Effects of Conditioned Medium upon Proliferation, Deoxynucleotide Metabolism, and Antimetabolite Sensitivity of Promyelocytic Leukemic Cells In Vitro

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ABSTRACT

In order to investigate the interaction of factors from leukocyte-conditioned medium with leukemic cells, effects of an ammonium sulfate-precipitated conditioned medium concentrate were tested upon HL-60 cells. This preparation increased tritiated thymidine incorporation into DNA of HL-60 cells markedly, an effect which was found to be attributable in large part to greater thymidine accumulation in the intracellular nucleotide triphosphate pool. A modestly expanded population of DNA synthetic phase cells was also demonstrated by flow cytometry. Similar effects were noted of the K562 and KG-1 cell lines and were also demonstrable with giant cell tumor-conditioned medium. These effects were not demonstrable with a purified preparation of granulocyte-monocyte colony-stimulated factor. Because of the altered pattern of nucleotide metabolism noted, the effect of the conditioned medium concentrate upon 5-fluorouracil sensitivity was tested. Following continuous 24-h exposure to 5-fluorouracil at 5 x 10^-6 M, tritiated thymidine incorporation of HL-60 cells increased in parallel with depletion of endogenous thymidine. Conditioned medium concentrate markedly sensitized cells to this effect of 5-fluorouracil and also increased growth retardation, cytotoxicity, and cell cycle arrest as assessed by flow cytometry. These studies thus demonstrated marked effects of a factor in conditioned medium on deoxynucleotide uptake and metabolism of the HL-60 line. These effects occurred in conjunction with, but were relatively more marked than, effects upon cell cycle distribution and were found to influence chemotherapy sensitivity.

INTRODUCTION

The HL-60 cell line is a human promyelocytic leukemia cell line which has been useful as a model of several aspects of leukemic growth (2). Foremost among its properties of interest is its ability to exhibit alternate patterns of differentiation following exposure to a variety of chemicals, with both granulocytic (2) and monocytic patterns of such differentiation having been described (3-5). A monocytic or myelomonocytic pattern of differentiation following exposure to LCM has also been reported (5, 6). Mitogen-stimulated LCM is a source of several lymphokines, including CSF (7-9) and IFN-γ, which has recently been identified as a differentiation-inducing factor for HL-60 (10, 11). In apparent contrast to the differentiative responses of HL-60, initial exposure to LCM has been associated with increased proliferation of HL-60, as assessed by [3H]dThd incorporation (6), and CSF from various sources has been reported to enhance HL-60 clonal growth in semisolid medium (12). Others, using flow cytometry, have only noted inhibitory effects of LCM upon HL-60 proliferation (13, 14).

The purpose of this study was to clarify the nature of the early proliferative effects of LCM upon HL-60. Using ammonium sulfate precipitation of LCM, a highly concentrated partially purified preparation was obtained (LCM-C), which was capable of increasing [3H]dThd incorporation into DNA of HL-60 cells by 10-fold or greater. Analysis of this effect disclosed that the high degree of stimulation noted was based upon multiple effects, including greater intracellular accumulation of [3H]dThd and a larger proportion of S-phase cells following treatment. Study of this effect with alternative cell lines and sources of growth factors suggested that this may be an effect of a factor other than CSF-GM upon cells at an earlier stage of differentiation than the committed progenitor. Because of the prominent alterations of deoxynucleotide metabolism induced by LCM-C, this preparation was also tested as a modulator of chemotherapy sensitivity and found to markedly sensitize to effects of 5-fluorouracil by metabolic mechanisms distinct from changes in cell cycle distribution.

MATERIALS AND METHODS

Materials. All biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise noted. [methyl-3H]Thymidine (77 Ci/mmol), [8-3H]deoxyadenosine (21 Ci/mmol), [5-3H]deoxycytidine (20 Ci/mmol), [8-3H]deoxyguanosine (6.5 Ci/mmol), and [α-32P]deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine triphosphates (3000 Ci/mmol) were purchased from ICN Pharmaceuticals, Irvine, CA. 5-[6-3H]Fluorouracil (28 Ci/mmol) was purchased from Research Products International, Mount Prospect, IL. Hoechst 33342 was purchased from Calbiochem-Behring Corp., La Jolla, CA, and Pyronin Y was from Polysciences, Inc., Warrington, PA. GCT-conditioned medium was purchased from Gibco Laboratories, Inc., Santa Clara, CA. Purified human CSF-GM was obtained from Genzyme, Inc. (Boston, MA). Cell lines were passaged in modified McCoy's Medium SA with 15% fetal calf serum and harvested for experiments while in exponential growth.

Conditioned Medium. Phytohemagglutinin-LCM was prepared by incubating phytohemagglutinin-LCM with human leukocytes for 7 days in serum-free medium. Cell-free conditioned medium was harvested and concentrated by ammonium sulfate precipitation.

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2 The abbreviations used are: LCM, leukocyte-conditioned medium; LCM-C, leukocyte-conditioned medium, concentrated; CSF-GM, colony-stimulating factor-granulocyte, macrophage; IFN-γ, γ-interferon; dThd, thymidine; GCT, giant cell tumor; 5-FU, 5-fluorouracil; CSF, colony-stimulating factor.
cubating Ficol:Hypaque (specific gravity, 1.077) interface peripheral blood mononuclear cells at 2 x 10^6 cells/ml of complete culture medium with phytohemagglutinin (1 µg/ml) for 5 to 7 days in a humidified CO2 environment. The supernatant was harvested, brought to 55% (NH4)2SO4, and stirred in the cold overnight. The precipitate was harvested by centrifuging at 15,000 rpm and resuspended in approximately 10% of the original volume of hypotonic buffer [5 x 10^-2 M KH2PO4 - K2HPO4 (pH 7.5):10% glycerol:0.1% 2-mercaptoethanol]. The supernatant was brought to 85% (NH4)2SO4 and the precipitate was harvested and resuspended as above. Resuspended (NH4)2SO4 precipitates were then dialyzed against three changes of phosphate-buffered saline (pH 7.4), filtered using 0.2-µm Nalgene filters, assayed for protein content using the Bio-Rad method, and stored at -20°C.

Nucleotide Uptakes and Pool Analysis. Overall incorporation of [3H]thymidine into macromolecules was measured upon replicate cultures in 24-well plates (Falcon, Oxnard, CA), each of which contained 1 x 10^6 cells, in 0.4 to 0.6 ml of medium, with additions as indicated. After 24 h, 0.5 µCi of [3H]thymidine was added to each well, and the cultures were incubated for 2 more h. Incorporation was terminated with 1 ml of ice-cold 1% thymidine per well in phosphate-buffered saline, and cells were harvested onto glass fiber filters using a Model M-24 automatic cell harvester (Brandel, Rockville, MD). Counts per minute were determined by liquid scintillation. These experimental conditions were within the linear range for cellular and [3H]thymidine concentrations. More detailed analysis of uptake into subcellular compartments of the four [3H-deoxyribonucleotides and [3H]5-FU was performed as follows. Cells were exposed to each tracer (2 µCi/ml) for the indicated times. Cells were pelleted, and the supernatant was carefully removed. The pellet was then incubated for 20 min at 4°C in 1 ml of 0.5 n HClO4 and then centrifuged at 2000 rpm; the supernatant was removed and added to 0.05 ml of 1 M Tris (pH 8.0) with 0.1 ml of 5 n KOH; and the pH was adjusted to 7.5 to 8.0. The HClO4 pellet was resuspended in 1 ml of 0.3 n KOH and incubated at 37°C for 16 h. Sixty µl of 5 n KOH were added to neutralize each sample. Total (corresponding to DNA and RNA) cpm were determined upon 0.5-ml aliquots, and counts present in DNA were determined by measuring trichloroacetic acid-insoluble counts of the remaining 0.5 ml (15).

Neutralized, HClO4 extract supernatants were assayed for each dideoxynucleotide triphosphate pool using Micrococcus luteus DNA polymerase (16), the corresponding α-32P-labeled dideoxyribonucleoside triphosphate, and the appropriate template (poly (dA-T)-d(A-T) (17) or poly (dC)-d(C) (18, 19)) as previously described (15, 20, 21). In addition, the uptake and conversion of exogenously added [3H]-labeled nucleosides into the intracellular acid-soluble dideoxyribonucleoside triphosphate pools were determined using similar DNA polymerase:template incubations (15). Statistical comparisons were performed using the Student t test.

Flow Cytometry. Following the indicated incubations, cells were washed and resuspended in 1 part of 1 mw EDTA in phosphate-buffered saline to which were then added 3 parts of 95% ethanol. Cells were stored at 4°C until analysis. For single laser analysis of DNA distributions, cells were pelleted and resuspended at a concentration of 5 x 10^5/ml in mithramycin (0.1 mg/ml) in 12.5 mw MgCl2:0.9% NaCl solution (22). Staining of ethanol-fixed cells for simultaneous DNA-RNA analysis was performed using a modification (23) of a procedure by Shapiro (24). Cells were resuspended in Hoechst 33424 (0.5 µg/ml) in phosphate-buffered saline for 15 min. Subsequently, an equal volume of a solution containing Pyronin Y (2.0 µg/ml), Hoechst 33424 (0.5 µg/ml), and phosphate-buffered saline was added 5 min prior to analysis (25). Final cellular concentration was 5.0 x 10^6/ml. Simultaneous DNA and RNA analysis was performed by exciting sequentially the Hoechst dye in the UV using an argon laser, followed by excitation of the Pyronin Y using a krypton laser (530 nm) (25). Computer analysis of cell cycle distribution was performed as previously described (26).

RESULTS

Following 24 h of incubation, LCM induced dose-dependent increases of [3H]dTd incorporation into HL-60 cells (Chart 1). Concentration of LCM by ammonium sulfate precipitation allowed application of higher dosages of LCM proteins, which enabled demonstration of more marked stimulation of [3H]dTd incorporation. Slight inhibition was observed with some batches at very high treatment levels. There was additionally some purification of the factor responsible for this effect in the 55% (NH4)2SO4 fraction, hereafter referred to as LCM-C. Similar stimulation of incorporation was also noted using KG-1 (27) or K562 (28) cells as a target, but not U937 cells (29). A similar pattern of stimulation and (NH4)2SO4 concentration of activity was noted for GCT-conditioned medium (30) but not purified CSF-GM, at concentrations up to 200 units/ml. Cell recovery and trypan blue viability were not significantly affected by 24-h exposures to LCM-C at 2 mg/ml, nor was morphological evidence of differentiation noted. Cells, however, appeared slightly larger in size as examined in the hemocytometer.

In order to determine if the increase of [3H]dTd incorporation reflected a true increase in proliferation, cell cycle distribution was studied using mithramycin staining of DNA quantitated by flow cytometry (Chart 2). Increased percentages of cells with S-phase DNA content were noted to be induced by 4 h and were maximal at 24 h. Effects upon G2-M cells were less marked and were proportionately greatest at 48 h, suggesting cell cycle progression of a stimulated cohort. Maximal effects upon cell cycle parameters were approached at lower LCM-C concentrations than for [3H]dTd incorporation (Chart 1).

Because the effects upon [3H]dTd uptake were proportionately much greater than the changes in cell cycle distribution
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A. % S CELLS

B. % G2 / M CELLS

LCM-C PROTEIN ADDED mg/ml.

Chart 2. Cell cycle effects of LCM-C-treated HL-60 cells. Cells were cultured with the indicated concentrations of LCM-C for 4 (A), 24 (B), and 48 (A) h, and then harvested, fixed, and stained with mithramycin; cell cycle distribution was determined by flow cytometry. Results shown are the calculated percentages of S-phase (A) and G2-M (B) cells.

noted, a more detailed analysis of deoxynucleoside uptake, metabolism, and incorporation into macromolecules was undertaken (Table 1). LCM induced marked increases of intracellular [%H]dThd accumulation and its conversion to [%H]dTTP. Since dTTP pools exhibited no consistent change, [%H]dTTP specific activity markedly increased (P < 0.02, n = 4). Evidence for enhanced thymidine accumulation of LCM-C-treated cells was also obtained using cultures supplemented with unlabeled thymidine (Table 2). Under these conditions, incorporation of labeled thymidine was relatively less suppressed with LCM-C, suggesting greater extracellular depletion of the competing unlabeled thymidine during the preceding culture period. A slight difference was noted between dialyzed and undialyzed serum. Similar but much less marked effects were noted of the other deoxynucleosides (Table 1). Deoxyadenosine and deoxyguanosine exhibited significant RNA incorporation which was stimulated by LCM-C.

Further studies were performed to determine if LCM-C could modulate the sensitivity of HL-60 cells to the halogenated pyrimidine analogue, 5-FU. Following 24 h of incubation with 5-FU, HL-60 cells exhibited increased [%H]dThd incorporation, which was interpreted as reflecting inhibition of thymidine synthetase. Sensitivity to this effect was markedly enhanced by LCM-C (Chart 3), although sensitivity to 1-β-D-arabinofuranosylcytosine or hydroxyurea under similar conditions of exposure was not altered. Intracellular accumulation of [%H]5-FU was increased in the presence of LCM-C (Table 3). Considerable [%H]5-FU incorporation into RNA of the LCM-C-treated cells was moreover detected. Analysis of thymidine uptakes and pools (Table 4) documented more complete dTTP depletion of combination LCM-C:5-FU-treated cells than of cells treated with 5-FU alone (P < 0.05 in repeated experiments). Cells exposed to 5 × 10^-6 M 5-FU for 24 h exhibited significant cytotoxicity only when coincubated with LCM-C (Chart 4). Multiparameter flow cytometric analysis disclosed more complete G1-early S-phase block of the combination-treated cells and also greater variance of RNA content with a population of abnormally high RNA content cells present (Chart 4).

DISCUSSION

HL-60 has been extensively utilized mainly as a model of induced differentiation by chemical and biological agents. Leukocyte-conditioned medium has been reported to induce a monocyticoid pattern of differentiation (5, 6), characterized by many techniques including cytochemistry (6, 13) and cell surface marker analysis by monoclonal antibody and lectin binding (31–34). Although some evidence of stimulation of proliferation of HL-60 by LCM has been previously reported (6, 12), these effects have been less widely studied. In this study, LCM was subjected to an ammonium sulfate precipitation step which has enabled the study of a factor stimulating [%H]dThd incorporation by HL-60 at higher concentrations and in slightly more pure form than would be otherwise possible. The [%H]dThd incorporation stimulating effect was found to affect both HL-60, KG-1 (27), and K562 (28) cells but not U937 cells (29). Similar effects were producible using giant cell tumor-conditioned medium (30).

The premise that [%H]dThd incorporation stimulation of HL-60 by LCM-C is associated with increased proliferation was corroborated in this study by flow cytometry. Our results apparently contrast with those of others who have not noted increased proliferative cells induced by LCM (13, 14). The effects noted here were small, however, particularly at the low concentrations of LCM-C corresponding with levels attainable with crude LCM, and shorter exposure periods were emphasized. The effects upon stimulation of [%H]dThd incorporation were disproportionately great relative to the enhancement of S-phase cells actually observed by flow cytometry or to the amount of stimulation of proliferation which one would expect to be possible for an already rapidly proliferating cell line. This effect was therefore studied in greater detail. It first of all seemed possible that the apparent stimulation reflected depletion of thymidine (either intracellular or extracellular) of the LCM-C-treated cells. Supplementation of the medium with 10^-6 M thymidine, a nontoxic level (35), exaggerated even further the difference between stimulated and unstimulated cells (Table 2). This was seen to be related to increased suppression of labeled dThd incorporation by the untreated cells, suggesting more rapid uptake and consequent depletion of extracellular thymidine by LCM-C-treated cells. Fetal calf serum...
Table 1
Effects of LCM-C upon uptake, pool size, specific activity, and nucleic acid incorporation of deoxyribonucleosides

Cells were incubated for 24 h with and without LCM-C (2 mg/ml) as indicated, then pulsed for 30 min with 2 μCi of the indicated [3H]-deoxynucleoside per ml. Analysis of pools, uptakes, and specific activities was performed as described in "Materials and Methods." Results shown are representative of multiple similar experiments.

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Table 2
Influence of exogenous unlabeled thymidine upon pattern [3H]thymidine uptake by HL-60 cells

Medium used included fetal calf serum which had been dialyzed against phosphate-buffered serum as indicated. Cultures were supplemented as indicated with unlabeled thymidine at the beginning of the 24-h culture period and pulse labeled with [3H]-thymidine at the conclusion of this culture period.

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Table 3
Effects of LCM-C upon [3H]-dThd (TdR) incorporation of HL-60 cells

Cells were incubated with (•) and without (O) LCM-C plus the indicated concentrations of 5-FU for 24 h before pulsing, harvesting, and counting. Points, mean of ratios of [3H]-dThd incorporation with, compared to without, 5-FU, based upon quadruplicate cultures; bars, SE.

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Table 4
Effects of LCM-C upon thymidine metabolism of 5-FU-treated cells

Experiments were performed as described in Table 1 legend, except that 5 x 10⁻⁶ M 5-FU was added at the beginning of the culture period. Results shown are representative of three similar experiments.

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* dNTP, deoxynucleotide triphosphate(s); SA, specific activity.
independent of any change of the rate of DNA synthesis would be an expected consequence of such pool effects.

In addition to increased proliferation, \(^{3}H\)dThd uptake, and intracellular specific activity, there could be additional effects contributing to the increased thymidine incorporation stimulated by LCM-C. It is possible that relative differences in pool specific activity are even more marked in S-phase cells or may be affected by differences in age distribution within S (37). Alternatively, an increase in rate of progression through S or a decrease in cells with S-phase DNA content not actively synthesizing DNA might be induced under these conditions. Such kinetic differences may appear unlikely in view of the minimal increases of cell recovery induced by LCM-C. Analysis of the HL-60 growth pattern, however, has led to the detection of a significant spontaneous cell loss factor (40), and so it is possible that an increased rate of proliferation was balanced by an increased rate of cell death.

A further question of interest is the identity of the factor in LCM-C responsible for the observed effects. The effects described occurred rapidly following exposure, at which point the cells remained nondifferentiated. The effects were shared with the KG-1 cell line (27), which has been reported to exhibit similar \(^{3}H\)dThd incorporation stimulation in response to CSF-containing preparations (41). An alternative source of CSF, GCT-conditioned medium, produced similar effects (30). U937 (29) was insensitive to these effects, despite its responsiveness to IFN-\(\gamma\) (42). Although important interactions of IFN-\(\gamma\) with HL-60 have been described, these are accompanied by inhibition of proliferation (10). \(^{3}H\)dThd incorporation stimulating activity for purified myeloid progenitors from chronic myelogenous leukemia samples has recently been identified as CSF in GCT and leucocyte-conditioned media (43). The lack of a similar effect with purified CSF-GM and the occurrence of a similar effect with K562 suggests the action of a factor operating at an earlier stage of differentiation (44, 45). In order to settle this question, work on further purification and characterization of the factor(s) responsible for this effect is proceeding in this laboratory and will be the subject of a future publication. Other recent studies have also disclosed effects of hemopoietic growth factors, such as CSF, beyond the classic role of triggering quiescent stem cells to proceed along their differentiation pathway. These include activating effects upon mature cells as well as proliferating marrow cells, and effects upon RNA, protein, and energy metabolism (46–51). Support of proliferation and stimulation of \(^{3}H\)dThd incorporation of acute myeloid leukemic cells obtained directly from patients by sources of CSF has also been previously noted (7, 9, 52–54). Our results extend these observations to the widely used HL-60 model and provide further metabolic details.

In accordance with our observations of altered deoxynucleotide metabolism of HL-60 cells in response to LCM-C, the possibility that sensitivity of such cells to antimetabolite chemotherapy could be similarly modulated was further investigated. Twenty-four-h exposures to low concentrations of 5-FU increased \(^{3}H\)dThd incorporation, and sensitivity to this effect was augmented by LCM-C. Cytotoxicity of 5-FU has been related to two major effects: inhibition of thymidy late synthetase by 5-fluorodeoxyuracil monophosphate and incorporation into RNA (55). The former effect tends to predominate at lower concentrations and exposures of less than 24 h and was probably responsible for the increased uptake and incorporation of oxogenous \(^{3}H\)dThd noted. Deoxynucleotide pool analysis confirmed the nature of this effect with marked depletion of dTTP noted in the combination-treated cells. Pool-specific activity of \(^{3}H\)dTTP thus became indeterminately high, leading to extraordinary incorporation rates into DNA. Combination 5-FU:LCM-C treatment also

**CONDITIONED MEDIUM AND LEUKEMIC PROLIFERATION**

Chart 4. Dual parameter flow cytometric analysis of LCM-C and 5-FU effects. Cells were incubated for 24 h without additions (A), with LCM-C (2 ng/ml) (B), with 5-FU (5 \(\times\) 10\(^{-8}\) M) (C), and with 5-FU and LCM-C in combination (D). Cell recoveries (per ml) and trypan blue viabilities are shown in the corner of each panel; contour lines include regions with 25, 50, 100, 200, and 400 or more cells per channel, respectively. Nonviable cells and debris with significantly lower than Gi DNA content were gated out. Results of the computer fit to the DNA histograms are in the upper right-hand corners. For C, the large number of S-phase cells relative to G_{2}/M cells resulted in computationally indeterminate G_{2}/M content.
resulted in enhanced proliferation arrest and cytotoxicity. The RNA content distribution of these cells exhibited marked broadening, with the appearance particularly of cells having increased content. Although 5-FU is associated with inhibition of RNA synthesis (55), it is possible that under the conditions of these experiments the thymidylate synthetase inhibiting effect predominated. This would be expected to induce a transient megablastoid state, with RNA synthesis continuing despite the halt in DNA synthesis. A further abnormality noted of the combination-treated cells was the appearance of \(^3H\) from labeled dThd in RNA, an effect of 5-FU which has previously been described in other cell types (56). The enhanced effects of 5-FU upon LCM-C-treated cells was found to be associated with increased intracellular accumulation of 5-FU. No such differential sensitivity to 1-\(\beta\)-d-arabinofuranosylcytosine or hydroxyurea was noted, and it thus seems unlikely that the modest cell cycle effects of LCM-C could be alone responsible for the difference. The augmented dTTP depletion of combination-treated cells provided further evidence for a metabolic difference. Other effects upon intracellular metabolism of 5-FU are certainly possible but were not further investigated.

We have thus demonstrated and characterized alterations of deoxynucleotide metabolism, of a human leukemic cell line, by a growth factor, which are linked to, but independent of, cell cycle changes. This shift in metabolism was shown to be exploitable to augment chemosensitivity and have mainly focused upon cell cycle manipulation (57, 58); this study provides evidence for other metabolic effects which could be useful in the treatment of leukemia. Similarly, others have recently reported modulation by growth factors of 1-\(\beta\)-d-arabinofuranosylcytosine uptake (59) in analogous hematopoietic systems. It is possible that such principles could be extended to other tissue-specific growth factors or could be applied in such a way as to differentially protect normal marrow progenitors from chemotherapeutic drugs. 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