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Inhibition by Chemotherapeutic Agents of Human Bone Marrow Progenitor Cells and Clonogenic Cells of a Lymphoblastic Cell Line*

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Abstract—*Using methylcellulose based semi-solid medium enriched with complete alpha medium and undialysed fetal calf serum, we compared the cytotoxic effects between the clonogenic cell (CFU) of a lymphoblastic leukemia cell line (MOLT-4) and normal bone marrow granulocytic progenitor cells (CFU-C) of methotrexate (MTX), vincristine (VCR), hydrocortisone (HC) and daunorubicin (DNR), as well as the reversibility by leukovorin (LV) of MTX cytotoxicity. The neoplastic cells were more markedly inhibited by MTX, VCR and HC following either brief or continuous exposure of the cell to the drugs. Inhibition by DNR was identical for both cell types. In order to reverse the inhibitory effects of MTX in MOLT-4 CFU, LV concentrations needed were at least one log order higher than MTX concentrations. At higher concentrations of MTX, LV reversal was less effective. Only one of four different human bone marrows studied were significantly inhibited by MTX under the experimental conditions. It is suggested that the high concentrations of nucleosides and deoxynucleosides present in the medium aborted the MTX cytotoxicity on CFU-C of normal human marrow but not on MOLT-4 CFU, while the differential inhibition observed with VCR and HC was due to greater sensitivity of the leukemic cells.*

INTRODUCTION

WE HAVE recently reported that there was a striking difference in sensitivities between established human T- and B-lymphocyte cell lines to certain chemotherapeutic agents [1, 2]. On the basis of drug concentrations which produce 50% inhibition of cell growth. T-lymphocytes in culture were found to be 800–2000 times more sensitive to *Escherichia coli* asparaginase and 45–80 times more sensitive to cytosine arabinoside, whereas human B-lymphocytes were 10–20 times more sensitive to 5-fluorouracil. The observation of increased susceptibility of T-lymphocytes to asparaginase and cytosine arabinoside appeared consistent with their clinical activity against neoplastic diseases of T-cell lineage. The differential sensitivities observed were thought to be not related to the 'benign' or 'malignant' nature of the cell lines but rather to the differences in the phenotype.

Since chemotherapeutic efficacy is often expressed by the therapeutic index, that is, cytotoxic effects on tumor cell versus toxicity to the host tissues, it is important to evaluate whether effects of chemotherapeutic agents on tumor cells can be compared with chemotherapeutically vulnerable normal tissues.

One of the 'malignant' characteristics of MOLT-4 [3], a leukemic cell line derived from a patient with acute lymphoblastic leukemia, is its clonability [4] in a semi-solid medium, thus making it possible to evaluate the proliferative capacity of the cell population by scoring the aggregates of cells derived from single cells, i.e. colony-forming units (CFU). We were able to grow MOLT-4 CFU in a semi-solid medium routinely used for growing human granulocyte progenitor cells (CFU-C). The technique is a modification of an assay of human CFU-C described

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by Iscove *et al.* [5]. This method and another similar to it have previously been used to evaluate the effects of cytotoxic agents on murine and human CFU-C [6–9]. In the present study we evaluated the cytotoxic effects of antileukemic agents commonly used in the treatment of ALL, singly or in combination, on MOLT-4 CFU and normal human CFU-C.

MATERIALS AND METHODS

Bone marrow samples

Bone marrow was obtained from patients with non-hematologic neoplasms who were undergoing routine baseline evaluation prior to chemotherapy. None of the patients had prior exposure to any of the drugs studied. None of them had neoplastic bone marrow involvement. Buffy coats that were obtained from such specimens were washed once with ample quantity of cold MEM-alpha medium (GIBCO, Grand Island, NY).

Leukemic cell line (MOLT-4)

This cell line was established from a patient with acute lymphoblastic leukemia (ALL) in 1971 [3] and maintained in our laboratory as a suspension culture [1, 2]. The process of culture of colonies of MOLT-4 cells was performed in two steps: starting with a concentration of 10^5/ml viable cells in RPMI medium 1640 with 20% fetal calf serum (FCS) (Flow Laboratories, Rockville, MD., lot No. 29101004), the cells were allowed to grow in 7.5% CO_2 and humidified air at 37°C. Exponential growth pattern ensued after 36 hr of culture and satisfactory colony forming efficiency was observed if the cells were transplanted at about the middle of the exponential growth phase into semi-solid medium, as will be described later.

Drug solutions

The five drugs investigated in this study were obtained from the following sources: MTX (Lederle Laboratories, Pearl River, NY), LV (Lederle Laboratories), VCR (Eli Lilly, Indianapolis, IN), HC (Upjohn, Kalamazoo, MI) and DNR (Farmitalia, Milan, Italy). All drug solutions and dilutions were prepared within 4 hr of incubation with the cells: PBS (GIBCO) was the diluent used.

Drug exposure

Two modes of drug exposure were investigated: a brief exposure period of 1 hr for VCR and DNR, and 6 hr for MTX. The exposure time of 1 hr to VCR and DNR was arbitrarily chosen because the drugs are active by the i.v. bolus schedule. The 6-hr exposure time to MTX was based on our current clinical protocol for the study of high dose MTX followed by LV rescue. All three drugs above as well as the remaining two drugs (HC and LV) were also studied by the continuous exposure method.

For the short term mode of exposure, an incubation mixture of 1–2 ml consisting of a cell suspension in incomplete MEM-alpha medium (i.e., MEM-alpha medium without nucleosides added), 20% undialysed FCS and the drug solution were incubated for the appropriate period of time at 37°C in 7.5% CO_2 and humidified air. The drug solution was replaced by PBS for controls. Thereafter, the incubation mixture was diluted with 20–30 times its volume and the cells were washed once by centrifugation at 600 ***g*** for 10 min at room temperature.

For the continuous mode of exposure, the drug solution was added to the plating mixture as will be described below. This mode of exposure was used to study (a) the effect of LV on cells following brief exposure to MTX, (b) effect of simultaneous continuous exposure of the cells to MTX and LV, (c) effect of prolonged exposure to VCR, (d) effect of prolonged exposure to HC, (e) effect of LV alone on cells and (f) effect of prolonged exposure to DNR.

Plating mixture

The plating mixture consisted of cell suspension (final concentration: 2×10^5/ml for bone marrow, 5×10^3/ml for MOLT-4), 20% FCS, 20% leukocyte conditioned medium [5] for CFU-C assay, 40% of 2.25% methylcellulose (DOW Chemical Co., Midland, MI) enriched with complete MEM-alpha medium (i.e., supplemented with nucleosides), and for the study of continuous exposure modes, the drug solution(s). The viscid medium was then plated in quadruplicates, 1 ml per 35 × 10 mm Lux dishes (Flow Laboratories, Rockville, MD) and incubated at 37°C in 7.5% CO_2 in humidified air for 7–9 days. Bone marrow cells (2×10^5) and MOLT-4 cells (5×10^3) produced essentially similar numbers of colonies per control dish. The colonies were then enumerated for both the CFU-C and MOLT-4 colonies, using the criteria of Messner [10] whereby only cellular aggregates of more than 20 cells were scored.

RESULTS

Effects of MTX and LV on CFU-C and MOLT-4

Continuous exposure. The effects of prolonged exposure of bone marrow cells and MOLT-4 to various concentrations of MTX are shown in Fig. 1A. Only one of four bone specimens studied was inhibited at the concentrations of MTX studied with LD_{50} between 5×10^{8} and 5×10^{-7} M. In contrast, MOLT-4 CFU was markedly sensitive to MTX with the LD_{50} less than 10^{-8}M and the LD_{90} less than 10^{-7} M.

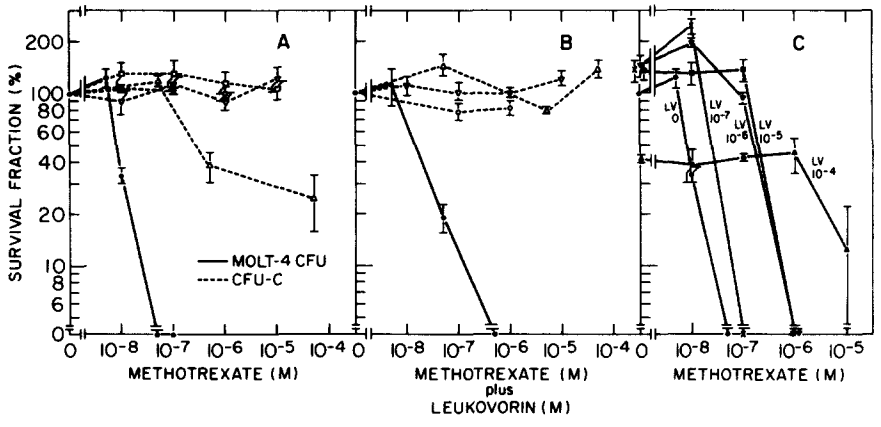

Fig. 1. Cytotoxic effects on MOLT-4 CFU and CFU-C of continuous exposure of (A) MTX alone, (B) MTX and LV at equimolar concentration and (C) MTX and LV at different concentrations. O----O; *mean of a quadruplicate experiment with bone marrow from different patients;* ●—●, *combined mean of once (C) to four times (A) repeated quadruplicate experiments with MOLT-4. In experiments (A) and (B) MOLT-4 CFU were studied concurrently with CFU-C assay. Bar represents S.E.M.*

On continuous exposure to LV concentrations of 10^{-8}–10^{-5} M, we observed no definite inhibitory effects of the drug on either CFU-C or MOLT-4 CFU. Simultaneous prolonged exposure of bone marrow CFU-C to equimolar concentrations of MTX and LV ranging from 5×10^{-9} to 5×10^{-5} M resulted again in significant cell kill of MOLT-4 cells, whereas all of three CFU-C studied were not affected by MTX plus LV (Fig. 1B). With increasing concentrations of LV the cytotoxic effects of MTX on MOLT-4 were progressively reversed. At lower concentrations of MTX at least one log or more higher LV than the MTX concentration was required for the reversal but the efficiency of reversibility lowered when MTX concentration increased (Fig. 1C). LV itself appeared stimulatory for MOLT-4 CFU. Thus, 10^{-5}–10^{-7} M concentrations of LV resulted in 150–200% increase in CFU as compared to control (Fig. 1C). The concentration of LV 10^{-4} M alone was markedly cytotoxic on MOLT-4 CFU. At this concentration of LV cytotoxic effects of LV overshadowed the reversibility against MTX.

Brief exposure. Bone marrow CFU-C was not inhibited by brief exposure to MTX at concentrations of 10^{-8}–10^{-5} M with or without subsequent prolonged exposure to LV at concentrations of 10^{-8} or 10^{-5} M. In contrast, MTX was again cytotoxic to MOLT-4 CFUs with LD_{50} between 10^{-7} and 10^{-6} M(Fig. 2). The brief MTX effect at 10^{-5} M was only partially, while that at 10^{-6} M was completely, reversed by prolonged exposure to LV at concentrations ranging from 10^{-8} to 10^{-5} M. As high as an 8-fold augmentation of the number of MOLT-4 colonies was observed when a brief exposure to non-inhibitory MTX concentrations of 10^{-7} and 10^{-8} M was followed by exposure to LV concentrations ranging from 10^{-8} to 10^{-5} M.

Effect of VCR on CFU-C and MOLT-4 CFU

On brief exposure (1 hr) to VCR MOLT-4 CFU is inhibited with the LD_{50} approximately 5×10^{-9} M. In contrast, VCR concentrations of up to 10^{-8} M had no significant inhibitory effect on CFU-C (Fig. 3A). Prolonged exposure of CFU-C to VCR resulted in a definite cytotoxic effect. The cytotoxic effects of VCR on MOLT-4 CFU was only moderately increased after the long exposure.

Effect of HC on CFU-C and MOLT-4 CFU

There was no remarkable inhibitory effect

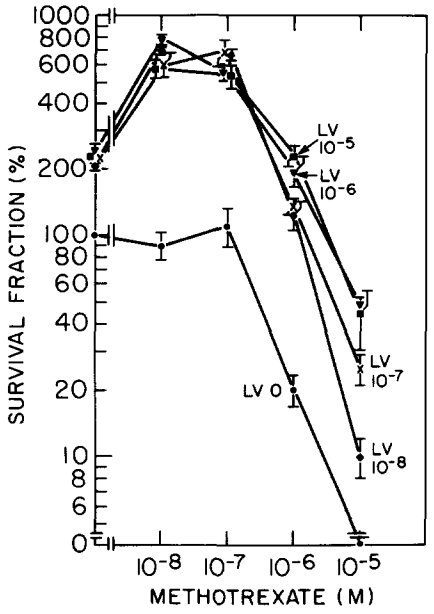

Fig. 2. Cytotoxic effects on MOLT-4 CFU of 6-hr *exposure of MTX followed by continuous exposure of L.V. Filled symbols and solid lines represent experiments with different LV concentrations. Bar represents S.E.M.*

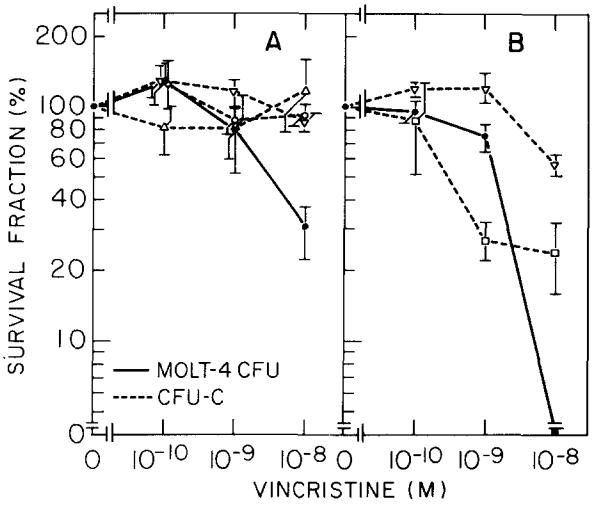

Fig. 3. Cytotoxic effects of MOLT-4 CFU and CFU-C of (A) brief (1 hr) *and (B) continuous exposure to VCR. See legend of Fig. 1 for explanation of symbols.*

of HC on CFU-C (Fig. 4). The leukemic clonogenic cells were, however, inhibited by HC with an LD_{50} of approximately 10^{-6} M.

Effect of DNR on CFU-C and MOLT-4 CFU

For the comparison of CFU-C and MOLT-4 CFU we were able to study only one marrow sample. There was no significant difference observed in the cytotoxic effect of DNR for CFU-C and MOLT-4 CFU following a brief (1 hr) exposure, the approximate LD_{50} being between 10^{-8} and 10^{-7} M (Fig. 5). With prolonged exposure, DNR cytotoxicity increased for both cell types. There was, again, no difference between both cell types.

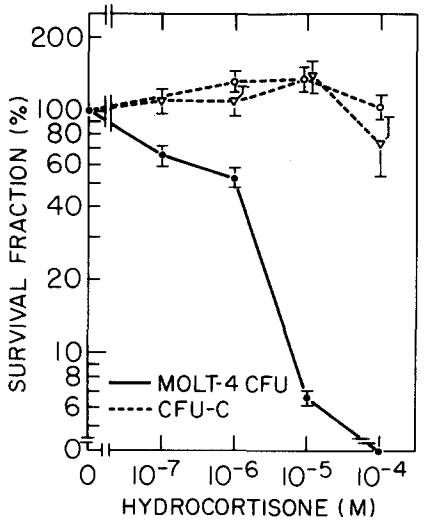

Fig. 4. Cytotoxic effects on MOLT-4 CFU and CFU-C of continuous exposure to HC. See legend of Fig. 1 for explanation of symbols.

DISCUSSION

The study of biological behavior of clonogenic cells within a tumor cell population is highly relevant to clinical oncology because such cells are most likely responsible for the progression and dissemination of cancer. In recent years, assays have been described for quantification *in vitro* of such neoplastic stem cells of mouse [11, 12] and human origin [13–15]. The predictive value of the bioassay of tumor stem cells for the outcome of experimental chemotherapy has been shown to be very good [12, 16]. Primary bioassay of the stem cell population of ALL has not been accomplished [17]. Thus, at present a direct application of stem cell bioassay to ALL patients is not possible.

We compared the cytotoxic effects on human acute lymphoblastic leukemia cells in culture and CFU-C of clinically active antileukemic agents. We observed a greater inhibitory effect of MTX, VCR and HC for MOLT-4 CFU as compared to CFU-C, whether the cells were exposed briefly to the drugs as was done with VCR and MTX, or continuously for 7–9 days as was done with VCR, MTX and HC. In contrast, there was no clear difference in the sensitivity of the two cell types to DNR. This observation, therefore, makes it unlikely that the differential drug sensitivity could be attributed to differences in suitability of the growth milieu for

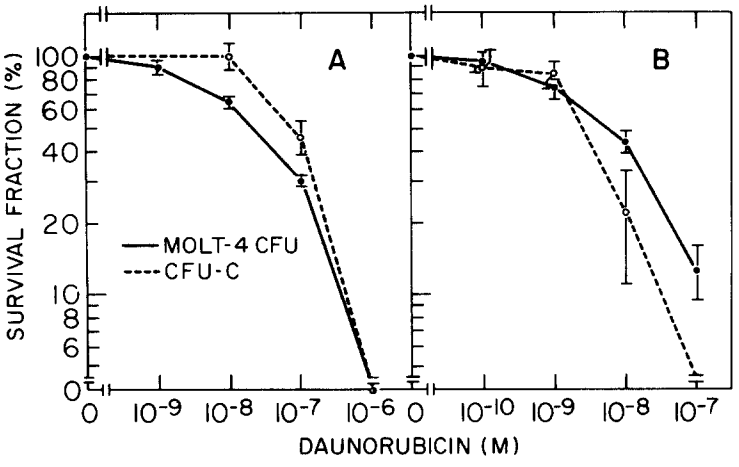

Fig. 5. Cytotoxic effects on MOLT-4 and CFU-C of (A) brief (1 hr) and (B) continuous exposure to DNR. See legend of Fig. 1 for explanation of symbols.

the two types. Since brief and prolonged exposures produced similar differential cytotoxicity for VCR, MTX and HC, the differences in the survival of MOLT-4 CFU and CFU-C following brief exposure to MTX and VCR cannot be explained on the basis of cell cycle-related differential activity of the antineoplastic agents [18]. By virtue of the scoring criteria used, the number of progeny cells produced per unit time, and, therefore, the minimum rate of cell proliferation, was similar for both cell types and constant from experiment to experiment. It appears, therefore, that some intrinsic properties of the different cell types which allow for differential cytotoxic effect of the drug, and/or differential repair capacity of such damages, may be responsible for the observed differences.

Selective toxicity of VCR and HC appears to be the most likely explanation for the marked inhibition of MOLT-4 CFU as compared to CFU-C. Though the experiments were limited to only one bone marrow sample, DNR did not appear to show such a selective effect. The marked difference in response to MTX may be explained not only on the basis of differential toxicity but also as a result of differential reversibility of the biochemical lesion caused by MTX. This view will be consistent with the opinion of others that the biochemical lesion or lesions caused by MTX may not only differ from cell to cell, but that the mechanism and degree of reversibility of such a lesion may also differ as well [19–21]. The specific type of biochemical lesion or lesions caused by MTX on MOLT-4 cells is unknown. However, in human leukocytes, the synthesis of DNA is inhibited to a greater extent than the RNA, thus suggesting that the inhibition of thymidylate synthesis is the most important mechanism of MTX cytotoxicity [22]. Credence is lent to this view by the fact that MTX cytotoxicity on mouse CFU-C is blocked in the presence of nucleosides but not in their absence. Thus, Pinedo *et al.*, in exposing mouse bone marrow continuously to MTX in the absence of nucleosides, observed a marked inhibition of CFU-C (LD_{50}:10^{-8} M) [23]. In contrast to their observations, and those of Holdener *et al.* [24] on human CFU-C, we observed a significant inhibitory effect of MTX on CFU-C in only one of four bone marrow specimens studied. The major difference between our assay and those mentioned earlier is that we used a medium containing large amounts of nucleosides (Table 1) in our culture system whereas nucleosides were either totally excluded [23] or reduced to a minimum (C.H. Park, personal communication) by the others. In effect, therefore, the marked difference that we have observed with MTX on MOLT-4 CFU and CFU-C appears to be a reflection of a difference in the reversibility of MTX toxicity on the two cell types by nucleosides rather than a pattern of selective toxicity to the leukemic cells. Although the basis for such a differential reversal of the MTX lesion(s) in the two cell types is not known, the observations suggest that a selective 'rescue' by nucleotides of the normal hemopoietic system from MTX toxicity in the course of management of T-cell ALL with high-dose methotrexate chemotherapy is a feasible proposition. While endogenous plasma concentrations of thymidine and uridine have recently been reported as 1.6×10^{-7} M [24] and 5×10^{-6} M [25], respectively, thymidine levels in human

Table 1. *Composition of complete MEM alpha medium in* mg/l

L-Alanine	25.0	L-Ascorbic acid	50.0
L-Arginine	105.0	Biotin	0.1
L-Asparagine H_2O	50.0	D-Ca pantothane	1.0
L-Aspartic acid	30.0	Cholin chloride	1.0
L-Cysteine $HClH_2O$	100.0	Folic acid	1.0
L-Glutamic acid	75.0	*i*-Inositol	2.0
L-Glutamine	292.0	Nicotinamide	1.0
Glycine	50.0	Pyridoxal HCl	1.0
L-Histidine (free base)	31.0	Riboflavin	0.1
L-Isoleucine	52.5	Thiamine HCl	1.0
L-Leucine	52.4	Vitamin B_{12}	1.36
L-Lysine (free base)	58.0	Adenosine	10.0
L-Methionine	15.0	Cytidine	10.0
L-Phenylalanine	32.0	Guanosine	10.0
L-Proline	40.0	Uridine	10.0
L-Serine	25.0	2′-Deoxyadenosine	10.0
L-Threonine	48.0	2′-Deoxycytidine HCl	11.0
L-Tryptophan	10.0	2′-Deoxyguanosine	10.0
L-Tyrosine	36.0	2′-Deoxythymidine	10.0
Valine	46.0		

serum can be raised therapeutically at least 6-fold [24] or as high as 10^4-fold [26] of the endogenous concentration. Thus, the equivalent concentration of thymidine present in our culture system (approximately 5×10^{-5} M) is achievable therapeutically in man.

Our experience suggesting that there is an approximately direct relationship between MTX concentrations and the amount of LV needed to repair their lethal effects on MOLT-4 CFU confirms similar observations made on mouse hemopoietic cells [23]. In the only case where MTX caused a significant inhibition of CFU-C, we observed a complete reversal with equimolar concentration of LV (see Figs. 1A and B, open triangles on broad base). This is in contrast to the experience of others [27] who needed a much higher LV concentration to reverse the MTX effect on human CFU-C. Again, these discrepancies are probably related to differences in the constitution of the growth medium used for these experiments.

We observed a moderate stimulatory effect of low doses of LV on MOLT-4 CFU but not on CFU-C. When a brief exposure to low concentrations of MTX (10^{-8} and 10^{-7} M) was followed by prolonged exposure to similar concentrations of LV, we also observed a marked increase in the number of MOLT-4 CFU. The explanation for this tremendous augmentation of proliferation of MOLT-4 CFU in not clear. It is possible that such *in vitro* manipulations have led not only to inducement of potential but ordinarily non-proliferating CFU's to enter into the mitotic cycle but also to enhancement of proliferation of CFU's in general.

Continuous exposure of MOLT-4 to MTX augmented the cytotoxic effect of the drug approximately 200-fold. Although on concentration × time basis there was only 30-fold difference between the two modes of the experiments, this marked increase in biological effect is consistent with MTX's unique characteristics that the duration of exposure rather than concentration is the critical determinant of the biological activity [28].

The question is often asked as to the relevance of data gathered in vitro to the *de novo* involving human neoplastic diseases. VCR and corticosteroids are the two most useful agents in remission induction chemotherapy of human ALL. These two agents in combination are able to induce remission of ALL without major bone marrow toxicity. The findings made in the present investigation are in accord with the clinical observation of these agents. Buick *et al.* have reported a patient-to-patient variation in the sensitivity of leukemic progenitor cells of acute myelogenous leukemia to adriamycin and DNR [29]. It is, therefore, possible that our observations with DNR on MOLT-4 represents only one particular type of dose response to DNR. Since there was little or no difference in the inhibitory activity of DNR on MOLT-4CFU and normal CFU-C (Fig. 5), one could speculate that an ALL patient whose leukemic lymphoblasts and normal CFU-C response

identically to DNR *in vitro* would not be expected to improve with DNR chemotherapy. The value of DNR in the management of this disease is currently investigated. It is of interest to note that while DNR is active in inducing remission in patients with ALL, DNR was unable to augment remission induction rate produced by VCR, corticosteroids and asparaginase (CALGB, unpublished observation). MTX is an effective agent for maintenance of remsiion in patients with ALL. High dose MTX and LV rescue is active in inducing remission in patients with ALL in relapse and in prevention of CNS relapse [30]. VCR infusion was tested in man [31] but whether or not infusional treatment is therapeutically superior to i.v. bolus is not known. Our *in vitro* observations suggest that a brief exposure to VCR would afford a better differential inhibitory activity on the leukemic cells while a prolonged exposure would only diminish the 'therapeutic differential' and increase the toxic effect on the bone marrow cells.

In general, the results derived from our *in vitro* chemotherapy of ALL appeared consistent with experiences gathered from clinical trials of these agents. Development of a direct clonogenic assay for human acute lymphoblastic leukemia using techniques described in this experiment may be potentially valuable in attempts to predict therapeutic outcome and in evaluation of new anti-leukemic agents.

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