Adriamycin-induced Delayed Erythropoietic Injury Expressed following Anemia Stress

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ABSTRACT

The present studies were undertaken to compare anemia-induced erythropoietic responses in femoral marrows and spleens of mice pretreated with Adriamycin (ADR) or 1-β-D-arabinofuranosylcytosine with those of untreated age-matched controls. Mice were bled 45 or 120 days after drug treatment. The erythropoietic response to bleeding was quantitated by morphological, gravimetric, and radioiron methods 48 hr after bleeding. At 120 days after ADR, prebleeding base-line cellularity parameters were, in general, similar to those found in untreated age-matched controls. The response to the anemia stress was compared in drug-treated animals and in age-matched untreated controls, and the response deficit was expressed as residual injury (Rl). At 120 days, ADR-induced Rl was observed to be dose dependent in both femoral marrow and spleen. ADR-induced Rl in femoral marrow and spleen were similar at 45 and 120 days, with no significant recovery. Although marrow Rl was noted 45 days after 200 mg 1-β-D-arabinofuranosylcytosine per kg, there was no Rl at 120 days. The results indicate that ADR can induce a long-lasting hematopoietic injury which is not obvious from measures of homeostatic cellularity, but which can be expressed after induction of an acute proliferative demand.

INTRODUCTION

Hematopoietic toxicity is often the single most important complication that must be considered by the clinical oncologist. Drug dosages and fractionation intervals in most protocols are designed to minimize acute toxicity and permit recovery of this critical normal tissue. The acute effects of radiation and cytotoxic chemotherapy on the hematopoietic system have been exhaustively studied and recently reviewed (26, 27). With the advent of new drugs and the elucidation of more innovative and exhaustively studied and recently reviewed (26, 27). With the advent of new drugs and the elucidation of more innovative and often more aggressive ways in which to utilize chemotherapeutic agents (e.g., in sequence, in combination or in concert with radiotherapy), a larger proportion of patients are attaining long-term remission and attendant increases in survival. In light of these advances, it has become important to consider possible delayed or latent toxicity potentials of chemotherapeutic agents so that delayed complications might be predicted and possibly minimized or avoided.

Latent hematopoietic toxicity has been documented after ionizing radiations. In split-dose studies, reduced values for a 50% lethal dose at 30 days were observed in animals previously exposed to a sublethal conditioning dose of X-irradiation (24, 25). Baum (3, 4) showed decreased radioiron utilization in rats exposed to split-course X-irradiation. Gong et al. (10, 11), utilizing radioiron incorporation to quantitate marrow cellularity, showed that an erythropoietic Rl could be detected in rats up to 60 weeks after an acute dose of 170 R and up to 10 weeks after only 1.0 R. In these studies, the erythropoietic response to anemia stress in the irradiated rats was compared to that in unirradiated controls.

In patients, late toxicity in the form of marrow aplasia and reduced CFUt, (29) have been observed following intensive radiotherapy. Latent hematopoietic toxicity, especially in terms of stem cell depletion, has been studied in mice after treatment with a variety of chemotherapeutic agents. Morley and Blake (30) observed that busulfan could induce late marrow aplasia, and Trainor and Morley (37) showed that busulfan and 1,3-bis(2-chloroethyl)-1-nitrosoure a decreased both CFUc and CFUe compartments 60 days after intensive fractionated treatments. Botnick et al. (6, 7), using a serial transplantation technique to assess the "proliferative capacity" of surviving CFUc, showed that treatment with busulfan and L-phenylalanine mustard resulted in decreased proliferative capacity of the CFUc as long as 95 weeks after treatment. Unlike the studies of Gong et al. (10, 11), these authors utilized exogenous test systems to assay for latent damage, and, as such, the responses observed were independent of homeostatic control mechanisms which may be altered in the treated animals.

In the present studies, we have utilized radioiron methods to evaluate the erythropoietic response to anemia stress (induced by bleeding) in femoral marrows and spleens of mice pretreated with the cycle stage-nonspecific anthracycline, ADR, and the S-phase-specific agent, ara-C. This report is a continuation and elaboration of previously reported preliminary results (33).

MATERIALS AND METHODS

Animals. Female Ha/ICR mice (Schmidt) used in these studies were housed 5 mice/cage and maintained on a 12-hr photoperiod in temperature- and humidity-regulated quarters. They were fed acidified tap water and Purina laboratory chow (autoclavable) ad libitum.

Drug Treatments and Anemic Stress. Mice were randomly assigned to various treatment groups (20 animals/group) at 8 weeks of age and treated with ADR (Adria Laboratories, Inc., Wilmington, Del.) at doses of 1.3, 3.3, 6.7, or 10 mg/kg or with ara-C (The Upjohn Co., Kalamazoo, Mich.) at a dose of

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2 To whom requests for reprints should be addressed, at Cancer Research Laboratories, Allegheny-Singer Research Corp., 320 East North Ave., Pittsburgh, Pa. 15212.
3 The abbreviations used are: Rl, residual injury; CFUt, colony-forming unit(s)-spleen; ADR, Adriamycin (NSC 123127); ara-C, 1-β-D-arabinofuranosylcytosine (NSC 63878); TNC, total nucleated cells; NRBC%, fraction of nucleated erythrocyte precursors, expressed as a percentage of the total nucleated cells; %ID, percentage of injected dose; NRBC, nucleated erythrocyte precursors; ERI, erythroid response index.
200 mg/kg body weight. Drugs were dissolved in sterile 0.9% NaCl solution and injected i.p. at 0.1 ml of solution per g body weight. Drug-induced mortality was observed only following the 10-mg/kg ADR dose level. Approximately 20% of these mice died within 30 days. There were no drug-associated deaths in any treatment group from 30 to 150 days after treatment. Concurrent untreated age-matched controls were also utilized in these studies.

At 45 or 120 days after drug treatment, one-half of the mice from each group were lightly anesthetized with ether and bled from the postorbital venous plexus with calibrated, heparinized Pasteur pipets. Approximately one-third of the calculated blood volume (estimated as 6.4% of the body weight) was removed on each of 2 successive days and replaced at each bleeding session by an equal volume of warm 0.9% NaCl solution i.p. to prevent hypovolemic shock. Such bleeding resulted in a prompt reduction of the hematocrit by approximately 50%. Mortality from the bleeding procedure was generally less than 10%.

The mice were killed 48 hr after bleeding and 6 hr after an i.p. injection of 1 ¡Ci of 59Fe citrate (20 Ci/g; New England Nuclear, Boston, Mass.). Spleens and femurs were immediately removed, cleaned of adherent soft tissue, and placed in iced 0.9% NaCl solution.

**Marrow Cellularity Assay.** The right femurs were stripped of loosely adherent connective tissue, the epiphyses were removed, and the diaphyseal marrow was expelled by flushing the medullary cavity with 1 ml of fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). The marrow clumps were dispersed by repeated aspiration of the suspension through a 24-gauge needle attached to a plastic tuberculin syringe. This cell suspension was diluted with isotonic Saline Diluent (Fisher, Pittsburgh, Pa.), and aliquots were taken for the determination of radioactivity/ml, TNC/ml, and the NRBC%.

Radioiron incorporation, determined in a well-type gamma scintillation spectrometer, was compared to separately prepared injection standards and expressed as %ID/ml. TNC/ml were determined with a Coulter Counter after lysis (Zaponin; S/P, McGraw Park, Ill.) of the mature erythrocytes. The NRBC% was determined from Wright-Giemsa-stained cytocentrifuge (Shandon Southern, Inc., Sewickley, Pa.) preparations. The contralateral femur was used to determine the radioactivity per femur, expressed as the %ID/femur.

The total erythroid cellularity (NRBC/femur) was calculated from the following relationships:

\[
\text{NRBC/femur} = \frac{\text{NRBC/spleen}}{0.064} \times \frac{\text{TNC/femur}}{\text{NRBC/femur}} = \frac{\text{NRBC/spleen}}{0.064} \times \frac{\text{TNC/femur}}{\text{NRBC/femur}}
\]

\[
\text{NRBC/spleen} = \frac{\text{NRBC/mg}}{\text{TNC/mg}} \times \frac{\text{TNC/mg}}{\text{TNC/femur}} \times \frac{\text{TNC/femur}}{\text{TNC/ml}} \times \frac{\text{TNC/ml}}{\text{TNC/femur}} = \frac{\text{NRBC/mg}}{\text{TNC/mg}} \times \frac{\text{TNC/mg}}{\text{TNC/femur}} \times \frac{\text{TNC/femur}}{\text{TNC/ml}} \times \frac{\text{TNC/ml}}{\text{TNC/femur}}
\]

\[
\text{NRBC/mg} = \frac{\text{NRBC/spleen}}{0.064} \times \frac{\text{TNC/femur}}{\text{TNC/ml}} \times \frac{\text{TNC/ml}}{\text{TNC/femur}} = \frac{\text{NRBC/spleen}}{0.064} \times \frac{\text{TNC/femur}}{\text{TNC/ml}} \times \frac{\text{TNC/ml}}{\text{TNC/femur}}
\]

\[
\text{TNC/ml} = \frac{\text{TNC/femur}}{\text{TNC/femur}} \times \frac{\text{TNC/femur}}{\text{TNC/ml}} \times \frac{\text{TNC/ml}}{\text{TNC/femur}} = \frac{\text{TNC/femur}}{\text{TNC/femur}} \times \frac{\text{TNC/femur}}{\text{TNC/ml}} \times \frac{\text{TNC/ml}}{\text{TNC/femur}}
\]

**Spleen Cellularity Assay.** The resected spleen was cleaned, weighed, and then bisected transversely. One half was then weighed and counted for radioactivity (expressed as %ID/mg). The other half was minced with fine scissors in fetal calf serum. Residual tissue clumps were removed by filtering through nylon gauze. The resultant suspension was diluted with phosphate-buffered saline, and aliquots were taken for %ID/ml, TNC/ml, and NRBC% (spleen) determinations as described for the marrow samples. Parameters of splenic cellularity were calculated from the following relationships:

\[
\text{ERI} = \frac{\text{RI}}{100} \times (1 - \text{ERI})
\]

The ERI was calculated from

\[
\text{ERI} = \frac{(R_B - R_NB)}{(R_B - CB)/(CB - CNB)}
\]

where \( R_B \) and \( R_NB \) are the cellularity parameters (NRBC/spleen or NRBC/femur) for bled and nonbled drug-treated mice, respectively, and \( CB \) and \( CNB \) are the cellularity parameters for the untreated bled and nonbled controls, respectively.

RESULTS

Gross observations of the animals in each treatment group showed no differences between any of the drug-treated groups and the untreated controls. Body weights at 120 days were similar in all groups [37.6 ± 1.2 (S.E.) g] except for the group given 10 mg ADR per kg, which was significantly subnormal (29.8 ± 0.8 g). Hematocrit values in nonbled mice were also similar in all groups (44.2 ± 1.0%). At 48 hr after the induction of the anemia stress, hematocrit values were routinely reduced to approximately 50% to 20.3 ± 0.9%.

Chart 1 shows the anemia-induced changes in spleen weights 120 days after ADR pretreatment. The data have been normalized to the values obtained for drug-treated nonanemic control spleens. The data indicate a dose-dependent decrease in spleen weight response to anemic stress. In untreated mice, 48 hr of anemic stress resulted in a 75 to 85% increase in spleen weights. At the highest ADR dose, spleen weights were similar to those for nonanemic controls. In contrast, increases in spleen weight after anemic stress in mice pretreated with...
200 mg ara-C per kg 120 days previously were similar to those for untreated controls.

Chart 2 shows the anemia-induced changes in the nucleated erythroid precursor fraction in spleens and femoral marrow 120 days after ADR. Nonanemic NRBC%’s in both femoral marrow and spleens were significantly, although slightly, increased at the 2 highest ADR doses. Anemia resulted in increased NRBC%’s in all instances; however, this increase was reduced in a dose-dependent manner in both spleens and femoral marrows by ADR pretreatment. NRBC%’s in spleens and femoral marrows from anemic and nonanemic mice pretreated with 200-mg/kg ara-C were similar to their respective untreated controls.

Chart 3 shows the incorporation of $^{59}$Fe (as %ID) in femurs and spleens from bled and nonbled mice 120 days after ADR or ara-C. $^{59}$Fe uptake in spleens from non-drug-treated anemic mice was nearly twice that of the nonbled counterparts. Radioiron uptake in the marrow of untreated anemic mice was, however, somewhat less than that in the nonanemic controls. In both femoral marrow and spleen, $^{59}$Fe incorporation decreased with increasing ADR dose. Radioiron uptake in ara-C-pretreated mice was similar to that in untreated controls.

Chart 4 shows the anemia-induced changes in total splenic cellularity at 120 days after ADR or ara-C. In untreated mice, total splenic cellularity was increased by only about 38%. Inasmuch as spleen weights increased by 85% (see Chart 2), TNC/g decreased (19.2 ± 2.1 x 10$^8$ and 14.8 ± 1.7 x 10$^8$ cells/g for nonanemic and anemic controls, respectively). This is probably a reflection of an increase in cell volume and mass of the less mature cells observed after the anemic stress.

Splenic TNC were similar in all nonbled groups except those pretreated with 10 mg ADR per kg. The apparent decrease, however, is due primarily to the subnormal body weights in this group. When the splenic cellularity was normalized to the body weights, the nonbled cellularity was similar in all groups (e.g., 3.85 ± 0.47 and 3.93 ± 0.38 x 10$^8$ TNC/spleen/kg body weight for nonanemic controls and nonanemic mice pretreated with 10 mg ADR per kg, respectively).

ADR pretreatment resulted in a dose-dependent decrease in the total spleen cellularity that was seen 48 hr after anemia stress. Total splenic cellularity in bled and nonbled ara-C-pretreated mice was similar to that in the respective untreated controls.

Chart 5 shows the anemia-induced changes in splenic nucleated erythroid cellularity in mice 120 days after ADR or ara-C. The results are presented both as NRBC/spleen and NRBC/mg spleen. Unlike TNC/mg, which showed little increase within 48 hr after anemic stress, erythroid cellularity (NRBC/mg spleen) increased dramatically (more than 7-fold) in untreated mice. The data also indicate an ADR-induced dose-dependent decrease in the anemic erythroid cellularity. In mice pretreated with 10 mg ADR per kg, the NRBC/mg spleen in the bled group was indistinguishable from that in the nonbled controls. This dose-dependent decrease in anemia-stimulated splenic erythropoiesis is also apparent if splenic cellularity is expressed as NRBC/mg spleen. NRBC cellularity in bled and nonbled mice 120 days after ara-C was similar to that in the respective untreated controls.

Chart 6 shows the femoral erythroid cellularity response to anemic stress 120 days after ADR or ara-C. The femoral erythroid cellularity in all nonanemic ADR-treated groups was similar to that in untreated controls. Within 48 hr after bleeding, femoral marrow erythroid cellularity in untreated mice increased by more than 2.5-fold. However, ADR pretreatment resulted in a dose-dependent decrease in the anemia-stimulated erythropoietic response. At the 10-mg/kg dose level, the
but, in the spleen, saturation may have been accomplished at a lower drug dose.

The RI's in femoral marrow and spleens 45 and 120 days after 10 mg ADR or 200 mg ara-C per kg are compared in Table 1. ara-C-induced RI in bone marrow repaired rapidly, with no significant damage remaining at 120 days. There was no detectable ara-C-induced RI in the spleen at either study interval. In contrast, ADR-induced RI in bone marrow and spleens was comparatively high and showed no significant reduction between 45 and 120 days.

**DISCUSSION**

The present studies were initiated to evaluate the effect of ADR and ara-C on the erythropoietic response to acute anemia stress 45 and 120 days after drug treatment. The anemia-induced changes in erythroid cellularity following drug pretreatment were evaluated by gravimetric, morphological, and radiotracer methodologies and compared to anemia-induced changes seen in untreated age-matched controls. This approach has been used previously to evaluate radiation-induced RI (10, 11) but differs from earlier work in which radiation was used both to induce the RI and as the nonspecific stress for its detection (3, 4, 24, 36).

The acute effects of ADR and ara-C on the hematopoietic system have been studied (5, 27). Whereas both ADR and ara-C kill cycling cells (27), the 2 drugs act by different mechanisms. The lethal effects of ADR are mediated by intercalation of the drug with DNA and the subsequent arrest of cells in G2 (2, 19, 25), in which the S-phase specificity of ara-C is mediated by inhibition of DNA polymerase (12). Twenty-four hr after a single dose (250 mg/kg or less) of ara-C, stem cell

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**Table 1**

<table>
<thead>
<tr>
<th>Substance</th>
<th>45 days</th>
<th>120 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR (10 mg/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral marrow</td>
<td>89.3 ± 10.2 (10)</td>
<td>98.1 ± 7.8 (10)</td>
</tr>
<tr>
<td>Spleen</td>
<td>87.6 ± 9.4 (10)</td>
<td>78.8 ± 5.6 (10)</td>
</tr>
<tr>
<td>ara-C (200 mg/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral marrow</td>
<td>46.3 ± 6.0 (10)</td>
<td>7.3 ± 10.2 (10)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
* Numbers in parentheses, number of animals.
survival has been reported to be 80 to 50% (8, 9, 27, 28, 38). CFUs and rapidly proliferating CFUs, however, are more sensitive (27, 28). Twenty-four hr after 5 or 10 mg ADR per kg, stem cell survival was estimated to be approximately 60 and 30%, respectively (20). In other studies, similar stem cell sensitivities have been noted (1, 5, 18, 31). CFUc survival was reported to be 80 and 30% after 5 and 10 mg ADR per kg, respectively (20). Thus, the relative levels of RI noted 120 days after ADR or ara-C treatment cannot be totally explained on the basis of acute stem cell killing.

Botnick et al. (6, 7), using a serial bone marrow transplantation technique, showed that busulfan and, to a lesser extent, L-phenylalanine mustard induced a slowly or nonrepairing proliferative defect in the pluripotent stem cell compartment. Trainer and Morley (37) demonstrated that intensive fractionated treatment with busulfan and 1,3-bis(2-chloroethyl)-1-nitrosourea, but not with cyclophosphamide, 5-fluorouracil, vinblastine, methotrexate, or 6-mercaptopurine, resulted in normal marrow CFUs and CFUc levels 60 days after treatment. These and other studies have led to the suggestion that late hematopoietic effects of cytotoxic chemotherapy may be a consequence of drug-induced stem cell depletion and limited repopulation potential (17).

Hanna (13, 14) has shown that anemic stress, induced by bleeding, results in an increase in the number of divisions for the erythroid elements present in the recognizable pool prior to the anemic stress. This response was attributed to the shortening of the cell cycle times for these cell populations after bleeding (15). Erythropoietin has been shown to stimulate cell proliferation in non-heme-producing as well as heme-producing erythrocyte progenitors within 8 hr after erythropoietin injection (16). During the first 48 hr after anemia, however, the earliest erythroid precursor, the burst-forming unit is probably not responsive to erythropoietin stimulation (21). The deficit in the 48-hr anemia-induced erythroid cellularity response in ADR-pretreated mice (i.e., the RI) may therefore be not a direct manifestation of ADR-induced reductions in stem cell or burst-forming unit cellularity (multiplicity) but an expression of damage residing in the more differentiated erythroid cell compartments. This would, however, not limit the RI to the more differentiated precursors, since any long-lasting cellular abnormality in these rapidly proliferating populations would of necessity be fixed in their less-differentiated precursors.

Although nonanemic base-line erythroid cellularity in the spleens and femoral marrows at 120 days after ADR or ara-C was similar to that in age-matched untreated controls, an earliest erythroid precursor, the burst-forming unit is probably not responsive to erythropoietin stimulation (21). The deficit in the 48-hr anemia-induced erythroid cellularity response in ADR-pretreated mice (i.e., the RI) may therefore be not a direct manifestation of ADR-induced reductions in stem cell or burst-forming unit cellularity (multiplicity) but an expression of damage residing in the more differentiated erythroid cell compartments. This would, however, not limit the RI to the more differentiated precursors, since any long-lasting cellular abnormality in these rapidly proliferating populations would of necessity be fixed in their less-differentiated precursors.

From the present data, it is not possible to discern whether ADR-induced RI is permanent or, as in X-irradiated rats (11), is slowly repaired. No significant RI recovery was observed between 45 and 120 days after 10 mg ADR per kg. Although a significant RI was noted after ara-C in the femoral marrow at the early study interval (45 days), no injury was detected in the spleen. ara-C-induced bone marrow RI recovered rapidly, and no damage could be detected at 120 days. In contrast, no recovery of RI was observed between 45 and 120 days after 10 mg ADR per kg, and the level of RI induced by ADR was dose dependent at 120 days.

In our previous studies with intestinal epithelium, high-dose ara-C apparently induced no long-term effects, but ADR was shown to induce significant delayed toxicity (33, 34). In these studies, the integrated jejunal cell production following 1000 R of abdominal X-irradiation was subnormal at 28 and 49 days after ADR treatment, even though stem cell multiplicity and crypt cellularity were within normal limits prior to radiation stress. Interestingly, in this system, the response to the 1000-R test dose at 48 days was significantly reduced from that seen at 28 days, indicating that the ADR-induced damage was not immediately apparent after drug treatment.

Unlike previously reported studies which have quantitated the number of CFUs (37) or their proliferative potential (7) after drug treatment, the erythroid proliferative response to anemia was quantitated in drug-treated mice up to 120 days after treatment. As such, the responses we measured could also be indicative of other effects of drug treatment, such as renal or hepatic toxicity, which could retard erythropoietin production, slowly repairing stromal toxicity, or other as yet unidentified factors. In this context, although cyclophosphamide treatment failed to significantly affect the proliferative capacity of the CFUs (7) or their numbers 60 days after intensive treatment (37), our initial results with this drug suggest a subnormal response to anemic stress in the bone marrow and spleens 120 days after a single 200-mg/kg treatment (33). Studies are currently under way to investigate the role of possible erythropoietin production and/or recognition defects involved in the expression of erythropoietic RI.

The significance of the subnormal erythropoietic response in ADR-treated mice for resumption of normal homeostasis is not known. One might speculate, however, that during a chronic hematopoietic stress, as has been described during cancer in animals (22) and humans (23, 35, 39), hematopoietic responses may be subnormal and, thus, the tolerance to a delayed therapy may also be subnormal. It is well appreciated that patients undergoing chemotherapy for recurrent disease often have a lower drug tolerance to this therapy than that observed during initial treatments.

In light of the available information on the action of antineoplastic agents, it is not surprising that alkylating agents have been shown to have the greatest potential for induction of delayed toxicities. These agents, however, are also some of the most effective cancer chemotherapy drugs. By definition, delayed toxicity implies that the host survives the immediate problems associated with malignant disease and its treatment, and it may be successfully argued that the potential for delayed toxicity is a necessary sacrifice accompanying an effective therapy that induces a significant and thus worthwhile disease-free interval. Inasmuch as recurrent disease is still a major problem, ways in which to screen agents as to their effectiveness in a particular tumor (32) and the most effective ways in which to administer these agents (e.g., in combination, in sequence, or in combined modality protocols) should be actively sought to minimize unnecessary delayed as well as acute toxicity to critical normal tissues.

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