Effects of 1-β-D-Arabinofuranosylcytosine Hydrochloride on Regenerating Bone Marrow[1]

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SUMMARY

1-β-D-Arabinofuranosylcytosine (ara-C[3] or cytosine arabinoside), a pyrimidine nucleoside analog, is capable of inhibiting marrow regeneration in lethally irradiated mice in which marrow recolonization by donor marrow cells from normal mice occurs. When administered on the 4th day after irradiation and marrow injection, the drug induces significant marrow suppression. Depending upon the relative amounts of the compounds and the intervals of time between their administration, reversal of the effect of cytosine arabinoside on the bone marrow can be accomplished by deoxycytidine. When the metabolic block induced by cytosine arabinoside is circumvented by deoxycytidine, with release of the biosynthesis of DNA, the administration of certain cytotoxic agents yields enhanced suppression of the regenerating bone marrow of mice.

Suppression of regenerating bone marrow, a very actively proliferating tissue, may be indicative of potential anti-neoplastic effect, since such suppression reflects an agent's growth-inhibitory properties. Practical use of the marrow-regenerating system in assaying for anti-tumor effect is further suggested by the observation that effective cytotoxic agents frequently demonstrate effects on normal tissues that undergo rapid proliferation.

Hollingsworth (6) demonstrated that heavily irradiated mice treated with homologous bone marrow immediately after exposure to irradiation showed re-colonization of the marrow with cells that possessed high susceptibility to cytotoxic drugs. The sensitivity of marrow cells to cytotoxic agents was maximal 3–5 days after the irradiation and the i.v. administration of suspensions of normal marrow cells. At this time the femoral marrow count was reduced to from $\frac{1}{3}$ to $\frac{1}{6}$ of normal values. Once the logarithmic phase of marrow regeneration was attained, usually 6–8 days after irradiation and marrow replacement, the increased susceptibility to cytotoxic drugs disappeared.

1-β-D-Arabinofuranosylcytosine hydrochloride (cytosine arabinoside, ara-C) possesses certain biologic properties that make it interesting to study in the regenerating marrow system of the rodent. On the basis of experiments utilizing murine lymphoblast cells, Chu and Fischer (2) postulated that this pyrimidine anti-metabolite, by suppressing the formation of deoxycytidine 5′-triphosphate, inhibits synthesis of deoxyribonucleic acid (DNA). Cardeilhac and Cohen (1), however, in studies with isolated enzyme preparations failed to demonstrate incorporation of arabinosylcytosine into polynucleotide. Evans *et al.* (4, 5) have described anti-tumor effects after treatment with ara-C in animals bearing transplanted neoplasms.

In mammalian cells the effects of ara-C can be reversed by CdR, the availability of which has been shown to be a limiting factor for growth of neoplasms in certain animal systems (2, 4). Welch (15) has postulated that in cells surviving the lethal effects of ara-C, synthesis of DNA would be arrested. In such cells, reversal with CdR could result in temporary synchronization of the synthesis of DNA, as surviving cells could be released by CdR simultaneously from the block in synthesis of DNA. After such reversal and synchronization, the introduction of cytotoxic agents could yield increased incorporation of these agents.

In the present experiments the effects of ara-C on marrow regeneration, as well as the effectiveness of CdR in reversing these effects, have been determined. The effectiveness of other cytotoxic agents in suppression of marrow regeneration, after deoxycytidine reversal of the effects of ara-C has occurred, also have been investigated.

MATERIAL AND METHODS

Male and female hybrid mice of strain C57BL, 10–12 weeks old and weighing 20–25 gm, were utilized in all experiments.

Irradiation was carried out with 850 r, a supralethal dosage. The radiation factors were: 250 kv, 15 ma, 0.5 cm Cu– 0.5 Al filter, and 75 cm tube distance.

Within 1–2 hr after irradiation, each recipient was injected i.v. with a suspension of normal homologous bone marrow obtained from an appropriate F_1-hybrid donor. Donor bone marrow was obtained from femoral marrow of

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[3] The abbreviations used are: ara-C, cytosine arabinoside (1-β-D-arabinofuranosylcytosine hydrochloride); DNA, deoxyribonucleic acid; CdR, 2′-deoxycytidine; IUdR, 5-iodo-2′-deoxyuridine; HN2, nitrogen mustard.

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decapitated animals by attaching a syringe with a 23-gauge needle to one end of the femur and using 1 ml of Hanks's solution to flush the marrow from the bone. A Coulter electronic particle counter was used to determine cell counts. Each irradiated recipient received the marrow contents of 1 femur of a normal mouse; this injection supplied from 4 to 8 million cells and was given 1–2 hr after irradiation.

Drugs were administered by the i.p. route on the 4th day after irradiation, since a 3- to 5-day interval after irradiation has been established as the period of maximal sensitivity to drugs (6). Sterile saline was administered to the controls.

The animals were sacrificed for femoral marrow counts on the 7th or 8th day after irradiation, although in a few experiments groups were sacrificed from 10 to 14 days after irradiation. The femur counts were performed separately for each extremity in the individual animals. Comparison of the individual extremity counts for both control and treated animals by the χ^2 test ($P < 0.01$) revealed no significant differences. Each experimental group comprised 10–20 animals.

RESULTS

a) Effects of cytosine arabinoside on marrow regeneration.—Inhibition of marrow regeneration after a single injection of ara-C was dose related. An effect on the regeneration of marrow was observed first at a dose of 100 mg/kg, while 150 mg/kg caused approximately 50% depression of marrow counts, as compared with those of control animals (Chart 1). Each point on the dose-response curve is derived from femoral marrow counts of 10 animals except the 200 mg/kg group, in which 4 of 10 animals died. A lethal effect occurred in 9–10 animals given a single injection of ara-C, 300 mg/kg.

Statistical evaluation of the dose-response curve according to the method of analysis of regression was carried out. With this method, the relationship of marrow count to drug dose is significant at the 5% level (correlation coefficient, −9.23°; S.E., 4.68°).

Although a single injection of ara-C of 150 mg/kg given on the 4th day after irradiation elicited a significant effect on marrow regeneration, the same amount given in divided daily doses (50 mg/kg daily for 3 days) did not inhibit marrow regeneration significantly. In order to achieve a significant depression of the regenerating marrow, doses of 50 mg/kg given on 4 consecutive days were required (Table 1).

When ara-C was given as 50 mg/kg 3 times daily, the marrow depression observed was equivalent to that noted with the single injection of 150 mg/kg.

b) Reversal of effects of cytosine arabinoside by deoxycytidine.—The ability of CdR to reverse the effects of ara-C on the regenerating marrow was observed initially by administration of ara-C, 150 mg/kg, as a single dose, followed immediately by CdR, 150 mg/kg. On the 8th day after irradiation and marrow injection, the marrow counts of the treated animals were 30% less than controls. With a dose of 200 mg/kg of CdR given immediately after ara-C (150 mg/kg), however, the marrow counts on Day 8 were not significantly different from those of control animals (untreated animals).

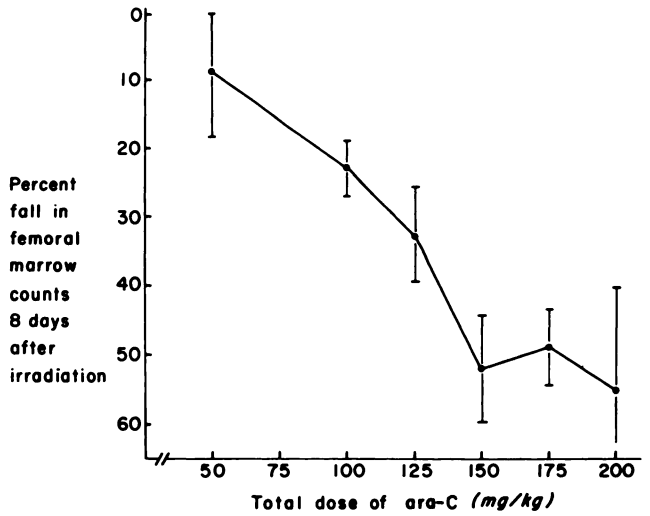

CHART 1.—Effect of cytosine arabinoside (ara-C) on regenerating bone marrow of mice. Each *point* represents the mean value of marrow counts at that dose level as compared to control, untreated animals. *Vertical lines* represent the range of values at each point.

TABLE 1

EFFECTS OF DIVIDED DOSES OF CYTOSINE ARABINOSIDE (ara-C) ON MARROW REGENERATION

DOSE OF ara-C (mg/kg/day) GIVEN BEGINNING ON DAY 4	MARROW COUNT IN MILLIONS (Day 8)	
	Mean	S.E.
Saline control	6.6	±1.0
50, 3 times	7.0	±1.3
50, 4 times	1.6	±0.6
75, 3 times	3.1	±0.7
50, once	5.8	±0.9

When CdR, 200 mg/kg, was given 2 hr after ara-C, 150 mg/kg, reversal of the effects of ara-C also occurred. Deoxycytidine administration 4 hr after ara-C did not completely reverse the effect of ara-C, however, and when the interval was increased to 6 hr negligible reversal occurred (Table 2).

When ara-C was administered 3 times daily, 50 mg/kg, and CdR was given 2 hr after the last dose of ara-C, complete reversal ensued.

c) Effects of cytotoxic agents on marrow regeneration after cytosine arabinoside and deoxycytidine.—Prior to evaluating the result of synchronization in increasing the effectiveness of cytotoxic agents, a number of compounds were studied singly for their effect on marrow regeneration; these were 5-iodo-2′-deoxyuridine (IUdR); porfiromycin; and the nitrogen mustard, mustargen (HN2). IUdR at a dose of 3.0 mg/kg yielded 50% reduction in marrow counts on Day 8, as compared to those of control animals, but 1.5 mg/kg was ineffective in inducing marrow suppression (Chart 2). Porfiromycin, at a dose of 10 mg/kg, had no significant effect on marrow regeneration on Day 8, but 15 mg/kg caused a 40% reduction in marrow counts as compared to those of controls. A dose of 0.1 mg of HN2/kg did not produce depression of marrow counts on Day 8, and 0.2 mg/kg produced only a slight suppression.

TABLE 2

EFFECT ON MARROW REGENERATION OF DEOXYCYTIDINE (CdR) ADMINISTRATION AT SPECIFIC INTERVALS AFTER CYTOSINE ARABINOSIDE (ara-C)

MARROW COUNT IN MILLIONS 8 DAYS AFTER IRRADIATION		TIME INTERVAL (hr) BETWEEN ara-C AND INJECTION OF CdR
Mean	S.E.	
2.99	±1.6	Saline control
3.5	±0.14	0
3.2	±0.143	2
2.2	±0.180	4
1.8	±0.316	6
1.5	±0.112	24

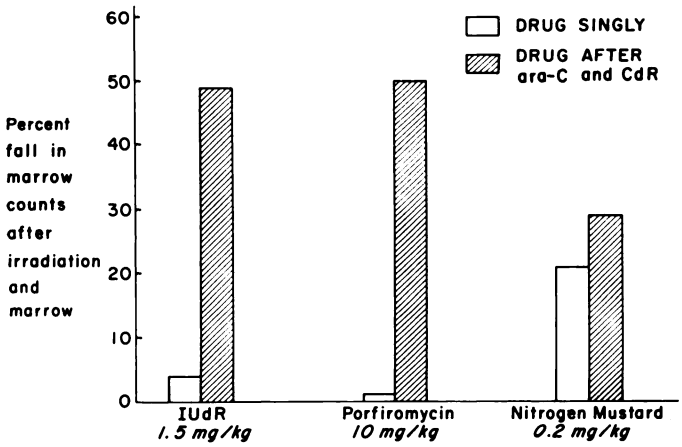

CHART 2.—Effect of cytotoxic drugs on regenerating marrow of mice that have received irradiation, followed by i.v. injection of donor marrow. Ara-C, 1-β-D-arabinofuranosylcytosine hydrochloride; CdR, deoxycytidine; IUdR, iododeoxyuridine.

Assuming that temporary synchronization of synthesis of DNA might follow the administration of ara-C and its reversal with CdR, various cytotoxic agents were given immediately after CdR. The doses utilized were those found to be ineffective when given singly. Ara-C was administered in divided doses (50 mg/kg 3 times daily), and CdR then was given as a single injection (200 mg/kg) immediately after the last injection of ara-C. IUdR, 1.5 mg/kg, under these circumstances induced a 40 % reduction of marrow counts, as compared to those of control animals on Day 8. After the ara-C and CdR, porfiromycin, 10 mg/kg, yielded a greater than 50 % reduction in marrow counts, and this persisted until Day 14. HN2, unlike the other agents studied, did not exhibit a significant increase in marrow suppression after the prior use of ara-C and CdR.

DISCUSSION

The pyrimidine analogue, cytosine arabinoside, is capable of inhibiting bone marrow regeneration in the irradiated mouse in a dose-related manner. This occurred when the drug was administered either as a single injection or in divided doses. When the drug was given during a 4-day period, the total dose that inhibited marrow regeneration to the same degree was greater than that given as a single injection. This is related presumably to its metabolic breakdown, since very rapid deamination to uracil arabinoside occurs in the catabolism of ara-C (4, 9, 11).

The reversal of the effects of ara-C on marrow regeneration by CdR is consistent with the findings of other investigators. Renis and Johnson (13) showed that the antiviral effects of ara-C, which cause an inhibition of the production of vaccinia virus, could be prevented by CdR. Chu and Fischer (2) demonstrated reversal of effects of ara-C by CdR in murine lymphoblast cells L5178Y. Evans *et al.* (4, 5) showed that profound interference with the anti-tumor effects of ara-C on transplanted animal neoplasms could be attained with CdR.

In the regenerating marrow system, the effectiveness of CdR in reversing suppression by ara-C was dependent upon the relative amounts of the compounds given and the intervals of time between their administration. An excess of CdR was necessary for complete reversal to occur. If the time interval between administration of ara-C and CdR exceeded 3 hr, complete reversal did not result.

The data presented demonstrate that the administration of ara-C followed by reversal with CdR provides a feasible method for increasing the effectiveness of cytotoxic agents. The theoretical basis for this approach derives from the supposition that the conversion of ribonucleotides to deoxyribonucleotides, particularly the conversion of derivatives of cytidine to derivatives of deoxycytidine, which is critical to the synthesis of DNA, may be markedly inhibited by ara-C. Deoxycytidine-induced reversal, in releasing the block in synthesis of DNA induced by ara-C, should then result in a spurt of temporarily synchronized synthesis of DNA. Exposure of proliferating cells to cytotoxic agents after release by CdR should enhance the attained cytotoxic effects, if such agents either are capable of incorporation into DNA during synthesis or if vulnerability to their action is greatest during the DNA synthetic phase of cellular proliferation. The data obtained indicate that an enhanced effect has resulted from the use of ara-C followed by CdR, although it has not been demonstrated that increased incorporation is the responsible mechanism.

In the experiments with IUdR, the evidence obtained does indicate that the prior use of ara-C and CdR enhanced the agent's effect on the regenerating marrow. Since the mechanisms of action of IUdR include competition with the utilization of thymidine and its phosphorylated derivatives, as well as actual incorporation into DNA (12), it is reasonable to assume that prior synchronization of the biosynthesis of DNA could augment the potency of this drug.

Porfiromycin, an antibiotic substance identified as an *N*-methylmitomycin, also demonstrated increased effect on marrow regeneration after ara-C and CdR. All details of the mechanism of action of mitomycin and porfiromycin have not been clarified, but from the recent work of Dyer and Szybalski (3) it is clear that a very reactive, biologically formed derivative of mitomycin (and almost certainly, therefore, of porfiromycin) among other things can cross-link adjacent strands of DNA, as well as attach as units to single strands of DNA. Porfiromycin and mitomycin are known to inhibit the incorporation of thymidine into DNA and are presumed to interfere also with

purine biosynthesis because of the partial reversal of their effects by guanylic acid (10, 14). The duration of this marrow-suppressive effect of porfiromycin exceeded that achieved with IUdR.

In the studies with the nitrogen mustard, mustargen (HN2), prior use of ara-C and CdR did not cause an enhancement of its effect on the regenerating marrow. The results observed suggest that, in the regenerating marrow system, the DNA-synthetic phase of cellular proliferation may not be most susceptible to the effect of nitrogen mustard. In other systems, namely Ehrlich ascites tumor cells and the RCP strain of guinea pig kidney cells, there is evidence that depression of synthesis of DNA may be an indirect effect of mustard (7, 8) and that the synthetic phase of the proliferative cell cycle is not the phase most vulnerable to the effects of mustard.

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