Effects of Cyclophosphamide and of Busulfan on Spleen Colony-forming Units and on Hematopoietic Stroma

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SUMMARY

The effects of cyclophosphamide (CY) and busulfan (BU) on the hematopoietic stromal function (HS-P) of mouse marrow were evaluated. Stromal function of femoral marrow was assessed by implanting the test femur s.c. into an isogenic host and determining the number of CFU-S in the implant 6 weeks later. Since the CFU-S have been shown previously to be primarily of host origin, this presumably measures the ability of donor hematopoietic sites to harbor host CFU-S.

After injection of CY, the number of CFU-S in the marrow fell but recovered to normal within 6 weeks. The HS-P function fell to half-normal after 10 mg of CY and did not regenerate detectably in 6 weeks. On the other hand, 2 mg of BU i.p. caused a lesser initial decline in the number of CFU-S, but recovery was still incomplete after 6 weeks. BU given p.o. had a more marked effect on CFU-S and caused a significant decline in the HS-P function.

Doses of CY (5 mg/dose) given intermittently appear to cause cumulative damage to HS-P function. HS-P function did not, in any experiment, recover significantly in the 6 weeks following the last dose of CY.

This result suggests that large doses of the alkylating agent CY causes prolonged and perhaps permanent HS-P damage. This damage to the HS-P is cumulative when the CY is given at weekly intervals. Despite lack of HS-P recovery, CFU-S regenerate rapidly after CY therapy is stopped.

On the other hand, BU also causes damage to the HS-P. However, even when BU is given at a dosage that does not significantly affect the HS-P, CFU-S recovery is delayed, suggesting that BU affects the CFU-S in a manner that differs qualitatively from that of CY.

INTRODUCTION

CY* and BU are commonly used alkylating agents, capable of causing severe hematopoietic suppression. Their effects on the proliferation, differentiation, and maturation of hematopoietic stem cells (CFU-S) and their progeny have been intensively studied (5, 9, 18, 21, 22). There are, however, also microenvironmental elements which determine the extent to which CFU-S can proliferate and differentiate in hematopoietic sites (1, 7, 8, 10, 16, 17). One form of microenvironmental influence is the so-called cell-cell interaction (7, 17), whereby proliferation of hematopoietic stem cells and also of myeloid committed precursor cells (11) is inhibited by proximity to large numbers of other cells at the same site.

Another variety of microenvironmental influence on CFU-S proliferation and differentiation is exemplified by the role of nonmobile (probably stromal) elements in hematopoietic sites in promoting the proliferation and differentiation of CFU-S (10, 23, 24). Wolf and Trentin (24) have defined as a hematopoietic-inductive microenvironment that portion of the hematopoietic stroma which influences the ability of CFU-S to differentiate. In addition, there are other factors which influence the ability of CFU-S to proliferate. Conditions that determine the ability of CFU-S to proliferate in a particular hematopoietic site are probably complex and may include the nature of the blood supply and the presence of cells that inhibit CFU-S growth, as well as the presence of cells that specifically promote CFU-S growth. The term HS-P will refer to the overall hematopoietic stromal function as manifested by its ability to support the growth of CFU-S.

The method used to assess HS-P function is based on the observation that all identifiable hematopoietic tissue initially disappears after s.c. implantation of a femur into an isogenic host (19). In the subsequent 6 weeks, however, it regenerates. At this time the differentiated hematopoietic cells and the CFU-S are predominantly of the host type, whereas the stromal elements are of donor type (8, 10). Accordingly, the HS-P function of a femur is assessed by assaying the number of CFU-S that reside in its marrow 6 weeks after implantation into an isogenic host. Defects of both the HS-P and the hematopoietic-inductive microenvironment contribute to the pathogenesis of the congenital anemia of S1/S14 mice (6, 16, 23) and have been postulated to play a role in the etiology of some forms of aplastic anemia in man (14). Damage to stromal factors probably also causes the long-lasting hematopoietic failure that occurs in heavily irradiated sites in man (4) and in rats (13). Some data are available on the effects of radiation on the HS-P (3, 15). However, the effects of alkylating agents on the hematopoietic stroma have not yet been determined. The studies to be reported here show the effects of BU and of CY on both the multipotential hematopoietic stem cells (CFU-S) and on the hematopoietic stromal factors that support CFU-S proliferation (HS-P).

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2 The abbreviations used are: CY, cyclophosphamide; BU, busulfan; CFU-S, spleen colony-forming units; HS-P, hematopoietic stromal factors that affect spleen colony-forming unit proliferation.

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MATERIALS AND METHODS

BALB/c X A F1 male mice weighing 20 to 25 g were used. CY was diluted with distilled water to a concentration of 5 mg/0.5 ml. One-half ml of this solution was injected i.p. into 5 mice/group according to treatment schedules indicated in the description of specific experiments. One hundred mg of BU in powder form were suspended in 10 ml of acetone and diluted with sesame oil to a concentration of 1 mg/0.5 ml. One-half ml of this suspension was either injected i.p. or was instilled into the stomach via a plastic, blunt-tipped catheter passed p.o. Controls received the appropriate vehicle in which the BU or CY was suspended or dissolved.

Radiation was delivered by a 60Co source with a unidirectional horizontal beam at a target distance of 36 inches and a rate of 76 rads/min. CFU-S were assayed by the method of Till and McCulloch (20). Marrow HS-P function was assayed by the method of Fried et al. (8) as follows. Five femurs to be assayed for HS-P were removed from 5 donor mice, and all attached muscle and tendon was removed. Both ends of the femur were then excised, and the shaft was implanted s.c. into a deeply undermined, 1-cm ventral skin incision on the abdomen of an otherwise normal syngeneic host. Each host mouse received 2 implants. One was a femur from either a BU- or CY-treated donor, and the other consisted of a femur from its corresponding control (both were implanted through the same incision; however, they were separated by placing them into an area undermined either to the right or to the left of the midline). The wound was closed with clips, which were removed after 5 days. Six weeks postimplantation, the host mice were sacrificed, and the ends of the femur were removed. Cells flushed from the 5 control femur implants were pooled and suspended in 50 ml of Hanks' balanced salt solution; the same was done with cells flushed from the 5 femur implants from CY- or BU-treated donors. The number of CFU-S in these cell suspensions was determined by injection of 0.5 ml of the cell suspensions (equivalent to the cells obtained from 1/femur implant) into each of 15 irradiated assay mice. Statistical significance of differences was tested by the Student t test. p values of < 0.01 were considered significant.

RESULTS

Effect of CY and BU on HS-P and CFU-S. Mice received either 5 mg CY i.p., 1 mg BU i.p., or 1 mg BU p.o. daily for 2 days. Controls received identical amounts of the vehicle in which these substances were dissolved or suspended. One and 42 days after receiving the last dose, batches of 5 mice from each group were sacrificed. The number of CFU-S in the femoral marrow and the HS-P function of the marrow were then assayed.

The results are shown in Chart 1 (CFU-S) and Chart 2 (HS-P function). The number of marrow CFU-S fell to less than 1% of that in controls 1 day post-CY and recovered to nearly control values in 42 days. On the other hand, the number of marrow CFU-S fell to 10% of that in controls (significantly less suppression than after CY) 1 day post-BU (given i.p.) and recovered to only about 50% of control values in 42 days (significantly less recovery than after CY). The number of CFU-S in marrows of mice that received 2 mg of BU p.o. fell to about 1% of that in controls after 1 day and recovered to about 50% of control values in 42 days. The marrow HS-P function of CY-treated mice was 50% of that in controls 1 day post-injection and did not recover significantly in the subsequent 42 days. The marrow HS-P function of mice that received BU i.p. declined only to about 85% of that in controls (difference from controls is not significant [p > 0.05]) and returned to control values within 42 days. The marrow HS-P function of mice that received BU p.o. fell to 40 to 50% of that in controls within 1 day after the last dose and did not recover significantly in 42 days.

The Effect of Various Amounts of CY on Marrow CFU-S and HS-P. Mice received either 1, 3, or 6 doses of CY (5 mg/dose) i.p. at weekly intervals. Controls received an equivalent volume of 0.9% NaCl solution. One and 42 days after the last injection, the number of CFU-S in the femoral marrow and the marrow's HS-P function were assayed in batches of 5 mice/group. The results are shown in Charts 3 (CFU-S) and 4 (HS-P function). The number of CFU-S in mice that received 1, 3, or 6 weekly injections of CY declined to about 10 to 15% of that in controls, 1 day after the final injection. The CFU-S population of mice given CY once and 3 times recovered to control values 42 days after the last
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Chart 2. The effect of VU and of CY on marrow HS-P function. Mice given CY and BU as indicated on the abscissa were sacrificed 1 or 42 days after the final dose, and the HS-P function of their femoral marrow was assessed after s.c. implantation of the femur into isogeneic hosts by the method described in the text. The HS-P function of marrows from CY- and BU-treated mice was compared to that of control mice that simultaneously received the appropriate vehicle. Each host mouse was implanted with 1 control and 1 test femur. The bars represent the mean results of assays of CFU-5 from pooled cells of 5 implants assayed in 15 assay mice and are expressed as a percentage of the results in controls. Brackets, S.E. The results of 2 experiments are shown.

injection. Mice given CY 6 times did not survive 42 days. Marrow HS-P function declined significantly to about 60% of control values after 1 injection of CY, to 10 to 20% after 3 injections, and to 0 to 2% after 6 injections. Significant recovery of HS-P function did not occur in the mice given injections either once or 3 times. The HS-P recovery of mice given CY 6 times could not be assessed since they did not survive 42 days.

DISCUSSION

Administration of both CY and of BU causes a decline in the number of CFU-S in the marrow. However, the CFU-S population regenerates more rapidly after damage by CY than by BU. The slow recovery rate of CFU-S after administration of BU has been attributed by Udupa et al. (21) to a predilection of BU for noncycling cells. This, however, does not explain the results of the studies reported here which indicate that the CFU-S population regenerates less rapidly after injection of 2 mg of BU than after injection of 10 mg of CY, even though the initial decline in the number of CFU-S is more marked after injection of CY than after BU. This observation suggests either that the CFU-S damage caused by BU is qualitatively different from that caused by CY.

The observation that 2 mg of BU, given p.o., cause more CFU-S damage than does the same amount given intraperitoneally and also results in detectable HS-P damage suggests that BU, suspended in acetone and oil, is either more effectively absorbed from the gastrointestinal tract than it is from the peritoneum or is less rapidly deactivated in the gastrointestinal tract than in the peritoneal cavity. Accordingly, exposure of the hematopoietic stroma to the amount of BU that is absorbed in the gastrointestinal tract causes significant HS-P damage, whereas exposure to the amount absorbed from the peritoneal cavity is insufficient to do so. The HS-P damage caused both by BU and CY is repaired slowly, if at all, as is also the case for radiation-induced HS-P damage (3).

The data reported here, as well as those previously published (3, 6), indicate a discrepancy between the ability of femoral implants to support host CFU-S growth 6 weeks postimplantation (presumably a measure of HS-P function) and the number of CFU-S in the femur before implantation. For example, the number of CFU-S in the femoral marrow of WCB6-S1/St1/mice is near normal (16), whereas the ability of this marrow to support the growth of CFU-S after implantation into WCB6-+/+ or WCB6-+SI/St1 hosts is markedly reduced (6). Also, the number of CFU-S in the femoral marrow 6 weeks after exposure to 950 rads and injection of 10^9 nucleated marrow cells is normal, whereas the HS-P

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they recover to only about 50% of normal after exposure to than in those of mice that receive 2 daily injections, indicat-
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covers to normal values after exposure to 300 rads, whereas
(3, 12).
celled marrow cells) 6 weeks before the 2nd radiation dose
of the observation that, although the number of marrow
sublethal dose of X-imradiation (12). This evidence consists
slowly than do those implanted into WCB6-++ mice (16),
imposed by the "shock" of implantation or by exposure to a
function of the femur (assessed by the implantation method
described above) is markedly reduced (3). In addition, ac-
cording to the data shown above, the number of CFU-S
recovers promptly after various amounts of CY are injected
into mice, whereas the marrow HS-P remains damaged for a
prolonged period of time. This suggests the possibility that
the marrow damage detected after implantation of a femur
does not critically affect CFU-S growth in the intact femoral
marrow. Although this possibility cannot be definitively re-
tected, there is evidence to support the contention that the
inability to support CFU-S after implantation does reflect
significant underlying HS-P damage which is not sufficient,
per se, to reduce the number of CFU-S in the femoral
marrow, but which limits the ability of CFU-S in the marrow
to recover completely from subsequent injury such as that
imposed by the "shock" of implantation or by exposure to a
sublethal dose of X-irradiation (12). This evidence consists
of the observation that, although the number of marrow
CFU-S in WC86-S1/S1++ mice and in mice that had been
exposed to 950 rads 6 weeks previously is normal, CFU-S
injected into irradiated WC86-S1/S1++ mice regenerate more
slowly than do those implanted into WC86-+/+ mice (16), and
the marrow CFU-S of previously unirradiated mice re-
covers to normal values after exposure to 300 rads, whereas
they recover to only about 50% of normal after exposure to
300 rads in mice that had received 950 rads (and 10⁶ nu-
cleated marrow cells) 6 weeks before the 2nd radiation dose
(3, 12).
A significantly larger number of CFU-S remain in the
marrors of mice that receive 3 or 6 weekly injections of CY
than in those of mice that receive 2 daily injections, indicat-
ing that substantial CFU-S recovery takes place in the 7 days
between doses. On the other hand, the amount of HS-P
damage is considerably more marked after 6 weekly in-
terons of CY than after 2 daily ones. This is probably due to
the very slow rate of HS-P repair, which results in cumula-
tive damage from successive doses of the drug.
In patients treated with large doses of CY (60 to 120 mg/
kg) repetitively for up to 6 doses, no cumulative effect was
observed on the severity of leukopenia and or early leuko-
cyte recovery. However, there was cumulative damage to
thrombopoiesis (2). This may be interpreted to mean that
the cumulative effects, if any, of CY given to man in doses
that are at about the tolerable limit (with regard to nonhe-
matopoietic toxicity) do not produce sufficient stromal
damage to markedly delay hematopoietic recovery. It
should be noted that the doses used in our studies in mice
are well in excess of those used in man (on a per weight
basis).

Further studies are now under way to detect the effects of
combinations of drugs on HS-P and to determine the effects
of drugs combined with radiation on HS-P.

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