasing their toxicity. With daunorubicin and cis-dichlorodiammineplatinum(II), ricin increased the leukemic cell kill in the bone marrow by factors of about 10 and 5, respectively. With vincristine, ricin increased the cell kill in bone marrow and spleen up to several hundred-fold, as measured by end point dilution. Also the life span of the animals was increased by ricin, but not corresponding to the increased cell kill in the bone marrow and spleen, probably due to inability of the drugs to pass the blood-brain barrier. Abrin-Adriamycin combinations were approximately as effective against leukemic cells in the bone marrow as has been observed previously for ricin-Adriamycin combinations.

End point dilution and spleen colony assays gave concordant results with respect to survival of leukemic bone marrow cells, except when vincristine was given alone. In this case, the apparent cell kill, as measured by spleen colony assay, was greater than that measured by end point dilution assay. The results suggest that vincristine may partially damage the leukemic cells, rendering them unable to colonize spleens, while they still retain the ability to multiply in the peritoneal cavity of recipient animals. When ricin was administered concurrently with vincristine, no discrepancy between the 2 assays was found.

The results demonstrate a true potentiation of combinations of ricin with cancerostatic drugs belonging to several different classes, and they suggest that the same may be the case with abrin combinations.

INTRODUCTION

The toxic proteins ricin and abrin represent a new type of cancerostatic agent. These toxins have been found to inhibit the growth of some murine tumors (5, 11) and a number of human tumors growing as xenografts in athymic mice (2, 3, 5, 17).

Since ricin and abrin inhibit cellular protein synthesis and thus have a mechanism of action different from that of the cancerostatic agents in current use, studies of the usefulness of the toxins in combination cancer chemotherapy were started. Previously, we have reported that abrin potentiates the effect of cyclophosphamide in Lewis lung carcinoma (9) and of 5-(dimethyltriazeno)imidazole-4-carboxamide in human melanoma xenografts (3). Studies in L1210 leukemia were also carried out with the combination of ricin and Adriamycin (6). The cells were injected i.v. to permit measurement of the cytostatic effect on the systemic disease. This is in contrast to most studies of L1210 leukemia in which the tumor cells, as well as the drugs, are injected i.p. and thus are essentially given i.t. Ricin and Adriamycin gave a synergistic effect (6, 7) that was particularly pronounced in the bone marrow but was also present in the spleen and in the liver. A synergistic effect on the life span of leukemic animals was seen after treatment with combinations that showed no increased toxicity in nonleukemic mice.

The question here is whether the strong synergistic effect of ricin and Adriamycin in L1210 leukemia is specific for this particular combination. To investigate this, we have studied, in mice with systemic leukemia, the effects of combinations of abrin and Adriamycin, as well as combinations of ricin with daunorubicin, cis-platinum, or vincristine. Daunorubicin is related closely to Adriamycin. cis-Platinum and vincristine were chosen because they both have mechanisms of action different from those of Adriamycin and ricin.

MATERIALS AND METHODS

Tumor and Animals. The L1210 leukemic cells were maintained by serial transplantation as an ascitic tumor in DBA/2 mice. Appropriate concentrations of leukemic cells were obtained from ascites fluid by dilution in 0.9% NaCl solution.

Chemotherapeutic Agents. Adriamycin was obtained from Farmitalia, Milan, Italy; daunorubicin was obtained from Rhone-Poulenc, Paris, France; vincristine was obtained from Eli Lilly and Co., Basingstoke, England; and cis-platinum was obtained from Bristol Laboratories, Syracuse, N. Y. Ricin and abrin were isolated from the seeds of Ricinus communis and Abrus precatorius, respectively, as described previously (13, 14).

Spleen Colony and End Point Dilution Assays. The assays were performed essentially according to the methods of Bruce and Van der Gaag (1) and Hewitt (10), as described previously (6).

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**RESULTS**

**Spleen Colony Assays**

Measurements of the ability of cells to form colonies in the spleen of recipient animals offer the possibility of studying the relative sensitivity to various treatments of leukemic cells in the bone marrow and of normal bone marrow cells.

**Effects of Ricin on the Cytostatic Activity of Daunorubicin.**

The first question raised was whether ricin potentiates daunorubicin, which is closely related to Adriamycin. Mice were treated with increasing doses of daunorubicin, alone and together with a fixed dose of ricin (1 μg/kg), and the number of surviving colony-forming cells in the bone marrow was measured by spleen colony assay. Such studies were carried out on normal animals, on animals in which the bone marrow cells had been induced to proliferate (see “Materials and Methods”), and on animals previously given injections of leukemic cells.

The results are shown in Chart 1. After treatment with daunorubicin alone, the normal resting bone marrow cells showed a dose-effect curve with a broad shoulder. The rapidly proliferating normal cells gave a close to exponential dose-effect curve. Clearly, these cells were more sensitive than were the normal resting cells. The leukemic cells gave an exponential dose-effect curve and were far more sensitive to daunorubicin than were the proliferating normal cells, in contrast to the situation found previously with Adriamycin (6). Comparison of the present data with our previous results (6) shows that daunorubicin has a much stronger effect than Adriamycin on leukemic bone marrow cells. Since the growth fraction of normal bone marrow cells injected into irradiated animals is very high (20), the present data suggest that the preferential effect of daunorubicin on the leukemic cells may not be entirely accounted for by the high rate of multiplication of the leukemic cells, in contrast to the situation with Adriamycin (6).

When ricin was given together with daunorubicin, the effect on normal resting and on proliferating cells was not greater than with daunorubicin alone. However, the combination was clearly more lethal to the leukemic cells in the bone marrow than was daunorubicin alone. At the highest dose of daunorubicin tested, the presence of ricin enhanced the cell kill by a factor of about 10. The effect of daunorubicin on leukemic cells in the bone marrow was thus clearly enhanced by the presence of ricin. However, the enhancing effect of ricin was several orders of magnitude lower than that reported previously in the case of Adriamycin.
Effect of Ricin on the Cytostatic Activity of cis-Platinum. With cis-platinum alone, similar results were obtained (Chart 2) as with daunorubicin. However, the dose-effect curve for normal cells was S-shaped and that for proliferating normal cells was biphasic, suggesting the existence of a resistant subpopulation. Concurrent administration of ricin did not further increase the toxicity of cis-platinum to the normal resting or proliferating cells. However, it did increase clearly the effect of cis-platinum on the leukemic cells. The results indicate that the addition of ricin increased the cell kill by a factor of about 5 at all dose levels of cis-platinum. Since the small dose of ricin used had virtually no effect when given alone, the results demonstrate a potentiation even though the dose-effect curves for the leukemic cells with and without ricin were parallel.

Effect of Ricin on the Cytostatic Activity of Vincristine. With vincristine alone, results were obtained (Chart 3) similar to those with cis-platinum. The dose-effect curves for normal and proliferating normal cells showed a decreasing slope with increasing dose. However, with leukemic bone marrow cells, the dose-response curve was exponential and very steep with no indication of a plateau in the dose range tested. When the doses used are related to the toxicity data (Table 2), it is clear that vincristine has a much stronger effect on the leukemic cells than does cis-platinum. In fact, the surviving fraction was brought almost down to $10^{-3}$ by vincristine doses that showed no toxicity as judged by the survival of nonleukemic animals. The addition of ricin did not increase the effect of vincristine on normal resting or proliferating cells, whereas it did enhance the effect on the leukemic cells. Within the limited dose range tested, the enhancement increased with the dose of vincristine up to a factor of about 10. For technical reasons, the spleen colony assays were not carried out with higher vincristine concentrations. As will be shown below, end point dilution studies indicate that the ability of vincristine alone to kill leukemic cells is far less than suggested by the curve in Chart 3, and that consequently the enhancing effect of ricin is much greater than that suggested by the data in Chart 3.

Effect of Abrin on the Cytostatic Activity of Adriamycin. The question was then raised whether the ability of ricin to enhance the effect of different types of cytostatic drugs is specific for this toxin or whether abrin, which has a similar mechanism of action, has the same ability. To elucidate this question, increasing concentrations of Adriamycin were administered together with a fixed nontoxic dose of abrin. Since abrin is 4 to 5 times as toxic as ricin (4), the dose of abrin used (0.2 μg/kg) corresponds closely in potency to the ricin dose used in the other experiments. Abrin was found (data not shown) to enhance the effect of Adriamycin on leukemic cells in the bone.
Synergistic Effect of Ricin in Combination Chemotherapy

Previously, we have reported (6) an experiment in which increasing doses of ricin were given together with a fixed nontoxic dose of Adriamycin (3.75 mg/kg). In Chart 4, an analogous experiment is shown in which increasing doses of abrin were given together with the same fixed dose of Adriamycin. It is apparent (Chart 4) that Adriamycin enhanced markedly the effect of abrin on leukemic cells, whereas no such effect was seen on normal resting or proliferating cells. The results are similar to those reported previously for ricin-Adriamycin combinations (6).

**End Point Dilution Studies**

The spleen colony assay is relatively insensitive, as only a small fraction of the leukemic and normal stem cells form colonies in the spleen of recipient mice. Moreover, this method which involves i.v. injection of a cell suspension is not suited for the assay of leukemic cells when their concentration in the tissues is very low, and hence the overwhelming number of cells are normal ones. Therefore, for the assay of very low-surviving fractions of leukemic cells in bone marrow, as well as for the study of leukemic cells in tissues other than the bone marrow, the more sensitive end point dilution assay was used.

The results are summarized in Table 1. The data confirm that ricin enhanced the effect of cis-platinum on leukemic bone marrow cells. The enhancement factor (A/B) was about 15, whereas with the spleen colony assay this factor was about 5 (Chart 2). In the spleen and brain, the enhancement factor was about 5 (Table 1).

The effect of vincristine on bone marrow cells, as measured by the end point dilution assay, gave results which differed significantly from those obtained with the spleen colony assay. Thus, when vincristine was given alone, the end point dilution data showed a much lower cell kill than was suggested by the spleen colony assay (Chart 3). This is particularly the case at the highest vincristine doses tested. In the end point dilution assay, there was already a clear plateau from the lowest dose tested (0.8 mg/kg), whereas in the spleen colony assay no indication of a plateau was seen at doses up to 1.2 mg/kg (Chart 3). The possible reason for this discrepancy will be discussed below. In contrast, when ricin was added, the end point dilution assay gave no plateau (Table 1) and the effect at high Vincristine concentrations was almost as great as that found with the spleen colony assay. The effect of vincristine alone on leukemic cells residing in the spleen also showed a plateau with increasing dose (Table 1). However, when ricin was present, the cell kill again increased with the vincristine dose.

In bone marrow and spleen, the antileukemic effect of the combinations involving vincristine was up to 2 to 3 logs greater than that of vincristine alone at the highest doses tested. In the brain, however, ricin increased the effect of vincristine by only a factor of about 2 to 5, and this effect did not increase with the vincristine dose. The modest effect of ricin on leukemic cells in brain tissue is probably secondary to its systemic effect, since the combination did not enhance the survival of mice inoculated with the cells i.e. (data not shown).

**Toxicity in Nonleukemic Animals**

To study whether ricin enhances the overall toxicity of vincristine and cis-platinum, the effect of various combinations on the life span of normal mice was studied (Table 2). The results indicate that the ricin dose used did not increase the lethality caused by cis-platinum, whereas the life span of those animals that died was slightly shortened. No indication was found that the addition of ricin increased the toxicity of vincristine. In fact, the life span of the animals that died was consistently somewhat longer than that of animals receiving vincristine alone.

**Survival of Tumor-bearing Animals: Effect of Ricin-cis-Platinum Combinations**

The results in Chart 5A show the effect of cis-platinum, alone and in combination with ricin, in mice inoculated i.v. with $10^5$ leukemic cells and treated i.v. 24 hr later with the doses indicated. As expected, a bell-shaped curve was obtained with a maximum corresponding to about 40% ILS. When ricin was administered concurrently, the curve was displaced to the left, showing that the same ILS could then be obtained with a lower...
Table 1
Effect of ricin on survival of L1210 leukemic cells in mice treated with vincristine or cis-platinum

Mice were made leukemic by i.v. injection of 1 X 10^6 L1210 cells. On Day 2, groups of animals were treated i.v. with the indicated doses of cis-platinum or vincristine, with or without ricin (1 μg/kg) given concurrently. All the mice were killed on Day 5, and cell suspensions were made from the tissues indicated. Appropriate dilutions were injected i.p. into recipient mice, and the life span was recorded. The fraction of surviving leukemic cells was estimated as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Bone marrow</th>
<th>Spleen</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. No ricin</td>
<td>B. Ricin (1 μg/kg)</td>
<td>A/B</td>
</tr>
<tr>
<td>cis-Platinum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>3.5</td>
<td>0.08-0.15</td>
<td>20-40</td>
</tr>
<tr>
<td>10.0</td>
<td>2.0</td>
<td>0.14</td>
<td>14</td>
</tr>
<tr>
<td>12.5</td>
<td>0.8</td>
<td>0.05-0.07</td>
<td>11-16</td>
</tr>
<tr>
<td>15.0</td>
<td>0.15</td>
<td>0.01</td>
<td>15</td>
</tr>
<tr>
<td>17.5</td>
<td>0.08</td>
<td>0.005</td>
<td>16</td>
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<tr>
<td>Vincristine</td>
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<td></td>
<td></td>
</tr>
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<td>30</td>
<td>2</td>
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<td>16-50</td>
</tr>
<tr>
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<tr>
<td>1.4</td>
<td>10-25</td>
<td>0.03-0.04</td>
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Table 2
Toxicity in mice of vincristine and of cis-platinum in the presence and absence of ricin

Increasing doses of cis-platinum and vincristine, alone or in combination with a fixed dose of ricin (1 μg/kg), were administered i.v. to healthy nonleukemic DBA/2 mice. The mean life span of the mice that died and the number of surviving animals at each dose level was recorded.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Survivors/total</th>
<th>Mean life span (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No ricin</td>
<td>Ricin (1 μg/kg)</td>
</tr>
<tr>
<td>cis-Platinum</td>
<td>12.5</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td>17.5</td>
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<tr>
<td></td>
<td>20</td>
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<td></td>
<td>2.50</td>
<td>6/8</td>
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<td></td>
<td>3.00</td>
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<td></td>
<td>3.25</td>
<td>0/6</td>
</tr>
</tbody>
</table>

DISCUSSION

The main finding in this paper is that administration of a fixed dose of ricin, which per se had virtually no effect on L1210 leukemic cells, enhanced considerably the effect on the cells of daunorubicin, cis-platinum, and vincristine, 3 drugs which act by different mechanisms and are in wide clinical use. The fixed, small dose of ricin gave a more than additive antileukemic effect by all the assays used, whereas it did not enhance the toxic action of the conventional drugs on resting or proliferating normal bone marrow cells or the overall toxicity as measured by the survival of nonleukemic animals. These results imply (8, 19) that the small dose of ricin gave a true potentiation of the effect of daunorubicin, cis-platinum, and vincristine in L1210 leukemia. Also, evidence was obtained that abrin potentiates the effect of Adriamycin on leukemic cells in the bone marrow, although to a lesser extent than the analogous effect of ricin reported previously (6).

The simplest assay of cancerostatic activity in experimental
animals is to record the increase in life span of tumor-bearing animals. Such experiments are easy to carry out, and the observed ILS is a measure of the net effect of cancerostatic action and host toxicity. However, when, as in our experiments, tumor cells are administered i.v. and hence are disseminated also in the central nervous system, ILS data alone may not give an adequate picture of the systemic cell kill (18, 21). For this reason, we used spleen colony and end point dilution assays to follow the effect of the drugs on the leukemic cells in bone marrow, spleen, and brain. It is of interest to relate the observed ILS to the cell kill in these tissues.

From the ILS data, it is possible to calculate the “overall leukemic cell kill” on the assumption that the leukemic cells have a generation time of 9.4 hr and that a 10-fold reduction in the number of tumor cells corresponds to an ILS of 1.3 (22). From Chart 5B, it can be seen that the addition of ricin increased the ILS by about 10% at the lowest dose of vincristine and by about 30% at the highest dose tested. Since the life span of the untreated leukemic animals was about 6.5 days, these ILS values correspond to about 15.6 and 46.8 hr, i.e., 1.66 and 5.0 doubling times, respectively. From this, it can be calculated that the addition of ricin increased the cell kill by vincristine by factors of 3.2 to 32 in the dose range tested. The enhancement factors in the bone marrow and spleen were 1 to 2 orders of magnitude higher (Table 1). The fact that the ILS was considerably less than that expected on the basis of the cell kill observed in the bone marrow and spleen is presumably explained by the finding that ricin enhanced the leukemic cell kill in brain tissue by only a small factor. The modest effect in brain tissue is probably secondary to the systemic effect of the drug combination, inasmuch as the drugs seem unable to penetrate the blood-brain barrier. Thus, i.v. administration did not enhance the survival of animals inoculated i.c. with the leukemic cells. This is in contrast to the situation with ricin and Adriamycin (6), in which the drugs given i.v. together increased the life span of such animals, whereas the drugs given separately had no such effect.

It was noted in “Results” that, when vincristine was given alone, the apparent cell kill was much greater as judged from the spleen colony assay than as judged by the end point dilution. This implies that many of the cells that were able to multiply in the peritoneal cavity of mice and eventually killed the recipient animals were unable to colonize the spleen after i.v. administration. Apparently, in this case, the spleen colony assay did not give an adequate measure of the number of surviving leukemic cells in the bone marrow. Possibly, this observation may be related to the recent finding that inhibitors of microtubule formation have 2 separate effects; namely, they inhibit directional migration and the invasive capacity of the cells, and also they inhibit cell multiplication (12). In view of this, it does not seem unreasonable that cells exposed to vincristine may suffer partial damage rendering them unable to invade the spleen and establish themselves in the foreign tissue to form colonies, while they do retain the ability to divide when injected into the peritoneal cavity. The fact that the 2 methods gave the same results when ricin was present suggests that under these conditions the cells suffer an all-or-none type of damage. Whatever the explanation of the discrepancy, the present results bear out the value of using several different assays for the purpose of assessing antitumor effects of drugs.

Synergistic Effect of Ricin in Combination Chemotherapy

The synergistic effects observed with the ricin combinations in this study were considerably less than that found previously for leukemic bone marrow cells with the combination of ricin and Adriamycin. Nevertheless, they are sufficiently large to be of clinical interest. The striking synergistic effect on leukemic bone marrow cells of ricin-Adriamycin combinations (6) may be related to the fact that Adriamycin alone has a particularly low effect in the bone marrow, compared, e.g., with daunorubicin.

Although the biochemical action of ricin and abrin has been studied extensively (for review, see Refs. 15 and 16), the mechanism of the anticancer activity of ricin and abrin is poorly understood. These proteins consist of 2 polypeptide chains joined by a disulfide bridge. The larger chains, the B-chains, serve to bind the toxins to cell surface receptors. Such binding is a prerequisite for toxicity in intact cells and animals. The A-chains inhibit protein synthesis by inactivating catalytically the 60S ribosomal subunits. The toxins inhibit protein synthesis in all eukaryotic cells. Therefore, the cancerostatic effects observed imply that at least some cancer cells are more sensitive to the toxins than are normal cells. Possibly, this difference is related to altered surface properties in cancer cells, facilitating the uptake of the A-chains into the cytosol.

The simplest interpretation of the synergism observed in this study is that somehow the presence of the one drug facilitates the uptake of the other one (6). In the above presentation, the synergistic effect on leukemic cells of different combinations has operationally been described as an ability of the toxin to enhance the cancerostatic effects of the conventional drug used in the combinations. However, the alternative possibility that the conventional drugs may enhance the action of the toxins has not been ruled out. Our data do not permit a distinction to be made between these possibilities. Since the first step in the uptake of the toxins is binding to cell surface receptors, such binding may conceivably alter the permeability of the cell membrane to other drugs.

ACKNOWLEDGMENTS

The valuable assistance of Unni Rønning and Anne Due is gratefully acknowledged.

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