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Differences in *in Vitro* Proliferative Responsiveness to Granulocyte Colony-stimulating Factor and Interleukin 2 of Bone Marrow Cells from Mice Treated with Chemotherapeutic Drugs[1]

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ABSTRACT

The toxicity effects of several anticancer drugs on normal mouse bone marrow (BM) were estimated using the *in vitro* proliferative responsiveness ([^{3}H]thymidine incorporation) of the treated BM cells to recombinant human granulocyte colony-stimulating factor (G-CSF) and recombinant human interleukin 2 (IL-2). From the response pattern of the treated BM cells to G-CSF and IL-2, the anticancer drugs were classified into three groups: (*a*) BM cells from cyclophosphamide- or nimustine hydrochloride-treated mice showed an increased responsiveness to G-CSF but a decreased responsiveness to IL-2; (*b*) BM cells from vindesine- or peplomycin-treated mice showed an increased responsiveness to both G-CSF and IL-2; and (*c*) BM cells from mitomycin C-treated mice showed a decreased responsiveness to both G-CSF and IL-2. These different response patterns may reflect qualitative differences in the myelotoxicity effects of these anticancer drugs.

INTRODUCTION

Treatment with almost all anticancer drugs produces adverse myelotoxic side effects. Whether different drugs have qualitative differences in their myelotoxic effects has not been studied. Biotechnology has enabled us to use a variety of hematopoietic factors to treat chemotherapy-related BM[3] suppression in cancer patients. A number of hematopoietic factors have been identified. These include CSFs such as G-CSF, M-CSF, GM-CSF, and interleukins such as IL-1, IL-2, IL-3 (multi-CSF), IL-4, IL-5, and IL-6 (1–11). These hematopoietic factors have the potential of accelerating bone marrow recovery following chemotherapy. In this study, we evaluated what effect treatment with chemotherapeutic drugs would have on the *in vitro* proliferative responsiveness of BM cells to G-CSF and IL-2. These studies were done to assess if the different drugs had different patterns of myelotoxicity. The drugs studied were those most commonly used for the treatment of lung cancer in Japan. The results show that anticancer drugs can potentially be classified into three separate categories based on the responsiveness of posttreatment BM cells to G-CSF and IL-2 *in vitro*.

MATERIALS AND METHODS

Mice. Male BALB/c mice were obtained from the Laboratory Division of the Shizuoka Agricultural Cooperative Association, Hamamatsu, Japan. The experimental mice were 10–12 weeks old and weighed 20–25 g. They were kept in specific-pathogen-free animal rooms.

Anticancer Drugs. ACNU (Sankyo Co., Ltd., Tokyo, Japan), CY, VDS, VCR, VBL (Shionogi Pharmaceutical Co., Ltd., Osaka, Japan), MMC (Kyowa Hakko Co., Lt., Tokyo, Japan), and PEP (Nippon Kayaku Co., Ltd., Tokyo, Japan) were dissolved in a 0.85% NaCl solution just before use. All drugs were injected into animals i.p.

G-CSF and IL-2. Recombinant human G-CSF (Lot T769109; specific activity, 5×10^7 units/mg protein) was provided by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan (12), and recombinant human IL-2 (5×10^4 units/mg protein) by Ajinomoto Co., Inc., Yokohmama, Japan. G-CSF was suspended in a solution which contained human albumin (0.1%), gelatin (0.05%), and mannitol (1%).

Preparation of BM Cells and Spleen Cells. BM cells were flushed from the femurs of mice with a 27-gauge needle using a 2-ml syringe which contained Eagle's minimum essential medium. To obtain spleen cells, the spleens were aseptically removed, and Eagle's minimum essential medium solution was injected into them by a 27-gauge needle with a syringe. RBC were lysed with Tris-NH_4Cl solution, and the remaining cells were placed into a culture medium. Our standard culture medium was RPMI 1640 supplemented with heat-inactivated fetal calf serum (10%), L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), penicillin G (50 μg/ml), gentamicin (60 μg/ml), and a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (20 mM).

***In Vitro* Proliferation Assay.** Bone marrow cells or spleen cells (2×10^5) were suspended in a volume of 0.1 ml into Microplate II flat-bottomed tissue culture plates (Falcon No. 3072). G-CSF or IL-2 in a volume of 0.1 ml was added to each well, and the plate was incubated at 37°C in 5% CO_2 for 72 h. Seven h before harvest, 1.0 μCi in a volume of 0.05 ml of [^{3}H]thymidine was added. The cells were harvested with a Mash sampler, and radioactivity was measured by a liquid scintillation counter. Each sample was assayed in duplicate or triplicate. The proliferation was calculated as

Proliferation (dpm) = dpm with G-CSF − dpm with vehicle for G-CSF,
or = dpm with IL-2 − dpm with normal medium

RESULTS

Proliferation of BM Cells and Spleen Cells to G-CSF and IL-2 *in Vitro*. We first examined the dose responsiveness of normal bone marrow cells to G-CSF as assessed by *in vitro* proliferation. We used dosages of 2×10^{-1}–2×10^{-4} μg/ml. As shown in Fig. 1*A*, normal BM cells responded to G-CSF in a dose-dependent manner.

We next examined the dose responsiveness of normal bone marrow and spleen cells to IL-2 as assessed by *in vitro* proliferation. BM and spleen cells responded to IL-2 in a dose-dependent manner at concentrations of 2×10^2–2×10^5 units/ml (Fig. 1*B*). From these studies, we decided to use the doses of 0.02 μg/ml of G-CSF and 2×10^4 units/ml of IL-2 for our future *in vitro* studies.

In order to see what cells are responsive to G-CSF and IL-2, the proliferative responsiveness was examined with BM cells, spleen cells, lymph node cells, and thymus cells. As shown in Table 1, IL-2 caused proliferation of all above cells, but only BM cells responded to G-CSF. The percentage of neutrophils (band + segment) in normal BM cells was around 20%, and it increased to over 50% of viable nonadherent cells after a 3-day

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[3] The abbreviations used are: CSF, colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; GM, granulocyte-macrophage colony-stimulating factor; IL-2, interleukin 2; BM cells, bone marrow cells; ACNU, nimustine hydrochloride; CY, cyclophosphamide; VDS, vindesine sulfate; PEP, peplomycin; MMC, mitomycin C; VCR, vincristine; VBL, vinblastine; LAK, IL-2-activated killer.

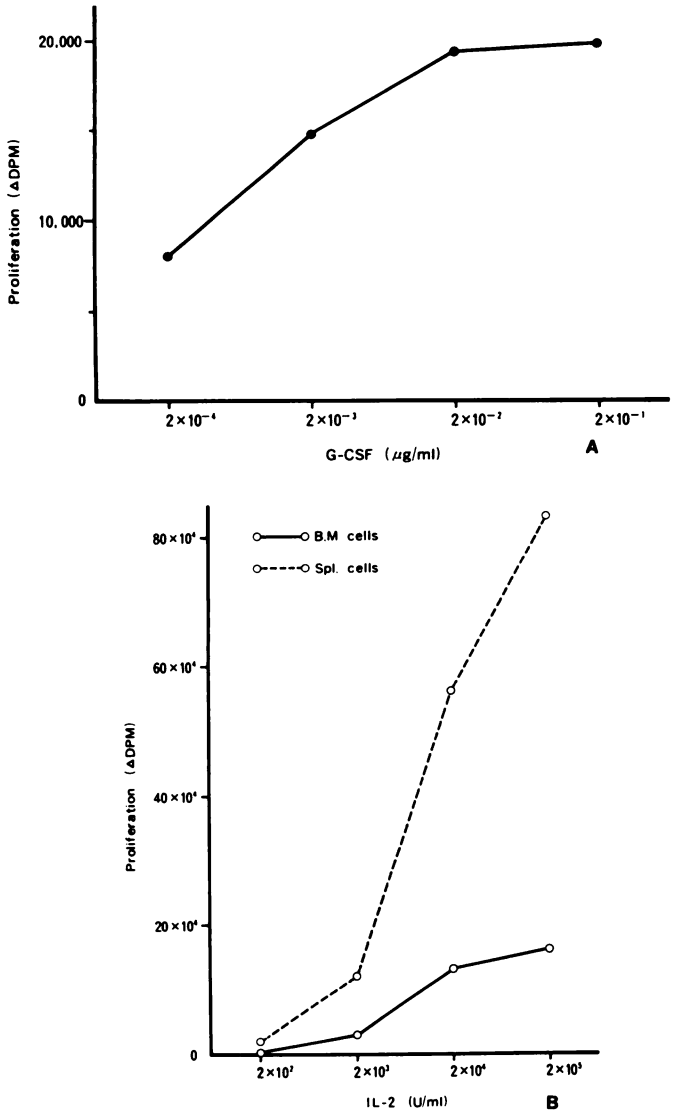


Fig. 1. *A*, effect of concentration of G-CSF on the proliferation of BM cells *in vitro*. *Points*, mean of 2 independent experiments. *B*, effect of concentration of IL-2 on the proliferation of BM cells and spleen cells *in vitro*.

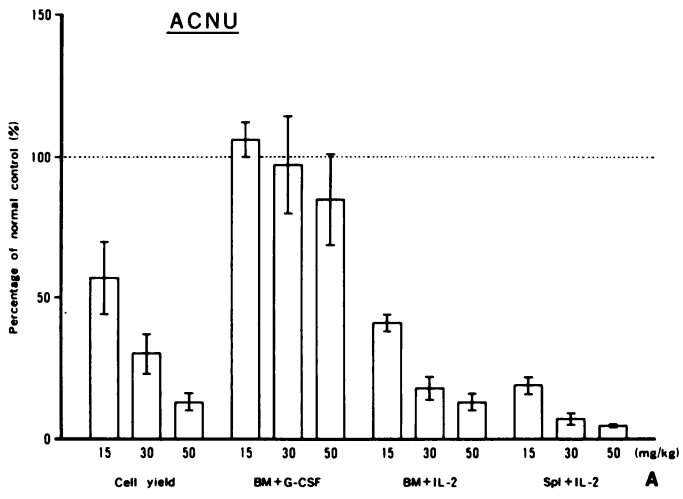
culture with G-CSF indicating that the G-CSF used in our study most affected the neutrophil lineage. Studies on what cell types in normal BM could be stimulated by IL-2 *in vitro* showed that natural killer, T-, and B-cells could respond to IL-2 more strongly in the order of natural killer > T > B (data not shown).

Proliferation to G-CSF and IL-2 *in Vitro* of BM Cells Obtained from Mice Treated with Various Kinds of Anticancer Drugs. Experiments were initiated to define if qualitative difference in myelotoxic effects of anticancer drugs could be delineated. This was done by assessing the proliferative responsiveness to G-CSF and IL-2 of residual bone marrow cells obtained from mice treated with various kinds of anticancer drugs. Cyclophosphamide (100, 150, and 200 mg/kg) was administered to normal mice. Two days later the bone marrow was removed and the *in vitro* proliferative response with and without G-CSF and IL-2 was assessed (Fig. 2*A*). The BM cell yield was 11–23% of normal control. The proliferative responses of treated BM cells and spleen cells to IL-2 were 13–40% and 13–50%, respectively. The proliferative response of treated BM cells to G-CSF was 316–527% of the untreated normal BM cells. A time course study was performed with the *in vitro* responsiveness to G-CSF and IL-2 of BM and spleen cells being assessed on days 2, 6, 10, and 14 after CY (150 mg/kg) treatment. As shown in Fig. 2*B*, the responsiveness to G-CSF of BM cells reached a peak on day 2. The responsiveness to IL-2 of BM cells and spleen cells was the lowest on day 2 but rapidly increased. A repeat study was performed and the

Table 1 *Proliferative response of BM cells to G-CSF and IL-2 in vitro*

	Proliferation (Δdpm)	
Responding cells[a]	G-CSF	IL-2
BM cells	34,590[b]	244,420[c]
Spleen cells	440	537,650
Lymph node cells	−1,020	738,190
Thymus cells	840	123,900

[a] Two × 10^5/well, 72 h of incubation, 1 μCi of [^{3}H]thymidine, 7 h.
[b] Δdpm = dpm with G-CSF − dpm with vehicle for G-CSF.
[c] Δdpm = dpm with IL-2 − dpm with normal medium.



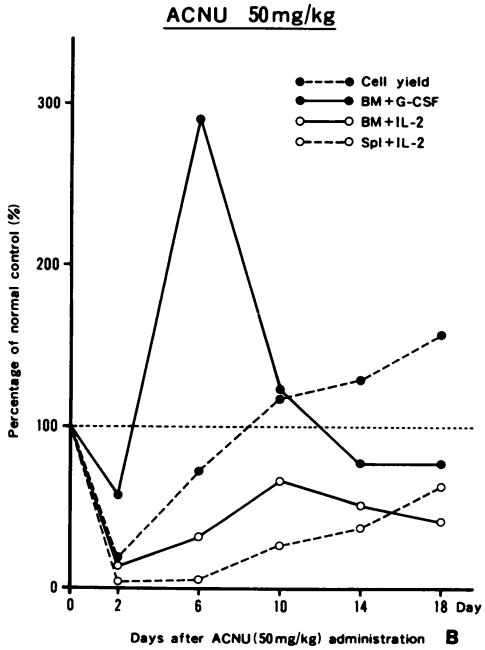
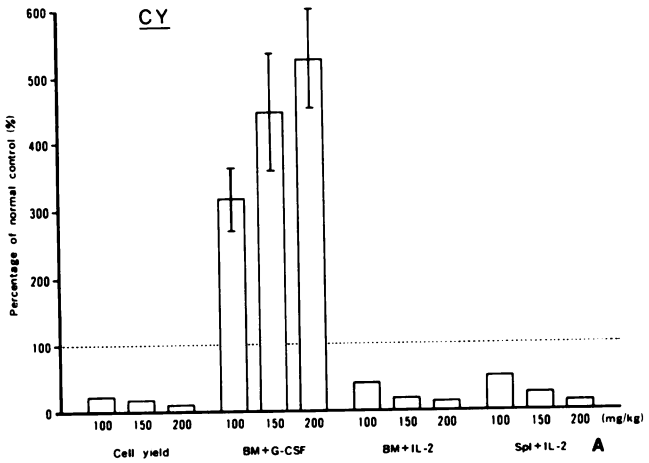


Fig. 3. *A*, effect of ACNU on BM cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells. Data for day 2 after ACNU administration. *Bars*, SEM for 3–5 independent experiments. *B*, changes in BM cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells after ACNU (50 mg/kg) administration. *Points*, mean of 2 independent experiments.



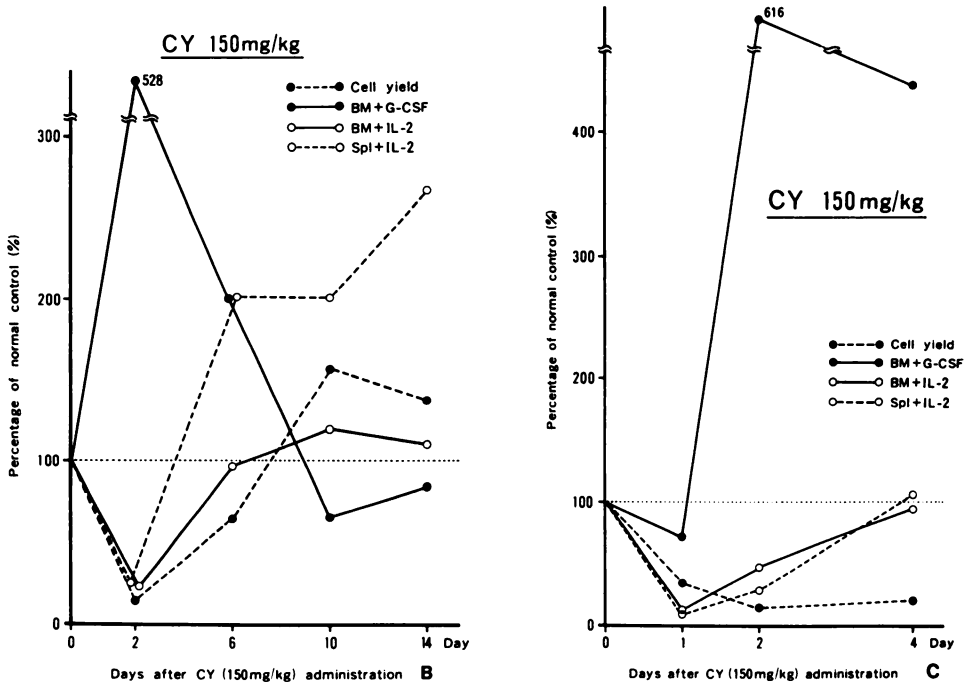


Fig. 2. *A*, effect of CY on BM cell yield, proliferation to G-CSF and IL-2 of MB cells and spleen cells. Data for day 2 after CY administration. *Bars*, SEM for 3–4 independent experiments. *B, C*, changes in BM cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells after CY (150 mg/kg) administration. *Points*, mean of 2 independent experiments.

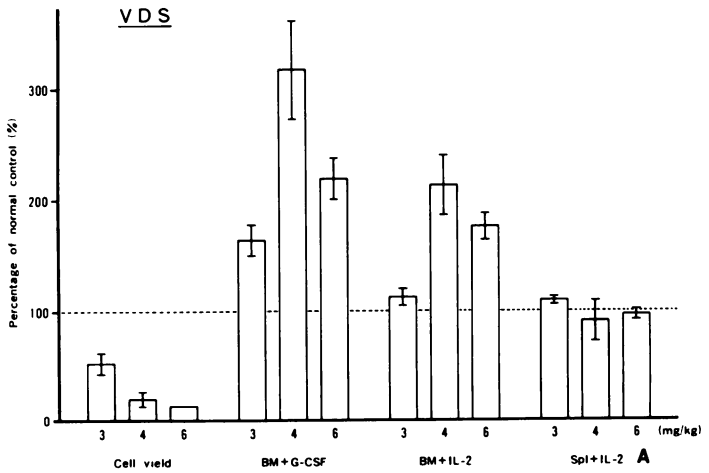
responsiveness on days 1, 2, and 4 was examined (Fig. 2*C*). A mildly decreased responsiveness of BM cells to G-CSF was noted on day 1.

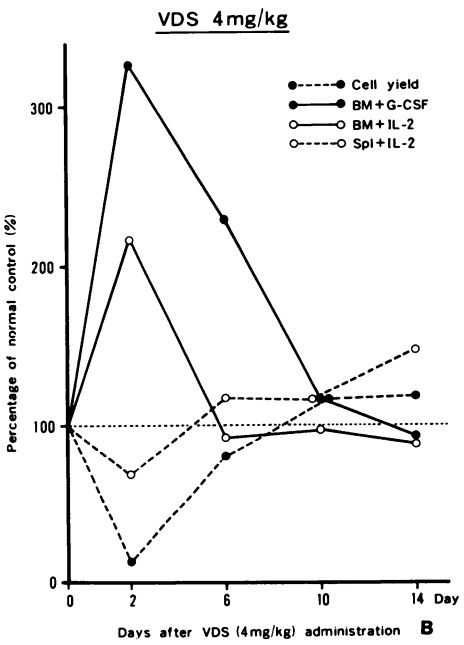
We next studied what effect treatment with ACNU (15–50 mg/kg) would have on these *in vitro* proliferative responses. As shown in Fig. 3*A*, cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells 2 days after ACNU treatment was generally lower than the normal control response. A kinetic study was done using a dose of 50 mg/kg of ACNU (Fig. 3*B*). BM cells from treated mice showed a response to G-CSF but the peak occurred on day 6 rather than on day 2 as was observed for CY. The proliferative responsiveness to IL-2 of both BM cells and spleen cells was less than that observed with CY treatment.

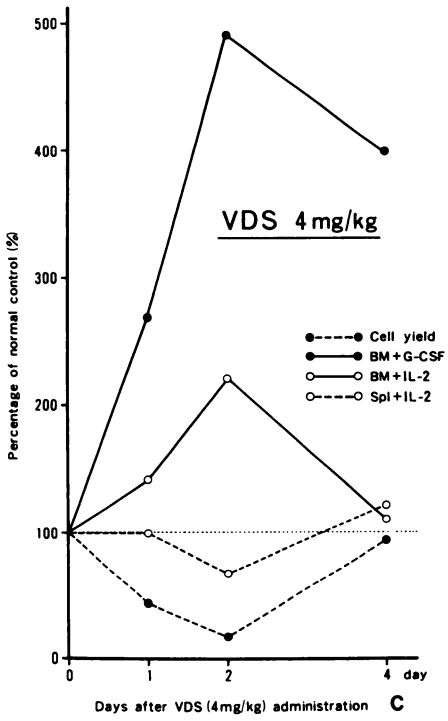
The myelotoxic effect of VDS (3–6 mg/kg) and proliferative responsiveness to G-CSF and IL-2 were studied in a similar way (Fig. 4, *A–C*). Similarly to CY, an increased responsiveness to G-CSF was observed with day 2 BM cells from VDS-treated mice. In kinetic studies (Fig. 4, *B* and *C*), we did not observe the decreased responsiveness as was observed with CY or ACNU. In contrast to CY or ACNU treatment, BM cells from VDS-treated mice showed increased responsiveness to IL-2 proliferation. The effect of VDS on the proliferation of spleen cells to IL-2 was not remarkable at the doses studied. We next assessed whether increased responsiveness of BM cells to both G-CSF and IL-2 would be also observed using BM from mice treated with two other *Vinca* alkaloid drugs (VBL and VCR) (Fig. 4*D*). BM cells from mice treated with VBL responded to both IL-2 and G-CSF and a similar but less dramatic pattern occurred with VCR.

The myelotoxicity of MMC treatment was studied (Fig. 5). The increased responsiveness of BM cells to G-CSF or IL-2 which we had previously observed was not found with day 2 BM cells from MMC (4–12 mg/kg) treated mice (Fig. 5*A*). Kinetic studies (MMC, 8 mg/kg) showed a marked decrease in the responsiveness of BM cells to G-CSF and IL-2 at all time points studied (Fig. 5*B*). Studies were repeated using 12 mg/kg of MMC and similar results were obtained (Fig. 5*C*).

The last drug studied was PEP (50–100 mg/kg), an anticancer drug with the least myelotoxicity (Fig. 6). BM cell yield was shown to be minimally affected by PEP. We observed an increased responsiveness to both G-CSF and IL-2 of BM cells from PEP (75 mg/kg)-treated mice which was similar to that observed following VDS treatment.







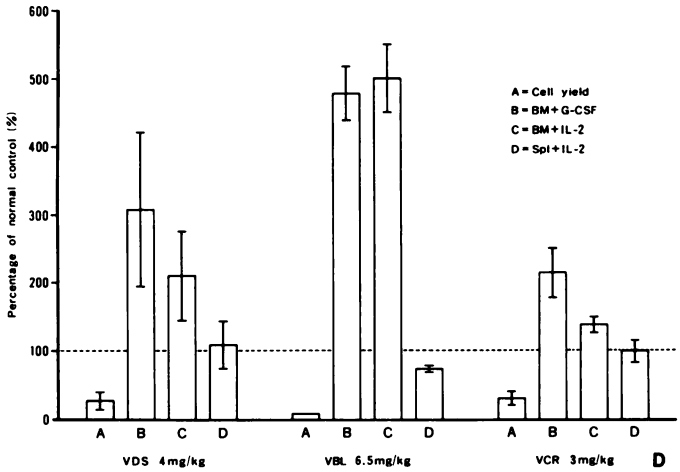


Fig. 4. *A*, effect of VDS on BM cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells. Data for day 2 after VDS administration. *Bars*, SEM for 4 independent experiments. *B, C*, changes in BM cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells after VDS (4 mg/kg) administration. *Points*, mean of 2 independent experiments. *D*, effect of other *Vinca* alkaloid drugs on the proliferation to G-CSF and IL-2 of BM cells and spleen cells. Data for day 2 after drug administration. *Bars*, SEM for 2 independent experiments.

DISCUSSION

In the studies reported here, we estimated the myelotoxic effects of anticancer drugs by assessing the *in vitro* proliferative responsiveness to G-CSF and IL-2 of residual BM cells. G-CSF stimulates the proliferation and differentiation of neutrophil linease *in vitro* and *in vivo* and has the potential of accelerating hematopoietic recovery after chemotherapy. IL-2 has been used to stimulate LAK cells used in lymphokine-activated killer therapy against neoplasms (13–15). These two cytokines

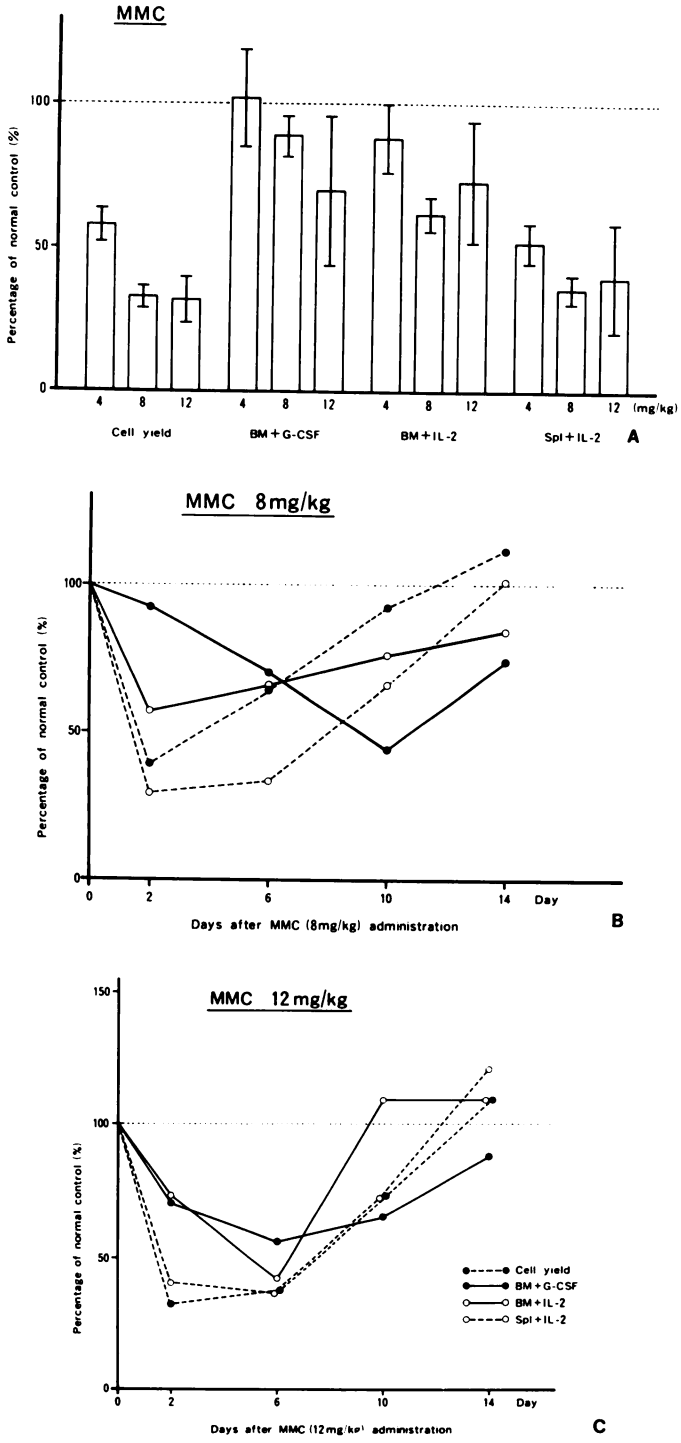


Fig. 5. *A*, effect of MMC on BM cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells. Data for day 2 after MMC administration. *Bars*, SEM for 4–6 independent experiments. *B*, changes in BM cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells after MMC (12 mg/kg) administration. *Points*, mean of 4 independent experiments. *C*, changes in BM cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells after MMC (8 mg/kg) administration. *Points*, mean of 3 independent experiments.

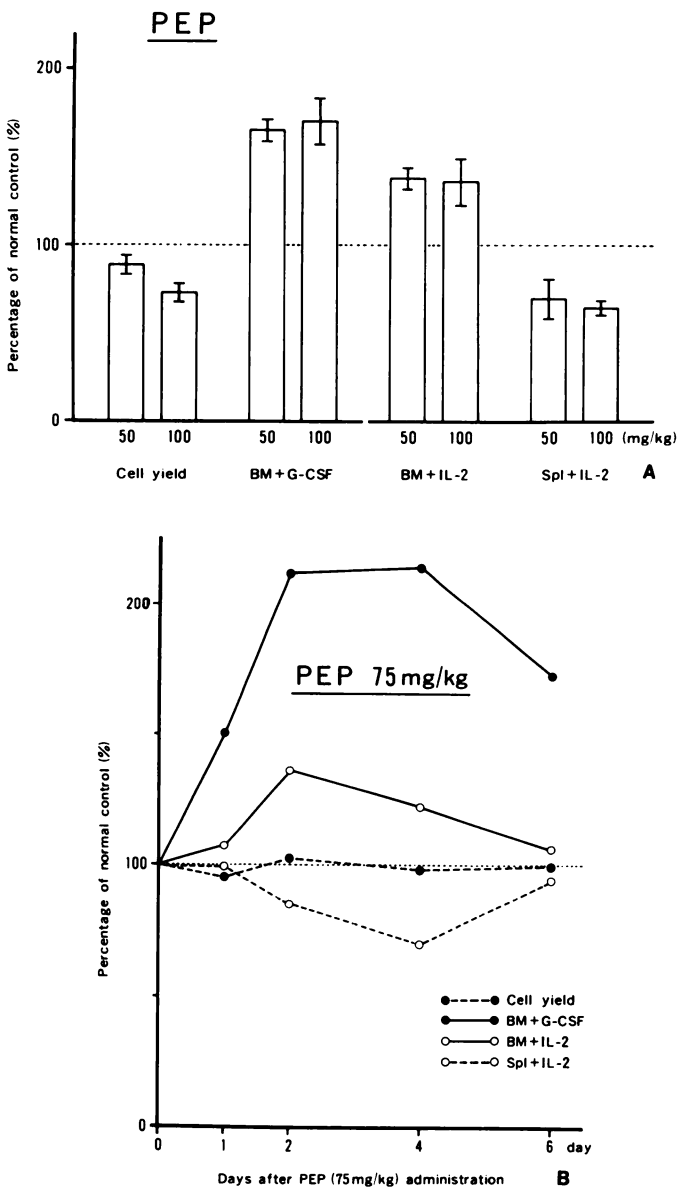


Fig. 6. *A*, effect of PEP on BM cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells. Data on day 2 after PEP administration. *Bars*, SEM for 2 independent experiments. *B*, changes in BM cell yield, proliferation to G-CSF and IL-2 of BM cells and spleen cells after PEP (75 mg/kg) administration. *Points*, mean of 2 independent experiments.

are now attracting much attention in cancer therapy; therefore we used these in the present studies. The colony-forming assay in agar medium or a methylcellulose medium is most commonly used to assess the effect various agents have on BM proliferation (16–18). This assay would not be useful for IL-2. We therefore decided to use the [^{3}H]thymidine incorporation assay in liquid medium to assess the effects of IL-2 and G-CSF on BM and spleen cells.

As summarized in Fig. 7, three response patterns were obtained for the drugs and doses examined. We did observe some minor changes at different drug doses. We speculate that the different patterns which we observed in response to G-CSF and IL-2 following chemotherapy are reflective of qualitative differences in the myelotoxicity of these drugs. We have been unable to identify other studies which show clearly that these chemotherapeutic compounds have different myelotoxicities. Major differences exist in how anticancer drugs are effective against neoplasms. CY and ACNU are alkylating agents, and MMC and PEP are antibiotic agents. These drugs are classified into the type I group because they show dose-dependent and cell cycle-nonspecific cytotoxicity. On the other hand, VDS, VCR, and VBL belong to *Vinca* alkaloid agents and are classified into the type II group which shows the time-dependent and cell cycle-specific (mainly S-phase-specific) cytotoxicity. The three response patterns we observed when cells were incubated with G-CSF and IL-2 did not seem to correlate with the above mentioned action patterns. The differences in response pattern we observed are most likely reflective of what differentiation

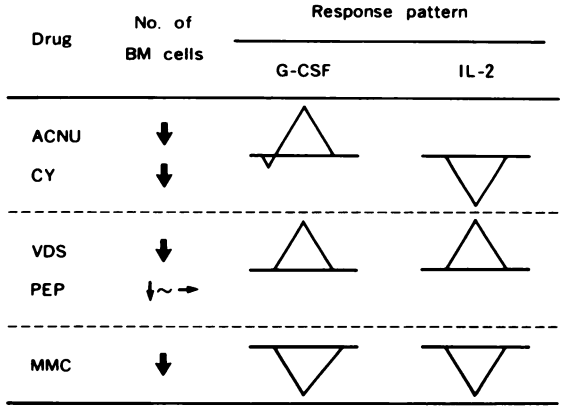


Fig. 7. Response pattern of BM cells to G-CSF and IL-2 *in vitro*.

stage cells were in when the drugs were administered. In the case of CY, BM cells showed an increased responsiveness to G-CSF and a decreased responsiveness to IL-2. Increased responsiveness to G-CSF was most likely due to the enrichment of G-CSF-responsive cells and the decreased responsiveness to IL-2 was due to the loss of IL-2 responsive cells (19, 20) in the BM after the treatment of the mice with CY. In other words, G-CSF-responsive cells were relatively resistant to CY, and IL-2-responsive cells were sensitive to CY. In the case of MMC, BM cells showed decreased responsiveness to both G-CSF and IL-2. This indirectly suggests that MMC might injure the BM cells at a stage closer to that of stem cells and therefore cells would be unresponsive to either G-CSF or IL-2. One could speculate that treatment with IL-3 or GM-CSF (early-acting, lineage-nonspecific factors) may be useful to decrease MMC-induced neutropenia.

It is interesting to note that BM cells from VDS, VCR-, VBL-, or PEP-treated mice showed an increased responsiveness to IL-2. Adoptive immunotherapy with LAK cells has been tried for the treatment of primary or metastatic tumors in man. Recently, studies have been done to induce LAK cells *in vivo* by IL-2 administration in cases of pleuritis or peritonitis carcinomatosa (14, 15). For optimal combination treatments with chemotherapy and LAK immunotherapy, our studies suggest that chemotherapy should be done with agents such as VDS, VCR, VBL, or PEP. These agents would be less toxic to IL-2 responsive cells and greater *in vivo* induction of LAK cells by IL-2 would occur.

Recently, high-dose chemotherapy has been performed and factors such as G-CSF or GM-CSF have been used to promote BM recovery in a variety of cancer patients (2, 7). As higher doses of chemotherapeutic drugs are used, we need to acquire additional information about the myelotoxicity. Is G-CSF effective for any kinds of myelotoxicities induced by chemotherapeutic agents? What is the most effective timing of G-CSF administration? Questions such as those raised in our studies need to be investigated so that optimal chemotherapy and hematorestorative treatment can be performed.

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