Pre–CFU-S Quiescence and Stem Cell Exhaustion After Cytostatic Drug Treatment: Protective Effects of the Inhibitory Peptide pGlu-Glu-Asp-Cys-Lys (pEEDCK)

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Pre–CFU-S are characterized by their ability to generate spleen colony-forming cells (CFU-S) and by their ability to repopulate the hematopoietic system after damage. We have investigated their response to three consecutive injections of cytosine arabinoside (ara-C), given at t = 0, 12, and 20 hours. Nine hours after treatment, the number of CFU-S and pre–CFU-S was reduced to 10% or 30%, respectively. No pre–CFU-S were in S-phase at this time, indicating that the pre–CFU-S losses were not caused by direct drug killing. Up to 1 year after treatment, pre–CFU-S were still depleted to 10% of normal, indicating that their proliferative quiescence was permanent. We have previously shown that inhibition of CFU-S recruitment with pGlu-Glu-Asp-Cys-Lys (pEEDCK) makes them ara-C resistant and prevents their decimation. We now found that this also **prevented the excessive drainage of the pre–CFU-S pool, suggesting that pre–CFU-S allocation into active hematopoiesis is triggered by the CFU-S deficit. pEEDCK may thus be applicable as a protector of the hematopoietic repopulation potential against cytostatic drug-induced aplasia. Postchemotherapeutic stimulator treatment with (pEEDCK)$_2$-dimer did not ameliorate pre–CFU-S losses. Long-term culture-initiating cells (LTC-ICs) showed a similar pattern of irreversible reduction after cytostatic drug treatment, which could be prevented by pEEDCK. Our results suggest, that certain subclasses of hematopoietic stem cells (pre–CFU-S) are permanently quiescent and exhaustible and that the capacity for self-renewal is not a necessary property of all stem cell–like cells.**
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$\mathbf{I}$N AN ADULT ORGANISM, a high output of functional blood cells is sustained by a slowly dividing population of primitive stem cells.[1] The size of the stem cell pool remains relatively constant over prolonged times despite a continuous drain through differentiation-accompanied cell divisions. To explain this apparent constancy, each stem cell is thought to have the ability to either divide and differentiate or to self-renew, with both daughter cells remaining in the stem cell compartment.[2] The balance between these alternatives seems to be determined by the requirements of the organism in such a way that the number of stem cells is kept constant.

The most important functional property of stem cells is their capability of long-term repopulation of the hematopoietic system.[3] Through this property they have recently become accessible to experimental investigation[4-8] but accumulating evidence indicates that the term stem cells comprises several distinct subclasses of primitive hematopoietic cells. Among these, pre–CFU-S[3,9] are defined by their ability to generate pluripotent spleen colony-forming cells (CFU-S) in two-step transplantation assays. It has been shown[4,8-11] that they share physical, antigenic, and biologic properties with other primitive cells detected by various in vitro and in vivo assays.[3-5,8,10-16] Most of these primitive cells are capable of resisting[7] the toxic effects of cycle-specific cytostatic drugs like 5-fluorouracil, but some subtypes are sensitive to repeated drug applications.[17]

We investigated the behavior of CFU-S–generating pre–CFU-S in mice under conditions causing CFU-S decimation and extreme proliferation of surviving CFU-S. Such conditions were induced by repeated cytosine arabinosine (ara-C) treatment and modulated by application of the hemoregulatory peptide pEEDCK as described earlier.[18] By combining the pre–CFU-S assay[4] with suicide techniques, we have found that (1) pre–CFU-S were reduced already shortly after treatment, (2) pre–CFU-S were proliferatively quiescent despite their drastic numerical reduction, and (3) pre–CFU-S remained depleted until at least 1 year after treatment. Long-term culture-initiating cells (LTC-ICs), which seem to be close relatives of the pre–CFU-S,[4,19] showed a similar irreversible depletion. This surprising result suggests that, contrary to

expectations based on the traditional stem cell concept, compensatory self-renewal is not a necessary property of all subclasses of hemopoietic stem cells. Pre–CFU-S seem to constitute an exhaustible reservoir of permanently quiescent primitive cells.

MATERIALS AND METHODS

Animals. All in vivo experiments were performed with 8- to 12-week-old (at the time of treatment) female BALB/c mice obtained from the breeding unit of the University of Vienna. They were kept under specific pathogen-free (SPF) conditions and had free access to (autoclaved) food and (acidified) water. The experiments we describe were performed in accordance with Austrian legislation on animal experiments under permit Nos. GZ68 205/7-12/88 and GZ68 205/259-12/88.

Chemicals. All chemicals and solvents were of reagent grade and were obtained from Merck (Darmstadt, Germany). Culture media and sera were from Boehringer (Vienna, Austria). Ara-C was a gift from Upjohn (Vienna, Austria).

Drug treatment protocol. Groups of five mice received intraperitoneal injections of ara-C (300 mg/kg, injection volume: 0.2 mL/mouse, solvent: pyrogen-free isotonic NaCl) with a 12-hour interval between them (t = 0 and 12 h). This resulted in maximal CFU-S proliferation 8 hours after the second ara-C administration.[18] A third injection of ara-C was therefore given at t = 20 hours. Control treat-

ment was with pyrogen-free 0.9% NaCl solution and the same time schedule.

Hemoregulatory peptide. An oxidation-resistant sulfur-protected derivative of pGlu-Glu-Asp-Cys-Lys (pEEDCK) was synthesized and activated as described previously.[20] Activation with dithioerythritol was performed immediately before use. This procedure prevents the oxidative formation of the disulfide bonded homodimer of pEEDCK, which is a strong stimulator of CFU-S proliferation.[21] pEEDCK was administered by intraperitoneal injection of 30 μg/kg in a volume of 0.2 mL. Subcutaneous or intraveneous injections had the same effect. The disulfide bonded dimer (pEEDCK)$_2$ was synthesized and purified as described before.[20] It was applied as continuous infusion with osmotic minipumps (type 1007, Alzet, Palo Alto, CA) at a rate of 1.4 μg/kg/d for 6 days. The pumps were filled and assembled under sterile conditions according to the instructions of the manufacturer and implanted under the dorsal skin under light ether anesthesia. The skin incision was closed with a standard surgical wound clip. Empty pumps were removed 1 day after their nominal running time.

Myeloid progenitors. Granulocyte-macrophage colony-forming cells were cultured by standard methods as described previously.[20] Colony formation was induced by suitably diluted mouse lung-conditioned medium in media composed of minimal essential medium (MEM)-alpha supplemented with 20% horse serum and containing methylcellulose (0.8%, 4,000 cps; Colorcon, Königstein, Germany). After 7 days at 37°C and 7.5% CO_2, aggregates of more than 40 cells were scored as colonies.

Determination of day-11 CFU-S numbers. Pluripotent hematopoietic cells (CFU-S) were determined by the spleen colony assay of Till and McCulloch.[12] Six to 10 recipient mice per group were irradiated with 8.5 Gy (1 Gy/min) from a ^{60}Co source and within 3 hours received 5.10^4 to 1.10^5 donor bone marrow cells (in 0.2 mL) into a lateral tail vein. After 11 days, the spleens were excised, fixed in Carnoy's solution, and the visible splenic nodules were counted under a stereomicroscope. The number of transplanted bone marrow cells was adjusted to give 15 to 20 colonies per spleen. The formation of endogeneous colonies was excluded in control experiments.

Pre-CFU-S. Pre-CFU-S were determined by a technique adapted from Ploemacher and Brons.[4] In short, 1.10^5 bone marrow cells were transplanted by intraveneous injection into seven irradiated BALB/c "primary" recipients as described above for CFU-S determination. After 11 days, the spleens and the bone marrow of these mice were obtained. The spleens were evaluated for day-11 CFU-S. Aliquots of the bone marrow were retransplanted within 1 hour into a second group of irradiated recipients. After another 10 days, the spleens of these "secondary" recipients were obtained, fixed, and evaluated for the number of macroscopic nodules. The pre-CFU-S number given is this number calculated per femur of primary recipient. It represents the number of day-10 CFU-S generated in the femurs of the primary recipients from pre-CFU-S contained in the original donor marrow.[4]

Determination of S-phase cells by suicide with ara-C. Proliferating CFU-S and pre-CFU-S were assayed by the suicide method using ara-C as S-phase killing agent as described previously.[18] In short, aliquots of the marrow cell suspension were incubated for 1 hour at 37°C with or without 10^{-3} mol/L ara-C in RPMI 1640. After washing twice, the cells were suspended in RPMI 1640 and the CFU-S or pre-CFU-S content was determined by the methods described above. The percentage of CFU-S or pre-CFU-S in S-phase was calculated as $100 \times (n_0 - n)/n_0$, where n and n_0 are the splenic nodule numbers in the suicided and the control group, respectively.

LTC-ICs. LTC-ICs were assayed by a modification of the methods of Eaves et al[19] and Van der Sluijs et al.[9] This two-step assay measures the production (after 3 to 4 weeks) of secondary clonogenic cells (CFU-GM) in a trypsinized and replated primary 3- to 4-week-

old long-term bone marrow (BM) culture. The basic strategy is similar to the in vivo pre-CFU-S assay described above. The following procedure was used. Feeder layers were prepared from normal BM cells (1×10^6 cells/cm^2 in 35-mm plastic Petri dishes) in enriched Van der Sluijs medium.[9] At weekly intervals, the cultures were fed by replacing 50% of the supernatant with fresh medium. On day 21, when a confluent adherent cell layer had formed, nonadherent cells were removed and the culture irradiated with 11 Gy (1 Gy/min) from a ^{60}Co source. The feeder layer cultures were then inoculated with 1×10^5 BM cells/mL obtained from the femurs of mice that were treated three times with ara-C as in the other experiments and maintained at 5% CO_2 in air, 33°C, and 98% relative humidity. Three weeks later, the BM culture on the first feeder layer was dispersed by consecutive treatment with trypsin-EDTA and collagenase. The digestions were terminated by the addition of serum-containing medium and washing with Hanks' balanced salt solution. The cells were resuspended in fresh medium, seeded onto irradiated second feeder layers (prepared in the same way as the first ones), and maintained for further 3 weeks under the above-mentioned conditions. Nonadherent cells were removed by washing and gentle agitation. The adherent layer was disrupted by treatment with trypsin and collagenase as above. The number of CFU-GM present in the adherent and nonadherent layers was determined by standard techniques as described above.

RESULTS

Long-term restoration of hematopoiesis after ara-C treatment. Three doses of ara-C (300 mg/kg) were injected into mice as described in the Materials and Methods section. pEEDCK (30 μg/kg) was given as three injections (2-h intervals) between the second and third ara-C application. This induced a severe but nonlethal leukopenia, after which the mice recovered. They were then left without further treatment until day 67. At this time, all cell populations down to the day-11 CFU-S level had returned to normal values (Table 1) and remained so for at least 1 year.

CFU-S and pre-CFU-S 9 hours after three ara-C injections. Three doses of ara-C were injected as described above. Nine hours after the last ara-C injection, the BM was obtained

Table 1. Hematopoietic Status of Mice Two Months After Treatment With Three Doses of Ara-C

Treatment	Nucleated Cells ($\times 10^{-6}$)	CFU-GM	Day-11 CFU-S
Control	13.7 ± 4.5	32,300 ± 1,800	3,150 ± 400
3 × ara-C	14.2 ± 2.5	24,300 ± 5,200	3,100 ± 650
3 × ara-C + pEEDCK	13.0 ± 2.1	31,800 ± 6,500	3,350 ± 200
3 × ara-C + (pEEDCK)$_2$	16.0 ± 5.0	39,200 ± 6,600	3,700 ± 950

Groups of five mice were treated with three doses of isotonic NaCl, three doses of 300 mg/kg ara-C (at 0, 12, and 20 h) with or without additional three doses of 30 μg/kg pEEDCK (or solvent) given 2, 4, and 6 hours after the second ara-C injection, or with three doses of ara-C followed by continuous infusion of the (pEEDCK)$_2$-dimer (1.4 μg/kg/d for 6 d). All animals were subsequently left without further treatment until day 67. At this time, the femoral BM was obtained and assayed for its content of nucleated cells, committed progenitors CFU-GM, and pluripotent spleen colony-forming units day-11 CFU-S. The results (means ± SD of three independent experiments) are expressed "per femur" of the ara-C–treated donor animals.

and assayed for CFU-S and pre–CFU-S. The cycling status of these cells was determined by a suicide experiment measuring their resistance to ara-C. The results (Fig 1) show that CFU-S were reduced to about one tenth of the normal value and that practically all of them (90%) were intensively proliferating (10% after control treatment). At the same time, the number of pre–CFU-S had decreased to one third, but none of them were found synthesizing DNA.

Long-term effects of ara-C treatment on pre–CFU-S numbers. The number of pre–CFU-S in untreated or solvent-treated mice was determined on day 0 and after 38 and 360 days. No age-related changes were found, as shown in Fig 2A. In ara-C–treated mice, the number of pre–CFU-S was determined 9 hours and 19, 67, and 360 days after treatment. Ara-C treatment caused an irreversible reduction to 10% to 20% of the normal value which persisted until at least 1 year after treatment. In some experiments, reductions down to 2% of the normal value were observed. The application of 30 μg/kg of CFU-S–inhibitory pEEDCK 2, 4, and 6 hours before the third ara-C injection preserved about 50% of the pre–CFU-S, whereas postchemotherapeutic treatment with the stimulatory dimer (pEEDCK)$_2$ did not result in improved recovery of pre–CFU-S (Fig 2B).

Correlation of CFU-S proliferation with pre–CFU-S losses. As we have shown previously, CFU-S are intensively proliferating after two injections of ara-C.[18] In our three-dose ara-C protocol, the third ara-C injection was given at the time of maximal CFU-S proliferation, achieving a maximal CFU-S kill, which was reduced to about half by pEEDCK application. The percentage of CFU-S in S-phase at the time of the third ara-C injection was correlated (Fig 3) with the number of pre–CFU-S present 67 days after treatment when

hematopoiesis had stabilized again. We find an exactly linear relationship between the two parameters ($r^2 = .987$).

Number of LTC-ICs after ara-C treatment. Three doses of ara-C were injected as described above, with or without additional pEEDCK injections. After 2 months, the BM was obtained and the number of LTC-ICs was determined in serial long-term cultures as described in the Materials and Methods section by measuring the number of CFU-GM present at the end of the second culture period. Figure 4 shows that the production of (fresh) CFU-GM from LTC-ICs was strongly reduced in cultures from ara-C–treated marrow. Considerably fewer CFU-GM were present in the adherent as well in the nonadherent culture layer. If the assay was performed with bone marrow cells from mice treated with ara-C plus pEEDCK, the reduction of LTC-ICs was not observed.

DISCUSSION

The most primitive hematopoietic cells are resistant to single doses of S-phase–specific cytostatic drugs, whereas more mature progenitors and precursors are killed.[7,22] Mechanisms apparently related to the reduction of marrow cellularity then induce compensatory proliferation in early hemopoietic populations. The phenomenon is best known for the CFU-S population[18,23,24] but has recently also been shown for more immature cells detectable by competitive repopulation assays.[17] In ara-C–treated mice, intensive CFU-S cycling is observed over prolonged times[18] indicating that either cohorts of CFU-S are successively recruited into proliferation, or that cells from an earlier compartment (pre–CFU-S) are allocated into CFU-S and start proliferating. Such a pre–CFU-S influx into active hematopoiesis is indicated by the data given in Fig 1 showing that already 9 hours after the
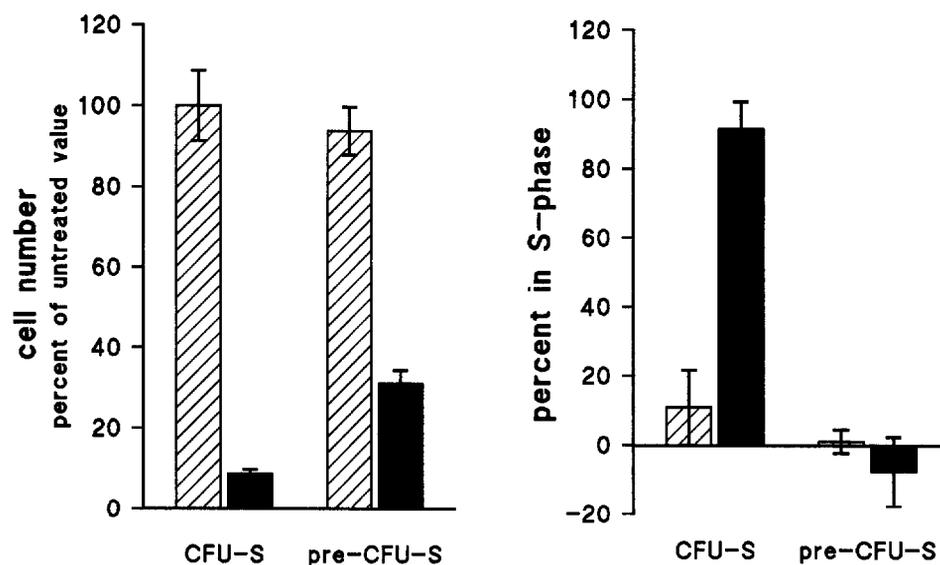
Fig 1. Determination of the proliferative status of pre–CFU-S and CFU-S after three injections of ara-C. Mice were treated three times with 300 mg/kg ara-C (■) (at 0, 12, and 20 h) or three times with isotonic NaCl solution (▨). Nine hours after the last ara-C injection, the BM was obtained. CFU-S were determined by transplanting into irradiated hosts (spleen colony assay) and pre–CFU-S by retransplantation into a second irradiated host. Values given are the percentage of CFU-S and pre–CFU-S in untreated mice. Cells in S-phase were eliminated by in vitro suicide incubation with ara-C for 1 hour. Percentage in S-phase were calculated as 100 × (N_0 − N)/N_0, where N and N_0 are colony numbers in the suicided and nonsuicided groups, respectively.
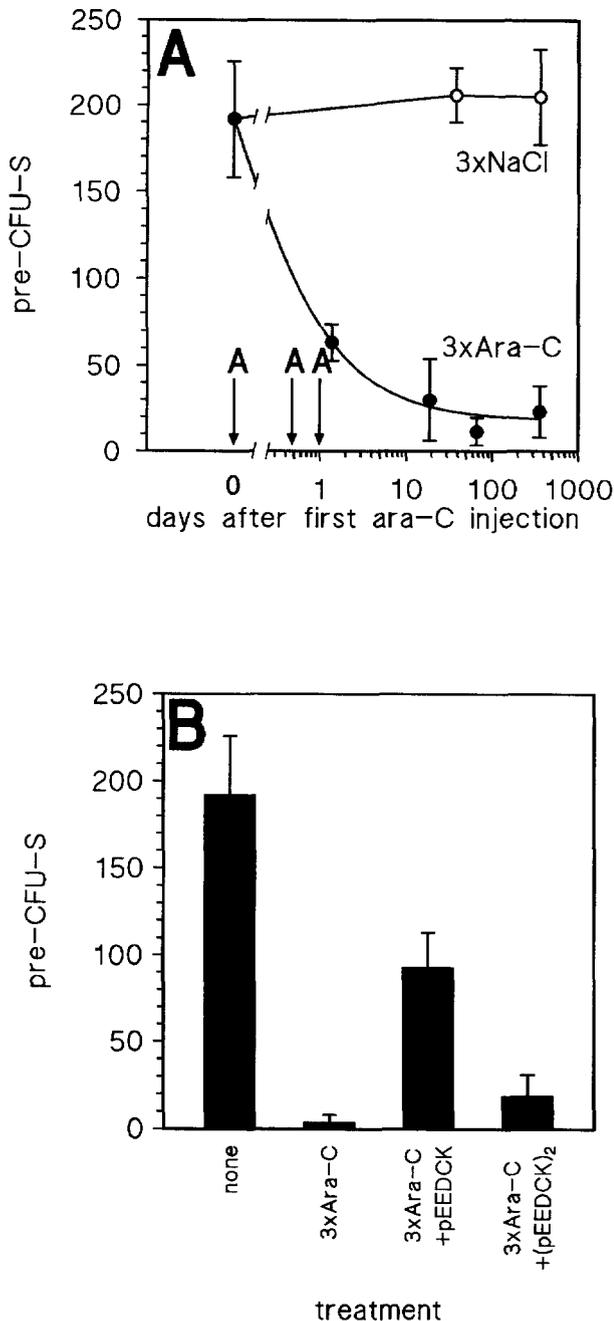
Fig 2. Pre–CFU-S status of mice after treatment with three injections of ara-C ± pEEDCK ± (pEEDCK)₂. Groups of five mice were treated intraperitoneally (IP) at 0, 12, and 20 hours with three doses of isotonic NaCl, or three doses of 300 mg/kg ara-C. Three IP doses of 30 μg/kg monomeric pEEDCK (or solvent) were given 2, 4, and 6 hours after the second ara-C injection. Dimeric (pEEDCK)₂ was given as continuous infusion with osmotic minipumps (0.14 μg/kg/d for 6 d) starting 4 hours after the last ara-C injection. All animals were subsequently left without further treatment. At selected time points, the femoral BM was obtained and assayed for its content of marrow-repopulating pre–CFU-S. The results (± SD) are from five independent experiments and are expressed per femur of primary recipient. (A) Time course of the pre–CFU-S decline; (B) influence of pEEDCK and (pEEDCK)₂ on the pre–CFU-S status 67 days after ara-C treatment. All later compartments had returned to their normal values by this time (see Table 1).

end of treatment, 70% of the pre–CFU-S have disappeared from their compartment. This numerical decrease is apparently not caused by direct ara-C killing because suicide experiments performed at the same time showed that the pre–CFU-S were completely resistant to ara-C, indicating their proliferative quiescence. It appears that the allocation of quiescent pre–CFU-S into active hematopoiesis does not involve proliferation events and that remaining pre–CFU-S do not rapidly start to self-renew. However, it is possible that the expected self-renewal proliferation of the pre–CFU-S occurred at later times[25] and thus remained undetected in our relatively early suicide experiments. Such proliferation should result in the eventual recovery of the pre–CFU-S population. The depletion was, however, irreversible. Pre–CFU-S numbers remained low until at least 12 months after drug treatment (Fig 2A). It is interesting to note that despite a 90% reduction of pre–CFU-S, all posterior populations (CFU-S and later) recovered completely and permanently (Table 1). It is known[10,17,26] that under steady-state conditions, only a small number of hematopoietic clones are active and sufficient for maintaining hematopoiesis over prolonged times. Relatively few remaining stem cells can thus sustain a normal level of hematopoietic activity, which is consistent with our observations (Fig 1A).

CFU-S cycling (ie, the degree of CFU-S killing by ara-C) and the number of pre–CFU-S remaining available are linearly correlated (Fig 3). This and other observations[26] indicate that the population deficit in the CFU-S compartment is the causal factor for excessive pre–CFU-S consumption. Keeping this deficit small, eg, by preventing CFU-S recruitment with the inhibitory peptide pEEDCK,[18] would require the allocation of fewer pre–CFU-S into CFU-S and result in their numerical preservation. Under these conditions, about 50% of the pre–CFU-S remain available 2 months after drug treatment (Fig 2B).

In vitro culture of FACS-sorted Thy^{lo}/lin$^-$/sca$^+$ cells in presence of various cytokine combinations, including c-kit ligand and/or interleukin-11, leads to a strong increase in the output of pluripotent clonogenic cells[27-29] suggestive of stem cell proliferation in these cultures. However, net cell production is transient, indicating[30] that LTC-ICs are consumed rapidly without adequate replacement. LTC-ICs are among the most immature, stem cell–like cells and share antigenic and physical properties with the pre–CFU-S and other stem cell subclasses.[3,9,19] We have found (Fig 4) reduced LTC-IC content in long-term cultures of BM from ara-C–treated mice, indicating that these cells did not recover during the 2 months between ara-C treatment and marrow sampling for long-term culture. pEEDCK prevented excessive LTC-IC losses similar to its protective effect on pre–CFU-S (Fig 2B). Until suitable assays for human pre–CFU-S are available, stem cell decimation may remain hidden behind a normally looking hematopoietic façade even after treatment modalities allowing complete recovery of the "visible" part of hematopoiesis. Culturing LTC-ICs from the bone marrow of patients with tumors treated with cytostatic drugs may allow detection of stem cell deficits before aplasia becomes evident.

Primitive hematopoietic (stem) cells are defined as self-renewing and capable of increased proliferation restoring a
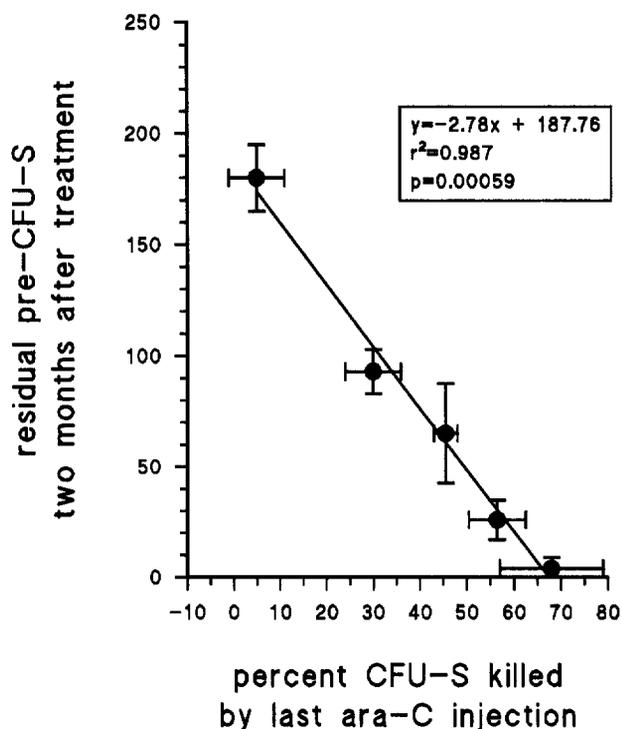
$$y = -2.78x + 187.76$$
$$r^2 = 0.987$$
$$p = 0.00059$$

percent CFU–S killed by last ara–C injection

Fig 3. Correlation between pre–CFU-S losses after three injections of ara-C and CFU-S proliferation at the time of the third ara-C injection. Data are from Fig 2 (67 days after treatment) and ref 18. The solid line was obtained by linear regression.

havior of the pre–CFU-S, their behavior in turn may not reflect stem cell behavior. Most stem cell–like populations, including CFU-S,[34] high-proliferative–potential colony-forming cells, hpp-CFU,[35] and cells detected by competitive repopulation assays,[17] can either be stimulated to proliferate in culture or can be triggered into cell cycle by demand situations in vivo. This is not the case with pre–CFU-S, which do not become suicide sensitive in a severe demand situation and do not recover with time. This does not exclude that some degree of very slow proliferation might be present below the detection limit. However, the physiologic significance of such extremely slow self-renewal (no recovery within a year, which is about half of the lifetime of a mouse) would be questionable.

Third, the question may be raised if the absence of self-renewal from one stem cell subpopulation is a unique property of this population (pre–CFU-S) or if other stem cell–like cells may behave in a similar way. The question of stem cell proliferation[36] has been addressed repeatedly. However, recent evidence[3,33] "precludes the requirement for self-renewal of hemopoietic stem cells."[1,37,38] Such views are supported by the exhaustability of the stem cell pool under various experimental and clinical conditions.[26,32,39,40] In a manner analogous to the function of the ovary, the hematopoietic stem cell compartment may consist of a limited reserve of primitive cells that are sufficient in number to maintain hematopoiesis over several normal life spans.[33] The possibility cannot be excluded that during ontogeny, an ample reserve of primary hematopoietic cells is formed from which the stem cell compartment is continually replenished. In mice, a large stem cell pool sufficient to maintain hematopoiesis for 15 to

normal population size after numerical reduction. In our experiments, we have reduced the pre–CFU-S number to a few percent of the normal level, but we have subsequently neither detected DNA synthesis nor numerical regeneration for at least 1 year. Pre–CFU-S seem thus to be a static, nonproliferative, population.

Our finding that at least one subpopulation of the stem cell family is permanently nondividing raises several questions. First, the pre–CFU-S assay measures the CFU-S–generating capacity and does not directly measure stem cell self-renewal. Essentially two types of assay are known to provide reliable data on stem cell proliferative potential[31]: competitive repopulation studies[5] and serial transplantation into irradiated mice.[32] Spangrude[33] has confirmed the validity of the double-transplant pre–CFU-S assay by showing that Ly-6A/E[pos], Rh-123^{low} cells contained long-term repopulating stem cells and generated the largest number of secondary CFU-S, whereas fractions depleted of long-term repopulating cells (Ly-6A/E[neg] Rh-123^{high}) generated only few secondary CFU-S. The combination of double-transplant assays with suicide techniques, as in our experiments, together with a long-term follow-up of the CFU-S–generating capacity may thus tell something about the proliferative behavior of stem cells after chemotherapy.

Second, the hierarchical or functional relationship of (competitive) long-term–repopulating cells or pre–CFU-S to the "real" stem cells is not clear. In the same way as the behavior of the CFU-S population does not reflect the be-
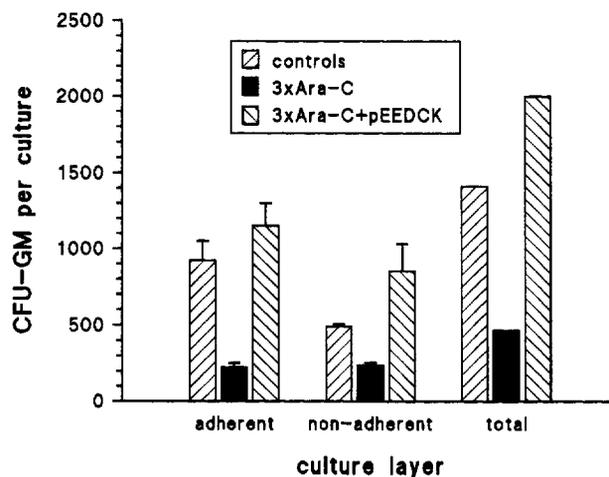

culture layer

Fig 4. LTC-ICs in murine bone marrow 8 weeks after three ara-C injections. Mice were injected three times with isotonic NaCl solution or three times with 300 mg/kg ara-C (at 0, 12, and 20 h) ± 30 μg/kg pEEDCK (at 14, 16, and 18 h). After 8 weeks, the BM cells were obtained and cultivated (for details, see text) for 19 days on a feeder layer of normal BM cells. Cells of the adherent layer (containing the LTC-ICs) were transferred onto a second feeder layer and further cultured for 21 days. Then the content of CFU-GM was determined in aliquots of the (secondary) adherent and nonadherent layers. The results given here (± SD) are from one of four independently performed experiments leading to similar results.

20 lifetimes has been found,[5] indicating that only one twentieth (5%) of the stem cell reserve will be used during a normal lifetime. This small decrease could be mistaken as a constancy that does not exist. Under such conditions, stem cells without self-renewal capacity cannot be excluded,[3] and, as Micklem[41] has pointed out, strong emphasis on self-renewal as the essential property of stem cells may even be misleading. It is neither necessary to assume that self-renewal occurs in this population of primitive cells[33] nor that proliferation events are necessarily involved when such a primitive cell is called into service. Subsequent[3,27,33] proliferation and differentiation result in a net production of multipotent precursors, with a concomitant loss of proliferative potential. According to such views, which are supported by our results, the prime source function of primitive hemopoietic cells seems to be dissociated from the traditional stem cell functions of self-renewal and differentiation and different subpopulations of the stem cell family may differently express these properties.

Our findings may have important clinical consequences[42] because it must be assumed that quiescence and exhaustability are also properties of human stem cells, with serious implications for cytostatic drug therapy. It is well known that repeated treatment cycles of alkylating cytostatic drugs may lead to aplasia and that surviving stem cells lack the ability to proliferate.[32] This has mainly been attributed to the specific cellular damage caused by alkylating drugs. Here we show that irreversible stem cell losses also occur after treatment with nonalkylating antimetabolites, like ara-C, which are commonly regarded as safe with respect to long-term damage.[43] Similar to the situation after alkylating drugs,[32] stem cells surviving repeated courses of ara-C treatment do not proliferate. Our results show that pre–CFU-S decimation is a direct quantitative consequence of the proliferative CFU-S disturbances caused by cytostatic drug treatment and not exclusively a result of qualitative stem cell damage, although such additional damage cannot be excluded for alkylating drugs. It is possible[31] that combining chemotherapy with hematopoietic growth factor treatment may increase the danger of stem cell exhaustion. Although short-term recovery is known to be improved by growth factors for relatively mature progenitors, hematopoietic stem cell (pre–CFU-S) depletion by repeated high-dose cyclophosphamide therapy is increased by application of granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF).[44] Our results with the CFU-S-stimulatory peptide (pEEDCK)$_2$ show that stimulator treatment after chemotherapy does not necessarily cause increased stem cell losses.

Because cytostatic drug-induced damage to the hematopoietic system is related to intensive proliferation of hematopoietic cells, the application of hemospecific inhibitors[45] during chemotherapy might have beneficial effects. We have systematically developed this concept[42] and have shown[18,24,21] that the application of the hemospecific CFU-S–inhibitory peptide pEEDCK prevents depopulation of the CFU-S compartment. Keeping the CFU-S population quantitatively intact not only ameliorates short-term neutropenia[18,21] but, as we show here, may also reduce the need for replacement from the stem cell pool. The highly hemospecific and easily

synthesized pentapeptide pEEDCK may therefore be a clinically useful supplement to conventional cancer chemotherapy ameliorating both short-term and long-term damage.

ACKNOWLEDGMENT

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