Differential Effects of Drugs upon Hematopoiesis Can Be Assessed in Long-Term Bone Marrow Cultures Established on Nylon Screens (43384)

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Abstract. **Hematotoxicity is associated with exposure to chemotherapeutic drugs and numerous other agents. Most measurements of the hematopoietic effects of prospective therapeutic drugs and environmental agents have been made in animal models. We tested the influence of various drugs on hematopoiesis in long-term cultures of Long-Evans rat bone marrow cells. These cultures were established on nylon screen-bone marrow stromal cell templates that were suspended in liquid medium. Previous phenotypic analyses of adherent zone cells of suspended nylon screen bone marrow cultures (NSBMC) using monoclonal antibodies and flow cytometry indicated that they maintain a multilineage character for extended periods in culture and display continuous proliferation of hematopoietic progenitors (colony-forming unit culture [CFU-C]). NSBMC of various ages were incubated for 21 hr with several concentrations of β-D-cytosine arabinofuranoside, 5-fluorouracil, cyclophosphamide, or methotrexate. Adherent zone cells were dissociated enzymatically, phenotyped by flow cytometry, and assayed for colony-forming unit culture content. β-D-cytosine arabinofuranoside, 5-fluorouracil, and methotrexate treatment of bone marrow cultures resulted in a dose-related diminution in colony-forming unit culture numbers in the adherent zones of NSBMC. Phenotypic analyses revealed similar trends but certain of these drugs manifested lineage specificities. Toxicity was also related to cyclophosphamide dose, but the presence of bone marrow stroma was necessary to demonstrate this effect *in vitro*. A subpopulation of these cells was found to metabolize ethoxyfluorescein ethyl ester to fluorescein after induction with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, an effect which was quantified by flow cytometry. NSBMC may be used to ascertain lineage-specific toxicities and evaluate the effects of drugs on the proliferation of hematopoietic progenitor cells.**

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Bone marrow is a target tissue for a wide variety of industrial, environmental, and therapeutic agents. The dosage and treatment duration of cell-cycle specific and nonspecific drugs for malignancy are often contingent upon the coincident hematopoietic suppression (1, 2). Although the use of hematopoietic growth factors to offset this suppression has become more widespread (3, 4), the possible co-stimulation of both malignant and normal hematopoietic cells cannot be discounted fully (5). The mechanisms underlying the inhibition of hematopoiesis resulting from drug exposure vary, but the majority of these agents interrupt DNA synthesis. Methotrexate (MTX), a folate analog, slows DNA synthesis by inhibiting thymine production (6). Other agents, such as cyclophosphamide (CP), require bioactivation to toxic metabolites by the cytochrome mono-oxygenases, an event that occurs pri-

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marily within the liver (7); CP metabolites exert their hematosuppressive effects ostensibly after transit to the bone marrow via the peripheral blood. Animal models are the primary tools to assess the potential hematotoxicity of prospective chemotherapeutic drugs and other agents. The benefit of such models is that they can be used to measure drug effects in an integrated organism, even though animal responses to drugs are not always identical to those in humans. However, the costs of animal experimentation are very high in this period of shrinking research funding and societal pressures are being placed upon the scientific community to limit the use of animals in research.

Several *in vitro* methods that measure potential hematopoietic toxicity have been published. Most of these procedures employ bone marrow progenitor-derived clonogenic assays to assess drug effects (8–10). Typically, the drug is added to semi-solid agar plates or suspensions of cells supplemented with serum or various hematopoietic growth factors. The ability of bone marrow cells to form gel colonies of the various lineages with and without the presence of the drug is compared. Another group has reported consistent prediction of toxicity to MTX by assaying progenitors derived from murine, long-term, bone marrow cultures (11). The putative benefit of the long-term bone marrow culture is that it contains not only hematopoietic cells, but stromal cells as well. Stromal cells have been reported to possess drug-metabolizing capabilities (12) and to support hematopoiesis by secreting growth and regulatory factors and extracellular matrix proteins. In this regard, MTX treatment failed to alter stromal cell colony-stimulating factor production in long-term culture, but still reduced the ability of stromal cells to support colony growth (11), possibly by inhibiting the synthesis of critical extracellular matrix components. In contrast to monolayer-based bone marrow cultures, we employ a nylon filtration screen-stromal cell matrix as a template for hematopoietic cell attachment and proliferation. This template, which is suspended in liquid medium, promotes active progenitor cell proliferation and maintains its multilineage character for extended periods in culture (13–15). These nylon screen bone marrow cultures (NSBMC) were used to study the differential effects of drug exposure on hematopoiesis. Hematopoietic potential was measured by the ability of the cells derived from drug-treated cultures to generate colony-forming units culture (CFU-C). The toxicity of MTX, β-D-cytosine arabinofuranoside (Ara-C), CP, and 5-fluorouracil (5FU) to cells of different lineages was assessed by phenotypic characterization using flow cytometry. In addition, the ability of hematopoietic or stromal cells to metabolize ethoxyfluorescein ethyl ester by cytochrome P-450-dependent enzymes was assessed by cytofluorographic methods.

Materials and Methods

Bone Marrow Cultures. The methods used to culture bone marrow cells from rats and other species have been reported in detail previously (13–15). Briefly, single-cell suspensions of Long-Evans male rat (250–400 g body wt) femoral bone marrow cells were plated into 25-cm^2 flasks containing 5–7 ml of Fischer's medium conditioned with 10% fetal bovine serum and 10% equine serum (HyClone Laboratories, Logan, UT) and supplemented with 10^{-7} *M* hydrocortisone hemisuccinate, (Sigma Chemical Co., St. Louis, MO), nonessential amino acids, fungizone, and penicillin/streptomycin (Gibco, Grand Island, NY). After incubation for 2 to 4 hr (33°C, 5% CO_2, humidity ≥90%), the nonadherent (hematopoietic) cells were removed and were frozen incrementally and cryopreserved in the vapor phase of liquid nitrogen (13). Adherent (stromal) cells were grown in monolayer culture until sufficient cell numbers were achieved to inoculate onto nylon screens (~three to four passes). Nylon filtration screens (3–210/36; Tetko Inc., Elmsford, NY) were pretreated with 1.0 *M* acetic acid, coated with solubilized Type IV mouse collagen (Gibco), and soaked in fetal bovine serum to enhance cellular attachment. Treated screens were inoculated with 10^6–10^7 stromal cells in Tissue Tek slide chambers (Nunc, Inc., Napersville, IL) following harvest from monolayer cultures and transferred 6–12 hr later to 25-cm^2 flasks. Stroma was allowed to grow until cytoplasmic processes projected across three to four out of every five mesh openings. At this point, the stromal cell-nylon screen templates were transferred to slide chambers and inoculated with $1–2 \times 10^6$ viable hematopoietic cells that were either freshly isolated or retrieved from cryopreservation (viability, 78–94% after thawing). Medium (2 ml) was added to each chamber and the cultures were transferred to 25-cm^2 flasks 6–24 hr later. The screens are suspended in the medium with sufficient density to remain below the surface, but with enough buoyancy not to touch the bottom of the flask. The co-cultures were fed twice weekly with complete medium.

Drug Treatment. At 10, 33, 100, 150, 170, or 208 days of culture, NSBMC were fed with medium containing 0.025% (0.4 mg/ml), 0.05% (0.8 mg/ml), or 0.1% (1.6 mg/ml) CP; 10^{-6} *M* to 10^{-4} *M* MTX; 0.01, 0.02, 0.05, 0.1, 0.2, 2.0, 3.0, or 5.0 mg/ml of Ara-C; or 0.05, 0.1, and 1.0 mg/ml of 5FU (Sigma). The cultures were removed 21 hr later, washed, and placed in flasks containing drug-free medium for 24 hr before measurements were performed. Adherent zone cells were dissociated into single-cell suspensions using a 1:1 mixture of collagenase Type IV and dispase and analyzed. Since the majority of hematopoietic progenitor and precursor cells were located in the adherent zones of NSBMC, only these cells were assessed in this study. Cells of the

nonadherent zone, which were mostly mature cells with low progenitor activity, were not assayed.

Methods of Analysis. *CFU-C assay.* Cells derived from the adherent zone of NSBMC were incubated in complete medium for 2 hr at 37°C and 5% CO_2. Nonadherent cells ($5–10 \times 10^4$) were plated into 10 mm × 35 mm Lux dishes (Nunc) containing 0.6 g of Noble agar in 100 ml of Dulbecco's modified Eagle's medium with 75 mg of DEAE dextran, 3 g of L-asparagine, and 250 ml of decomplemented rat plasma. Pokeweed mitogen rat spleen cell conditioned medium was used as a source of colony-stimulating activity (16). Since widely variant numbers of CFU-C were expected in NSBMC treated with various drugs and doses, colonies were scored several days earlier than usual to eliminate potential problems of enumerating colonies in overcrowded plates. For this reason, groups of ≥30 cells were considered to be colonies and were quantified after 8 days of culture. The CFU-C assay was only performed on cells derived from the adherent zones of NSBMC.

Flow cytometry. Phenotypic analysis. Adherent zone cells were incubated with 100 μl of the following monoclonal antibodies as described previously (17): MRC OX-33 (directed against the leukocyte common antigen on rat B cells [18]), MRC W3/25 (recognizing T_4 lymphocytes and some macrophages [19]), MRC OX-8 (against T cytotoxic/suppressor cells and natural killer cells [20]), MRC MOM/3F12/F2 (recognizing epitopes on mature granulocytes [21] and myeloid precursors [22]) (Serotec Inc., Cambridge, UK), or mouse IgG_1 (Coulter Immunology, Hialeah, FL). After washing, the cells were labeled for 35 min (4°C) with goat antimouse IgG_1 conjugated to fluorescein isothiocyanate (Cappel Inc., Cochranville, PA). Cells labeled with mouse IgG_1 followed by secondary monoclonal antibodies were used as indirect controls. Specimens were analyzed using an EPICS C flow cytometer (Coulter Electronics) tuned to a wavelength of 488 nm, with the fluorescence gain adjusted to exclude ≥98% of the control cells. Windows were established around the various cell populations using the forward light scatter (FLS) versus side scatter (SS) two-parameter histogram and the percentage of positively fluorescent events was determined. Absolute numbers were calculated as the product of this percentage and the adherent zone cell count.

Cytochrome P-450 assay. Monolayer cultures of bone marrow stromal cells and freshly isolated bone marrow nonadherent cells were assayed for cytochrome P-450 mono-oxygenases by flow cytometry. One nanomole of a 1-μ*M* stock solution of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Chemical Carcinogen Repository, National Cancer Institute, Kansas City, MO) in dimethylsulfoxide (Sigma) was added to cell cultures for 18 hr to induce enzyme activity (23). This nonfluorescent compound was found to be an ideal inducer for this assay. Cells in monolayer cultures were lifted using a trypsin-collagenase mixture (13), pelleted and resuspended in phosphate-buffered saline at a density of ~5×10^5 cells/ml, stored on ice for 1 hr, and gradually warmed to 37°C. Cells were analyzed for evidence of cytochrome mono-oxygenase activity by quantifying incremental fluorescein fluorescence in cells taking up ethoxy-fluorescein ethyl ester (EFEE) (24, 25). Cells were incubated with 50 n*M* EFEE (Molecular Probes, Eugene, OR) in phosphate-buffered saline for 5 min at 37°C and examined for green fluorescence on a flow cytometer with a 515-nm long-pass filter and tuned to the 488-nm band. Fluorescence was gated on various populations of cells based on differences in FLS versus SS characteristics and was measured once per minute for 8 min in samples maintained at 37°C. Fluorescein accumulation in cells over time was indicative of cytochrome P-450 activity (24–26).

Statistical analysis. Flow cytometry measurements were taken in triplicate on sample sizes of 5,000 to 10,000 events. Data representing percentages of controls were calculated using drug-treated versus untreated cultures from each time interval of culture. These percentages were pooled for each drug and dose. All results are expressed as mean ± 1 SE. Levels of significance (P) were determined using Student's t test. Data with $P < 0.05$ were considered significantly different from controls.

Results

The adherent zones of NSBMC contain stromal cells (fibroblasts, macrophages, adipocytes, and endothelia) and blasts that are representative of all of the hematopoietic lineages (13–15). Treatment of these cultures with increasing concentrations of drugs resulted in dose-related decreases in the absolute numbers of specific hematologic cell types, an effect which, in some instances, was exerted differentially. In this regard, myeloid (MOM/3F12/F2$^+$; 59% decrease) and B (OX-33$^+$; 67% decrease) cells were more sensitive to the highest dose of Ara-C (3 mg/ml) than either T_4 (W3/25$^+$; 28% decrease) or T_8 (OX-8$^+$; 43% decline) lymphocytes isolated from 33-day NSBMC (Fig. 1). However, myeloid cells appeared to be sensitive to Ara-C at lower doses than the other cells (Fig. 1). In general, these findings were demonstrable in individual cultures of the same age as well as pooled cultures of different ages, which were treated with the same drug and dose range (Tables I and II). In pooled cultures, Ara-C toxicity toward lymphoid cells was evident at doses ≥2 mg/ml, whereas significant ($P < 0.02$) myeloid toxicity was observed at the 0.2-mg/ml dose (Table I). Although CP caused a linear depletion in the percentages of W3/25$^+$ (T_4), OX-8$^+$ (T_8), and MOM/3F12/F2$^+$ (myeloid cells) in dose ranges of 0.025–0.1%, no linearity was

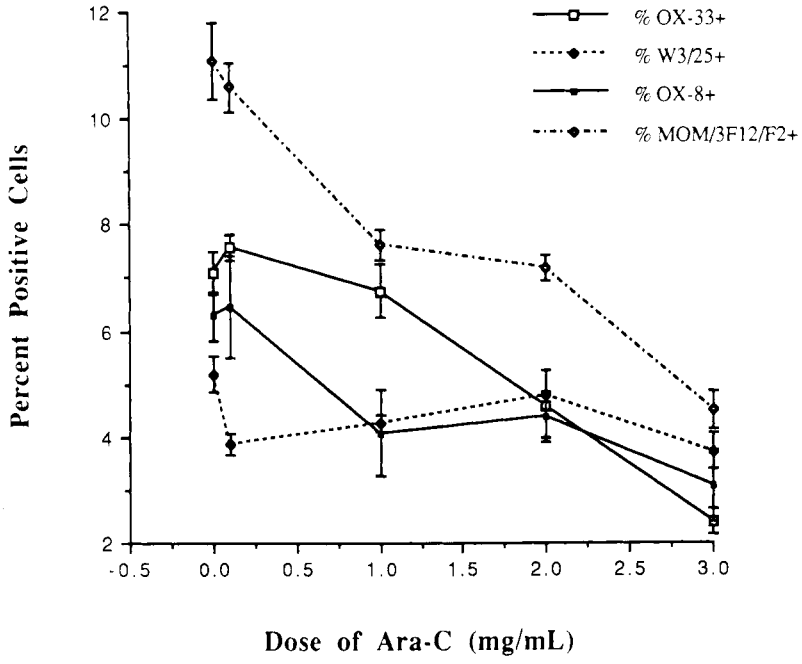

Figure 1. The effect of Ara-C on different hematologic lineages in NSBMC. Vertical bars through the means = ± 1 SE. MOM/3F12/F2, myeloid; OX-33, B; W3/25, T_4; and OX-8, T_8.

Table I. The Effects of Various Doses of CP, Ara-C, MTX, and 5FU on the Phenotypic Distribution of Hematopoietic Cells in the Adherent Zones of Rat Nylon Screen Bone Marrow Cultures[a]

Drug—dose	OX-33 (B)	W3/25 (T_4)	OX-8 (T_8)	MOM/3F12/F2 (myeloid)
Untreated	7.84 ± 0.99 [100]	5.62 ± 0.83 [100]	6.01 ± 0.96 [100]	12.88 ± 0.72 [100]
CP				
0.025%	8.09 ± 0.11 [103]	5.71 ± 0.71 [102]	5.94 ± 0.62 [99]	15.41 ± 4.72 [120]
0.05%	4.80 ± 0.96 [59][b]	3.74 ± 0.95 [67][b]	2.97 ± 0.39 [49][b]	8.26 ± 2.03 [64][b]
0.10%	5.09 ± 2.02 [65]	2.94 ± 0.91 [38][b]	2.73 ± 0.70 [45][b]	7.63 ± 1.25 [59][b]
Ara-C				
0.02 mg/ml	8.37 ± 1.10 [107]	5.96 ± 0.89 [106]	7.54 ± 0.84 [125]	16.35 ± 0.56 [127]
0.20 mg/ml	8.52 ± 1.93 [108]	4.41 ± 1.31 [78]	7.58 ± 0.90 [126]	8.46 ± 0.82 [66][b]
2.0 mg/ml	4.49 ± 0.77 [57][b]	4.08 ± 0.54 [73][b]	4.60 ± 0.73 [77][b]	5.77 ± 0.96 [45][b]
MTX				
10^{-6} *M*	7.02 ± 0.48 [90]	7.52 ± 1.70 [134]	6.03 ± 1.48 [100]	10.36 ± 1.10 [80]
10^{-5} *M*	8.19 ± 1.72 [104]	5.32 ± 0.11 [95]	9.51 ± 1.83 [158]	7.61 ± 0.54 [59][b]
10^{-4} *M*	5.53 ± 0.74 [71][b]	3.78 ± 0.72 [67][b]	3.93 ± 0.53 [65][b]	7.47 ± 0.83 [57][b]
5FU				
0.1 mg/ml	4.47 ± 0.92 [57][b]	5.41 ± 0.75 [96]	3.51 ± 0.89 [58][b]	9.52 ± 0.88 [74][b]
0.2 mg/ml	3.26 ± 0.61 [42][b]	4.39 ± 0.38 [78][b]	2.32 ± 0.14 [39][b]	8.75 ± 0.70 [68][b]
2.0 mg/ml	1.23 ± 0.24 [16][b]	0.82 ± 0.08 [15][b]	0.69 ± 0.09 [11][b]	2.42 ± 0.36 [19][b]

[a] Represents the mean positive fluorescent events for each antibody and each drug and dose level as pooled from cultures of various ages (10, 33, 100, 150, 170, and 208 days). n = 3–11. The bracketed numbers indicate percentage of control.
[b] Significantly different from controls.

noted in the B cell ($OX\text{-}33^+$) response to CP at these doses (Table I). Similar trends were evident with MTX. 5FU treatment, however, appeared to diminish the percentages of all of these cell types in a dose-related manner (Table I).

The effect of these agents on CFU-C progenitor cells was measured as well. Ara-C treatment of NSBMC in a dose range of 0.05–5.0 mg/ml resulted in a significant diminution in the concentrations of CFU-C, but only at doses ≥1.0 mg/ml. In this regard, 1.0, 2.0, and 5.0 mg/ml doses of Ara-C resulted in a drop of 29% ($P < 0.05$), 64% ($P < 0.01$), and 73% ($P < 0.01$) of CFU-C numbers, as compared with untreated control NSBMC (Table II). In addition, CFU-C colonies derived from adherent zone cells of NSBMC were markedly larger (>1 mm in diameter) when cultures were

Table II. Mean Numbers of CFU-C Derived from the Adherent Zones of Suspended Nylon Screen Bone Marrow Cultures after Treatment with Either Cytosine Arabinoside, Methotrexate, or 5-Fluorouracil[a]

Drug—dose	CFU-C (*n*)	Percentage of control[b]	Culture ages[c]
Ara-C			
0 mg/ml	2219	100	33/100
0.05 mg/ml	2332	105.1 ± 9.1	33/100
0.10 mg/ml	2096	94.5 ± 7.7	33/100
0.50 mg/ml	2070	93.3 ± 6.8	33/100
1.0 mg/ml	1575	71.0 ± 8.2	33/100
2.0 mg/ml	801	36.1 ± 4.4	33/100
5.0 mg/ml	590	26.6 ± 5.4	33/100
MTX			
0 *M*	2293	100	100/170
10^{-6} *M*	1650	71.2 ± 11.2	100/170
10^{-5} *M*	718	31.2 ± 1.5	100/170
10^{-4} *M*	598	25.0 ± 1.7	100/170
5FU			
0 mg/ml	534	100	10/33
0.05 mg/ml	423	79.3 ± 6.2	10/33
0.10 mg/ml	394	73.7 ± 5.6	10/33
1.0 mg/ml	214	40.0 ± 8.9	10/33

[a] Means are derived from cultures of various ages that were treated with the same dose of drug. Mean percentages of controls ± 1 SE are listed also.
[b] Calculated from the percentage of applicable controls for each time interval of culture.
[c] Indicates the cultures from which data were pooled for each dose level.

treated with high doses of Ara-C (Fig. 2). This effect was not observed with any of the other test agents. Concentrations of Ara-C ≤0.5 mg/ml had no significant effect on these progenitor cells. MTX in doses of 10^{-6} *M* to 10^{-4} *M* exhibited a dose-related inhibition of CFU-C numbers (29–75%; $P < 0.05$–$P < 0.025$). Similar findings were observed with the 5FU-treated NSBMC.

Although the number of CFU-C and the phenotypic distribution of cells within the adherent zones of NSBMC vary with age, all cultures exhibited a dose-related decrease in CFU-C numbers following treatment with CP when compared with untreated (0%) cultures of the same age (Fig. 3). Toxicity of hematopoietic cells to this synthetic alkylator was apparent only if stromal cells were present. Exposure of freshly isolated bone marrow nonadherent cells (depleted of adherent stromal cells) to CP did not significantly inhibit their ability to generate CFU-C colonies in agar culture (Fig. 3). Differences in CFU-C concentration were apparent in CP-treated NSBMC of different ages. Thus, the percentage of decrease in CFU-C in 10-day cultures evoked by 0.1% CP was 64%, as compared with 89% and 81% for the 170-day and 208-day NSBMC, respectively. In an effort to define the cell type(s) that was responsible for metabolizing CP, bone marrow suspensions that were depleted of adherent cells or monolayer cultures of bone marrow stroma that are used to establish the templates were treated with EFEE after induction with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Modulation of FLS and SS gain, as well as laser power, was used to produce histograms when a minimum of three distinct cell populations could be defined based upon their physical characteristics. For stroma, these attributes were: Map 1, small agranular cells; Map 2, moderately sized granular cells; and Map 3, large granular cells (Fig. 4). Log green fluorescence accumulation gated on each map was measured as a function of time. The larger, more granular subpopulation of bone marrow stromal cells displayed active metabolism of EFEE to fluorescein as indicated by the appearance, with time, of increased numbers of fluorescing cells and an increased fluorescence intensity (peak channel number) (Table III) (Fig. 4). The smaller, granular cells also exhibited a modicum of enzyme activity, but no ability to convert EFEE to its fluorescent product was found in the small to moderately sized agranular stromal cells (Map 1) or any of the bone marrow nonadherent (hematopoietic) cell populations (Table III).

Discussion

Toxicity to bone marrow can be induced by a wide variety of therapeutic drugs and industrial/environmental agents. Many of the anticancer drugs suppress hematopoiesis at the higher dose levels which are often required to achieve therapeutic benefit (1, 2). Although hematotoxicity of prospective therapeutic drugs is assessed in animal models prior to use in humans, relatively few laboratories attempt to measure the effects of drugs on hematopoiesis *in vitro*. The use of hematopoietic, progenitor-derived, clonogenic assays to predict cytotoxicity (8–10) primarily provides information concerning the direct effects of drugs upon hematopoietic cells. The relatively low numbers of stromal cells in these preparations is one potential problem with this approach, since these cells have been shown to exhibit drug metabolizing capabilities as well as important support functions for hematopoiesis (12, 27–30). The generation of colonies in these clonogenic assay systems is stimulated by the addition of exogenous growth and regulatory factors, many of which have been reported to originate from stromal cells (27, 30). Inhibition of the ability of stromal cells to elaborate trophic/regulatory factors, or to proliferate, may be indirectly responsible for the suppression of hematopoiesis that is noted following exposure *in vivo* to certain drugs. In contrast to the progenitor assay systems, long-term bone marrow cultures contain all of the cell types found in bone marrow *in vivo*, and provide a more natural milieu for hematopoietic proliferation and differentiation (27, 31). One group used a murine long-term bone marrow culture system to assess the toxicity of MTX, which

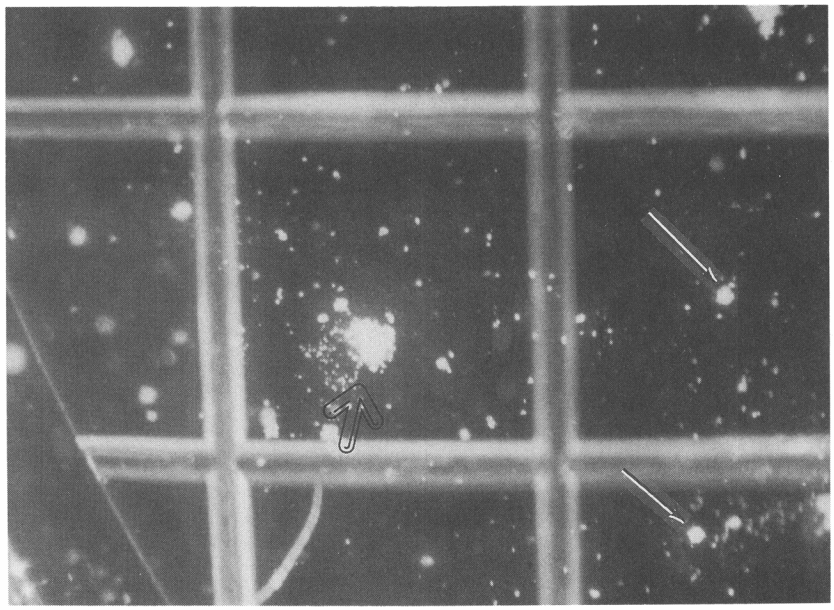

Figure 2. CFU-C assay plate showing a large colony that is >1 mm in diameter (large arrow) versus colonies containing ~30–60 cells (small arrows). The cross-hatched pattern at the bottom of the plate is used to facilitate counting. Cells were derived from the adherent zones of NSBMC treated with 5.0 mg/ml of Ara-C. A mean 17% of the total colony numbers were substantially larger. Total magnification, 30×.

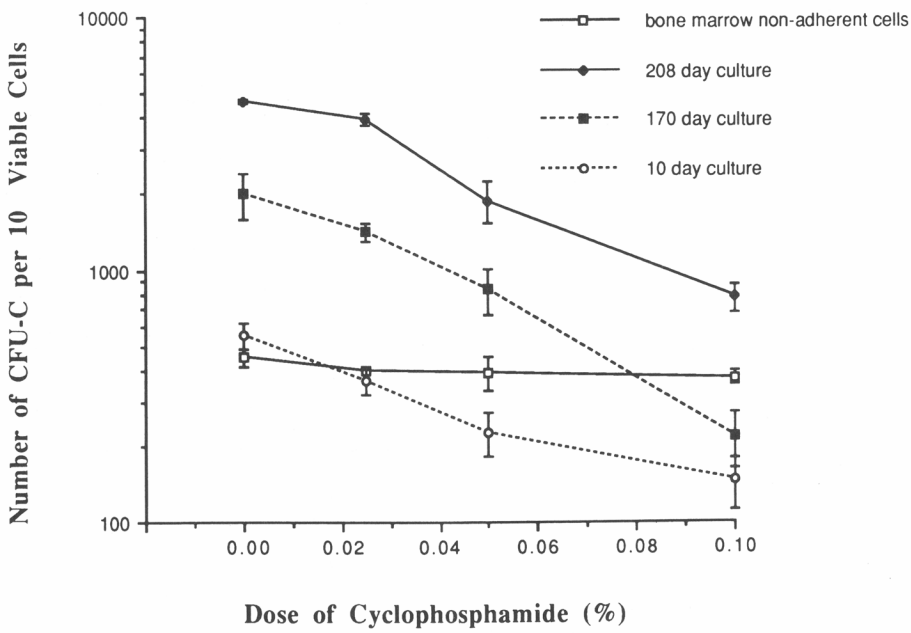

Figure 3. CP-induced decreases in the numbers of CFU-C derived from NSBMC of various ages as compared with its relative lack of effect on bone marrow nonadherent cells. The *y* axis is on log scale, but the numbers represented on this axis are actual. Vertical bars through the means represent ± 1 SE.

suppressed myelopoiesis (CFU-C generation) at dose ranges of 10^{-5}–10^{-3} *M* and also diminished the ability of stromal cells to support hematopoiesis. This effect, perhaps, was mediated by interference with the ability of stroma to synthesize extracellular matrix components, rather than their capacity to synthesize colony-stimulating activity, which was not altered by this drug (11). Monolayer-based bone marrow cultures primarily express myeloid cells; no attempt was made in this study to measure the effect of MTX on other cell types.

NSBMC contain actively cycling hematopoietic progenitors and produce blasts representing all of the hematologic lineages (13–15). The stromal cell content of these cultures is ~40–50% (14). A dose-related diminution in the numbers of CFU-C progenitor cells in NSBMC was demonstrable with all of the drugs used (Fig. 3 and Table II). The percentages of decrease in the concentrations of CFU-C induced by 0.1% CP in the 170-day and 208-day cultures were 89% and 81% of the untreated NSBMC of the same ages, respectively.

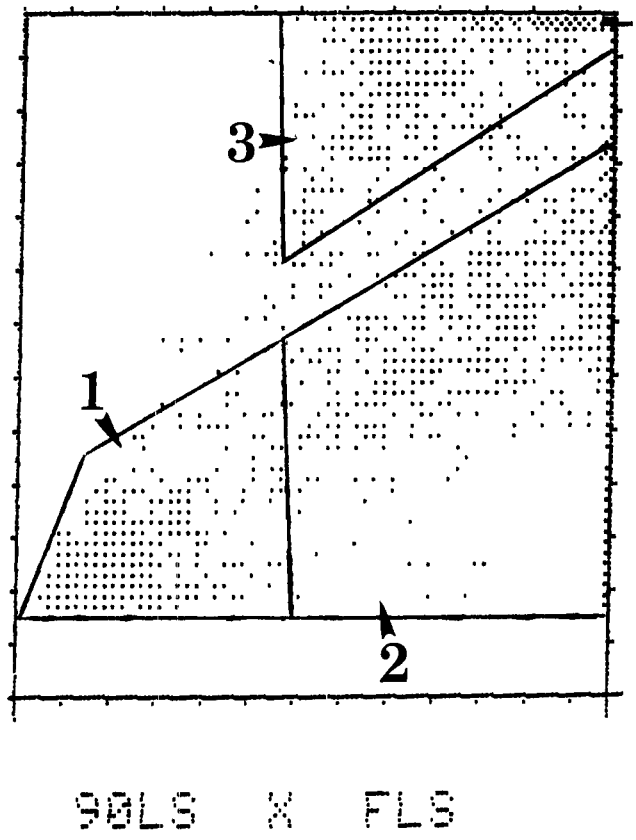

Figure 4. FLS versus SS histogram depicting the maps used to assess the conversion of EFEE to fluorescein in rat bone marrow stromal cells. FLS is an indicator of cell size, whereas SS measures granularity. Log green fluorescence accumulation by cells within each population was measured.

Table III. Fluorescein Fluorescence[a] as a Function of Time after EFEE Introduction in Rat Bone Marrow Stromal Cells or Freshly Isolated Stromal Cell-Depleted, Bone Marrow Nonadherent (Hematopoietic) Cells 21 hr after Induction with TCDD[b]

Time (min)	BMS-total population	CH	Map 1	Map 2	Map 3	BMNA-total population[c]	CH
0	0	40	0	0	0	0	40
1	1.89	42	0	0.27	0.40	0.14	45
2	1.93	50	0	0.37	4.21	0	40
3	3.73	62	0	0.73	8.15	0	48
4	3.16	80	0	0.62	6.89	0	40
5	2.70	47	0	0.50	5.59	0	40
6	2.52	49	0	0.49	5.49	0	43
7	2.26	47	0	0.30	4.03	0	42
8	2.09	42	0	0.17	2.55	0	40

[a] Log green fluorescence gain was adjusted so that the baseline channel for fluorescence measurements (40) was 0%.
[b] Peak channel numbers (CH) and the mean percentages of positively fluorescent cells in three populations of bone marrow stromal cells (defined based on the FLS versus SS histogram; the mean percentages of cells within each map were, 1. 49%, 2. 19%, and 3. 23%) are indicated also. Additional abbreviations used in this table: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; BMS, bone marrow stromal cells; BMNA, bone marrow nonadherent cells.
[c] No detectable positively fluorescent cells were observed either in the total bone marrow nonadherent cell population or any of the three subpopulations. The mapped regions are, therefore, not included in the Table.

In contrast, 0.1% CP diminished CFU-C in 10-day cultures by only 64%. This finding might be attributable to the presence of relatively fewer numbers of stromal cells in the 10-day as compared with the older cultures, or to a greater sensitivity of the recently seeded hematopoietic cells to activated products of this drug. The progenitor potential and phentoypic characteristics of cells within NSBMC are related to the age of the culture as well as the stromal cell "stock" culture that was used to establish the nylon screen template. Since bone marrow stromal cells are somewhat heterogeneous with regard to the type of hematopoietic cells which they support, additional variations may result from random seeding (attachment) of these cells to the pretreated nylon screen. For these reasons, the effects of drugs on cultures of different ages (Fig. 1, e.g.) were interpreted relative to the "0" dose level (untreated) control.

Leukocytic cells, present in the adherent zones of NSBMC, did not react in a uniform manner to all of the drugs used in this study (Table I). Myeloid cells exhibited a greater sensitivity to MTX and Ara-C than did lymphoid cells. Although a dose of 2 mg/ml of 5FU appeared to inhibit all of the leukocytes to a similar degree, CP exhibited greater inhibitory effects against T lymphocytes than either B or myeloid cells at the dose levels we employed (Table I). Since CP requires activation to its toxic metabolites by enzymes of the cytochrome P-450 system (7), this phenomenon may be attributed to metabolism of CP by stromal cells, which are relatively abundant in the adherent zones of these cultures. The toxic effects of CP were manifested only if stromal cells were present; bone marrow nonadherent cells that were treated with CP prior to inoculation into agar gel cultures displayed only nonsignificant decreases in their ability to generate CFU-C colonies (Fig. 3). CP did not appear to exert any direct effects on hematopoietic cells in this study, but we cannot preclude the possibility that this agent might influence a parameter that we did not measure. Cytochrome P-450 activity has been reported to exist in a number of different types of cells, including fibroblasts and macrophages (12, 32–34). In this regard, bone marrow stromal cells have been reported to metabolize benzene derivatives (29). In addition, human skin fibroblasts display cytochrome oxidase activity, a function that apparently is altered in individuals with Zellweger syndrome (32). Cytochrome P-450-dependent mono-oxygenases were induced in alveolar macrophages following exposure to benzopyrene (33) and macrophages derived from peripheral blood have been shown to metabolize ethanols, sodium phenobarbitone, chlorpromazine, and other xenobiotics (12, 34). This macrophage function was abrogated by the addition of SKF-525A or other agents known to inhibit cytochrome

mono-oxygenases. Developing hematopoietic cells may be uniquely sensitive to the generation of toxic metabolites by macrophages, since erythroid and myeloid cells often develop around these cells, ostensibly because they provide nutrients and cytokines essential to growth and differentiation (27). The metabolism of xenobiotics by bone marrow has been reported previously (35) and this capacity has been ascribed to hematopoietic cells (36). In this regard, the localization of arylhydrocarbon hydroxylase, a cytochrome mono-oxygenase, was observed in clonogenic murine erythroid and granuloid/monocytic colonies (36). The potential role of stromal cells in this phenomenon is difficult to assess since no adherent cell depletion was performed prior to inoculation of the bone marrow cells into the assay system. In addition, there may be some qualitative differences with regard to cytochrome oxidase activity between recently isolated bone marrow cells and cells that have been hormonally stimulated to form colonies *in vitro*. Bone marrow stromal cells exhibit inducible cytochrome P-450 enzyme activity. EFEE conversion to fluorescein was localized to the large sized, granular cell subpopulation and, to a lesser extent, the smaller granular cells (Fig. 4). Similar activity was not observed with other stromal cell populations or with any bone marrow nonadherent cells (Table III), although this does not necessarily rule out xenobiotic metabolism by these cells. More sensitive cytofluorographic methods, such as the benzo(a)pyrene fluorescence reduction assay (23, 25), may be required to demonstrate cytochrome P-450 activity by other populations of cells that express low levels of these enzymes. However, with the EFEE conversion assay, stromal cells display measurable drug-metabolizing capacity, whereas hematopoietic cells do not. The larger more granular cells in the stromal cell cultures were morphologically similar to macrophages and react with a monoclonal antibody, ED-1, specific for monocytic cells. In a previous study, the toxic effects of CP on bone marrow cultures were exacerbated by the presence of nylon screen hepatocyte cultures during the 21-hr exposure period (37).

In general, Ara-C, when introduced into NSBMC at higher dose levels, significantly diminished the numbers of CFU-C (Table I). However, many of the CFU-C generated after dosing with 5 mg/ml of Ara-C were considerably larger in size than "normal" colonies consisting of 30–60 cells (Fig. 4). The reason(s) underlying the production of these large colonies is unclear. Irradiated stroma or stroma derived from a cloned adherent cell line (TC-1) was reported to induce the formation of giant leukocytic colonies in an agar overlay system (38), ostensibly because of colony-stimulating activity production by the stromal cells. Although cells derived from NSBMC are adherent-cell depleted prior to being placed into the CFU-C assay, the potential contribution of contaminating stromal cells in eliciting this effect cannot be fully discounted. However, exposure to high levels of Ara-C may modify the differentiation of leukocytic cells derived from NSBMC or select for cells with an enhanced ability to proliferate. Cells derived from the bone marrows of 5FU-treated mice generated large colonies *in vitro* reportedly due to a higher percentage of cells within the colony with high proliferative potential (39). Immature cells have also been identified in giant colonies formed by cells taken from murine bone marrow regenerating after radiation exposure (40). Of interest to us in this study was the observation that these large colonies appeared during early stages of culture and became smaller with time. For practical reasons, our CFU-C assays were enumerated after only 8 days in culture. The larger colonies generated by high doses of Ara-C probably reflect enhanced proliferative activity, rather than megaloblastic alterations, since similar changes were not observed in the MTX-treated cultures.

The toxic effects of Ara-C, 5FU, MTX, and CP also were assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide and neutral red assays (41). In general, higher drug doses were required to demonstrate toxicity using the dye uptake/release assays as compared with the progenitor cell proliferation or phenotypic characterization indices (41). For example, MTX caused a significant diminution in the numbers of T and myeloid cells at doses $\geq 10^{-5}$ *M*, whereas significant ($P < 0.05$) decreases in CFU-C numbers were observed at doses of MTX as low as 10^{-6} *M* (Table II). In contrast, a MTX dose $\geq 10^{-2}$ *M* was required to kill 50% of the cultured cells as measured by the neutral red assay. Similar trends were noted with 5FU, Ara-C, malathion, and CP (41). The greater sensitivity of the progenitor cell assays and phenotypic characterizations in assessing hematotoxicity as compared with the dye uptake/release viability assays may be ascribed to the differential sensitivities of hematopoietic and stromal cell populations of our co-cultures. The latter are less sensitive to many drugs because they manifest a capacity to metabolize these agents and are less mitotically active than their hematologic counterparts. These findings indicate that indices of cellular proliferation and differentiation may be more sensitive indicators of the susceptibility of hematologic cells to toxic substances than dye uptake/release viability assays.

In summary, (i) NSBMC can be used to measure hematotoxicity; this was evaluated using CFU-C assay and flow cytometry; (ii) measurements of differential hematotoxicity are possible because NSBMC display the ability to produce several hematologic cell lines concurrently; (iii) the effects of Ara-C and MTX were most pronounced on myeloid cells, whereas CP primarily influenced lymphoid cells of NSBMC; (iv) although hematopoiesis in NSBMC varies with the age

of culture and other factors, hematotoxicity versus 0 dose level controls was still quantifiable; (v) CP exhibited toxic effects on hematopoietic cells only when stromal cells were present during exposure; (vi) because of their ability to metabolize EFEE to fluorescein, the larger, more granular stromal cells were identified as possessing cytochrome P-450 activity; and (vii) long-term bone marrow cultures may prove to be useful tools for the assessment of drug toxicities.

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