In Vitro Sensitivity to Steroid Hormones and Cytotoxic Agents of Normal and Malignant Lymphocyte Colony-forming Cells

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ABSTRACT

In vitro assays were used to assess the sensitivity of normal T-cells and malignant, chronic lymphocytic leukemia (CLL) lymphocyte colony-forming cells (CFC) to a panel of cytotoxic drugs and steroid hormones. Normal T-CFC were remarkably resistant to hydrocortisone, progesterone, estradiol, and testosterone at concentrations ≤10−9 M. Variable inhibition was seen at concentrations of 10−4 M, and prior exposure to phytohemagglutinin increased sensitivity only to sex steroid hormones. In contrast to T-CFC, which showed little variation in patterns of steroid hormone inhibition in vitro, CLL-CFC from individual patients displayed widely varying sensitivity to all hormones tested; 50% inhibitory dose varied by as much as 2 logs. T-CFC were fairly resistant to a 1-hr exposure to achievable concentrations of 1-β-d-arabinofuranosyl-cytosine, 5-fluorouracil, chlorambucil, melphalan, cisplatin, methotrexate, Adriamycin, and bleomycin. Prior exposure to phytohemagglutinin resulted in increased sensitivity only to low concentrations of Adriamycin, a phenomenon that appeared related to prior or concurrent lectin exposure and not to changes in cell cycle status. CLL-CFC showed variable sensitivity to Adriamycin and cisplatin, and concurrent exposure to lectin and Adriamycin did not increase sensitivity to that drug. CLL cells displayed much greater sensitivity to a 1-hr exposure to antimetabolites and bleomycin than to continuous exposure to the same drugs. In contrast to normal T-CFC, CLL-CFC exposed to methotrexate were not "rescued" by subsequent culture in media and fetal bovine serum. Incubation of T-CFC or CLL-CFC with melphalan and a source of protein (fetal bovine culture in media and fetal bovine serum) did not "rescue" the same drugs. In contrast to normal T-CFC, CLL-CFC showed variable sensitivity to hydrocortisone, progesterone, estradiol, and testosterone at concentrations <10−5 M. Variable inhibition was seen at concentrations of 10−4 M, and prior exposure to phytohemagglutinin increased sensitivity only to sex steroid hormones. In contrast to T-CFC, which showed little variation in patterns of steroid hormone inhibition in vitro, CLL-CFC from individual patients displayed widely varying sensitivity to all hormones tested; 50% inhibitory dose varied by as much as 2 logs. T-CFC were fairly resistant to a 1-hr exposure to achievable concentrations of 1-β-d-arabinofuranosyl-cytosine, 5-fluorouracil, chlorambucil, melphalan, cisplatin, methotrexate, Adriamycin, and bleomycin. Prior exposure to phytohemagglutinin resulted in increased sensitivity only to low concentrations of Adriamycin, a phenomenon that appeared related to prior or concurrent lectin exposure and not to changes in cell cycle status. CLL-CFC showed variable sensitivity to Adriamycin and cisplatin, and concurrent exposure to lectin and Adriamycin did not increase sensitivity to that drug. CLL cells displayed much greater sensitivity to a 1-hr exposure to antimetabolites and bleomycin than to continuous exposure to the same drugs. In contrast to normal T-CFC, CLL-CFC exposed to methotrexate were not "rescued" by subsequent culture in media and fetal bovine serum. Incubation of T-CFC or CLL-CFC with melphalan and a source of protein (fetal bovine serum or bovine serum albumin) resulted in decreased cell kill. Differences in in vitro sensitivity of normal and malignant lymphocyte CFC to steroids and cytotoxic agents can be demonstrated using these culture systems. CLL-CFC showed variable sensitivity to hydrocortisone, and much greater sensitivity to antimetabolites than normal T-CFC. Differences in conditions of drug exposure, such as concurrent exposure to lectin or inclusion of protein, may alter the in vitro sensitivity of lymphocyte CFC to some drugs.

INTRODUCTION

Growth of colonies of primary, human tumor cells in semisolid media has been achieved for both solid tumors and hematological cancers (7, 9, 14, 21). These assays have been used to assess in vitro sensitivity of leukemia and tumor CFC3 to antineoplastic agents (18, 28). Correlation of in vitro results with in vivo drug responses has suggested that such assays may be used either to predict the response of individual tumors to drugs (28) or as preclinical screens for new chemotherapeutic agents (1, 27). We have reported the adaptation of a colony-forming assay for B-lymphocytes (15) to growth of chronic lymphocytic leukemia cells (32), and demonstrated the feasibility of testing drugs in vitro using this assay system. We (33) and others (6, 24) have also developed colony-forming assays for normal lymphocytes which may be used to compare in vitro drug sensitivity of normal and malignant lymphocyte CFC.

In these studies, we have expanded our previous studies of CLL-CFC to examine effects of a panel of drugs and steroid hormones, and have used assays for normal T-lymphocyte CFC to compare responses by normal and malignant lymphocytes.

MATERIALS AND METHODS

Patients. Blood was obtained from normal donors or patients with CLL in heparinized syringes, according to procedures approved by the Committee on Human Subjects, University of California, San Diego. All patients had elevated circulating lymphocyte counts and a clinical syndrome consistent with CLL. Typing for surface antigens (surface immunoglobulin, λ-ligand antigen, κ- and λ-light chains) and for cytoplasmic immunoglobulin, performed as described previously (32), demonstrated the origin of leukemia cells from monoclonal B-lymphocyte populations. CLL and normal blood cells were initially separated on Ficoll-Hypaque (Ficoll/Paque; Pharmacia Chemicals, Piscataway, N. J.) as described previously (32).

Two cell fractions were subsequently separated on the basis of 2-amino-ethylisothiouronium bromide hydrobromide (Sigma Chemical Co., St. Louis, Mo.)-treated sheep erythrocyte (Colorado Serum Co., Denver, Colo.) rosette (E-rosette) formation (17). Briefly, a mixture containing normal Ficoll-Hypaque-separated mononuclear cells or CLL cells at 5 × 106/ml and 2-aminoethylisothiouronium-treated SRBC (final concentration, 1%) was centrifuged at 150 × g for 10 min, and the initial volume was reduced by aspiration; 0.5 ml of SRBC absorbed FCS (Flow Laboratories, Rockville, Md.) was layered over the cell pellet of 30 to 50 × 106 lymphocytes, and the cells were incubated at 4°C. CLL cells were incubated for at least 1 hr, and normal cells were incubated overnight. After gentle resuspension, the cells were separated a second time on Ficoll-Hypaque. The E-rosette-depleted (T-cell-depleted) B-CLL cells were removed from the interface. E-rosette-positive T-cells from normal donors (sheep erythrocyte rosette-positive cells) were recovered from the pellet by lysing SRBC with Tris-buffered NaCl (21, 32). The viability of T-cell-depleted B-CLL preparations and normal sheep erythrocyte rosette-positive lymphocytes exceeded 95% as determined by trypan blue exclusion.

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Preparation of Conditioned Medium. Media conditioned by PHA-stimulated, sheep erythrocyte rosette-positive cells (PHA-TCM) was prepared as described previously (15, 32). Normal sheep erythrocyte rosette-positive cells (1 x 10^6/ml) were cultured in Iscove’s medium with 10% FCS and PHA (2 Ìg/ml; Burroughs-Wellcome, Research Triangle Park, N. C.) for 48 hr. The cells were removed by centrifugation, and the conditioned medium was passed through a 0.25-Ìm millipore filter and stored at 4°C.

Colony-forming Assays. The colony-forming assays for B-CLL cells and normal T-lymphocytes were performed as described previously (32, 33). T-lymphocyte-depleted B-CLL cells or normal sheep erythrocyte rosette-positive lymphocytes (T-cells) were cultured at 5 x 10^5 cells/ml in a final mixture of 1% methylcellulose (Student Service Science, Glendale, Calif.), 20% FCS, 20% PHA-TCM, and Iscove’s medium. Feeder cells consisted of normal mononuclear cells (in T-cell cultures) or normal T-cells (in B-CLL cultures) irradiated with 2500 rads and added at a final concentration of 3 x 10^5/ml. Controls confirmed the loss of colony-forming capacity of irradiated cells.

Triplicate or quadruplicate 1-ml aliquots were incubated in 35-mm dishes (Lux Scientific, Newbury Park, Pa.) or quintuplicate 0.1-ml aliquots in microtiter plates (Falcon Plastics, Cockeysville, Md.) at 37°C for 5 to 10 days in 7.5% CO2. Aggregates of more than 40 cells were scored as colonies using an inverted microscope.

For experiments on PHA-stimulated T-cells, sheep erythrocyte rosette-positive lymphocytes were incubated with PHA as described in the preparation of PHA-TCM. After 48 hr, the cells were removed by centrifugation, a single-cell suspension was formed by passing the cells through successively smaller needles, and the cells were washed 3 times with media.

Drug and Hormone Exposure. Normal and leukemia lymphocytes were exposed to cytotoxic drugs at various concentrations for 1 hr at 37°C in HBSS, washed twice in media, and plated in colony-forming assays. Most drugs were tested over a 5-log range, spanning plateau and peak serum levels described by Salmon et al. (28). Drugs studied included 1-ß-D-arabinofuranosylcytosine (The Upjohn Co., Kalamazoo, Mich.), 5-fluorouracil (Hoffman-LaRoche Laboratories, Nutley, N. J.), chlorambucil and melphalan (Burroughs-Wellcome), Adriamycin (Adria Laboratories, Columbus, Ohio), methotrexate and bleomycin (Bristol Laboratories, Syracuse, N. Y.), and cisplatin (Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.).

In some experiments, the cells were exposed for 1 hr at 37°C to drug in HBSS supplemented with 10% FCS or 0.5% BSA, washed twice in media, and plated in the colony-forming assay or continuously exposed to drugs by adding them directly to the culture plates. Drug effects were assessed by percentage of reduction in colony-forming capacity compared to that of controls (6 to 10 colony counts).

Stock solutions of steroid hormones were prepared in ethanol, and ethanol controls were performed where appropriate. Hormones studied included: hydrocortisone (Sigma), progesterone (Sigma), 5ß-estradiol (Sigma), and 5ß-dihydrotestosterone (Sigma). Stock solutions were diluted just prior to plating and were added directly to cultures. A single lot of FCS used in these studies was assayed for estradiol, hydrocortisone, progesterone, and testosterone prior to use through the courtesy of Dr. Robert Rebar, University of California San Diego Cancer Center; all hormones were present in FCS at concentrations of less than 5 x 10^-5 M.

RESULTS

The effects of steroid hormones on growth of normal T-CFC are shown in Chart 1. Results shown represent the mean ± S.E. of 3 to 5 experiments on fresh T-CFC and T-CFC grown from cells previously exposed to PHA for 48 hr in liquid culture. Fresh T-CFC were remarkably resistant in vitro to all hormones tested including hydrocortisone. After exposure to PHA for 2 days in liquid culture, T-CFC showed greater inhibition by high concentrations of progesterone, estradiol, and testosterone.

When similar studies were performed on CLL-CFC, certain differences were noted. In Chart 2, each data point represents the mean of triplicate or quintuplicate colony counts on CLL-CFC grown from one of 8 patients. In contrast to normal T-CFC from different donors, which showed a narrow range of sensitivity to steroid hormones in culture, CLL-CFC showed a wide range of responses to all 4 hormones tested. There did not appear to be definite differences in sensitivity of CFC from patients previously treated with alkylating agents and those who were previously untreated, although the numbers of patients in each group were small.

The effects of a panel of cytotoxic agents on growth of normal T-CFC are shown in Chart 3. Cells were exposed to a 5-log concentration range of each drug for 1 hr, using serum-free conditions, and were washed and plated. Points represent the means ± S.E. of triplicate experiments on fresh T-CFC and means or mean ± S.E. of 2 to 5 experiments on PHA-stimulated cells. In general, T-CFCs were resistant to all but extremely high concentrations of the alkylating agents, melphalan and chlorambucil, and to bleomycin and cisplatin. Prior exposure to PHA did not alter sensitivity to chlorambucil, melphalan, or cisplatin. Increased sensitivity to bleomycin, a drug with relative specificity for cells synthesizing DNA (S phase) (4), was observed at extremely high drug concentrations.

Increased sensitivity of T-CFC to a 1-hr exposure to low concentrations of Adriamycin was observed after PHA stimulation (Charts 3 and 4). This increased sensitivity was most marked over the concentration range of 0.05 to 0.3 Ìg/ml used by
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Chart 2. Effect of various concentrations of steroid hormones on growth of chronic lymphocytic leukemia colonies. Various concentrations of steroid hormones were added to cultures containing E-rosette-depleted CLL cells, irradiated, normal T-lymphocytes, and media conditioned by PHA-stimulated T-cells. Results are means of triplicate or quintuplicate colony counts on individual untreated (○) patients or patients previously treated with alkylating agents (●).

Salmon et al. (26) in studies of solid tumor CFCs (Chart 4). Buick et al. (6) reported similar findings and attributed this observation to an increased number of CFCs in cycle at the time of drug exposure. Since our previous data showed no increase in T-CFCs in cycle after PHA exposure (33), we sought to expand our observations. When fresh T-CFCs were continuously exposed to Adriamycin by direct addition to the culture plate, the sensitivity of the CFCs was virtually identical to that of PHA-stimulated cells (Chart 4). Continuous exposure to Adriamycin of T-CFC grown from cells previously exposed to PHA was also similar to a 1-hr exposure of PHA-stimulated cells to this drug. The common denominator of these observations appeared to be concurrent or previous exposure to lectin and drug, although other explanations are possible.

Varying conditions of drug exposure to the alkylating agent, melphalan, also resulted in differing T-CFC sensitivity (Charts 3 and 4). Normal T-CFCs showed moderate in vitro sensitivity to a 1-hr exposure to melphalan, and prior exposure to PHA for 48 hr did not alter sensitivity to the drug (Chart 3). When T-CFCs were continuously exposed to melphalan in the presence of FCS, sensitivity to the drug was decreased (Chart 4). In 2 experiments, exposure to this drug for 1 hr in HBSS and 10% FCS resulted in identical decreases in cell kill (data not shown). Thus, in this model system (T-CFC), 2 different factors were found to alter in vitro drug sensitivity: prior or concurrent lectin exposure increased T-CFC sensitivity to Adriamycin, while drug exposure in the presence of serum decreased T-CFC sensitivity to melphalan.

Examination of in vitro T-CFC sensitivity to several antimetabolites was carried out, for simplicity, using the same 1-hr drug exposure. Under these conditions, and since these drugs are S-phase-specific agents, maximum percentage of inhibition could approach the percentage of cells in cycle (~60%) (11). In general, this was the case for 5-fluorouracil and 1-β-o-arabinofuranosylcytosine (Chart 3). Maximal inhibition, except at extraordinary concentrations, was approximately equal to the expected fraction of cells in cycle, as determined by thymidine “suicide” studies (32, 33). Furthermore, exposure of T-cells to PHA for 48 hr did not alter the sensitivity of T-CFC to these drugs, again supporting the contention that prior PHA exposure does not alter the S-phase status of normal T-CFC.

These observations were not relevant to the antimetabolite, methotrexate (Chart 3). In previous studies, we have shown that nucleosides (dThd and hypoxanthine) in FCS can alter or abrogate the in vitro toxicity of methotrexate (12) so that continuous exposure to the drug may be ineffective in vitro. Furthermore, exposure of normal marrow cells in vivo to adequate concentrations of dThd and hypoxanthine within 24 hr of methotrexate treatment “rescue” them from toxicity due to this drug (11, 34). It is, therefore, not surprising that a 1-hr exposure even to very high concentrations of methotrexate did not result in toxicity to either fresh or PHA-stimulated T-CFC (Chart 3) which were subsequently cultured in FCS containing dThd and hypoxanthine.
The in vitro sensitivity of CLL-CFC to a panel of antineoplastic drugs was also assessed (Chart 5). Because of our previous experience with T-CFC, CLL cells were either incubated with drug for 1 hr in HBSS, washed, and plated, or various concentrations of drugs were added directly to the culture plate. Results in Chart 5 represent the means ± S.E. of experiments on 3 patients with CLL. In vitro sensitivity to the non-cell cycle-specific agents, Adriamycin and cisplatin, was similar whether cells were exposed for 1 hr or continuously to these drugs. Exposure for 1 hr to drugs with absolute (antimetabolites) or relative (bleomycin) specificity for S-phase cells was markedly more toxic to CLL-CFC than was continuous drug exposure. In contrast to normal T-CFC, a 1-hr exposure to methotrexate resulted in substantial CLL-CFC kill, but continuous exposure to this drug was ineffective. These data suggest differences between T-CFC and CLL-CFC in effects of short-term exposure to methotrexate, followed by culture in FCS containing nucleosides. Thus, in this system, conditions of drug exposure to cell cycle-specific drugs markedly altered patterns of drug sensitivity of CLL-CFC.

The effect of inclusion of protein on a 1-hr exposure of CLL-CFC to Adriamycin and melphalan was also examined using cells from 2 patients (data not shown). When CLL cells were incubated for 1 hr in HBSS or in HBSS containing 0.5% BSA, the inclusion of BSA in a 1-hr exposure did not alter in vitro sensitivity to Adriamycin. As with T-CFC cultured with FCS, the presence of BSA decreased cell kill by melphalan at all concentrations tested.

DISCUSSION

In these studies, we have used colony-forming assays for CLL cells (32) and T-lymphocytes (33) to study in vitro sensitivity of these cells to drugs and hormones. In previous studies, we characterized CFCs and cells in colonies grown from normal and malignant lymphocytes (32, 33). T-CFCs arise from mature T-cells and give rise to colonies composed of >90% cells bearing mature T-lymphocyte markers (SRBC receptors, T101 and OKT3). CLL colonies contain cells which mark for mononuclear surface and/or cytoplasmic immunoglobulin light chains, consistent with monoclonal B-lymphocytes. When lymphocyte colonies were grown from E-rosette-depleted, normal peripheral blood lymphocytes, most of the cells in colonies were polyclonal B-cells as determined by surface immunoglobulin and immunoglobulin light chain staining (32). The composition of these colonies was, however, more heterogeneous than that of T-CFC, since macrophages and T-cells were also detected, suggesting that E-rosette-depleted CFC may derive from more heterogeneous cell populations. Therefore, we chose to compare the drug and hormone sensitivities of CLL-CFC to the more homogeneous and better-characterized T-CFCs. The choice of the T-CFC system as a normal “counterpart” of the CLL lymphocytes was also based on other considerations.

Although >95% CLL cells bear markers consistent with B-lymphocytes (surface immunoglobulin, Ig antigen) (2, 38), and some CLL cells can mature to plasma cells in vitro (36), many critical attributes of the CLL cell resemble T- rather than B-lymphocytes. We and others (20, 23) have shown that intracellular concentrations of cyclic AMP and the enzyme, adenyl cyclase, in CLL cells resemble those of normal T-cells rather than those of B-cells. Cyclic AMP and guanylate cyclase concentrations in CLL cells also resemble those of T-cells (23). Intracellular concentrations of enzymes such as 5' -nucleotidase and some isoenzymes of lactate dehydrogenase in CLL cells also resemble those of normal T-cells rather than those of normal B-lymphocytes (16, 25). T-cell surface antigens, such as T65 (40) and receptors for Helix pomatia A lectin (10), are also present on CLL cells and absent from normal B-cells. Finally, CLL cells can be induced in culture to express SRBC receptors (29, 39).

The use of T-CFC allowed us to compare in vitro responses of normal and malignant lymphocyte CFC, and gave us a model system allowing investigation of conditions of drug exposure on observed in vitro responses. The power of the T-CFC as a model was increased by ability to study cells from a resting (fresh) T-cell population, and the same cells when >90% are synthesizing DNA (5) after lectin stimulation.

In vitro studies of steroid hormones showed T-CFC to be essentially resistant to all but extremely high concentrations of these compounds, and prior lectin exposure affected in vitro sensitivity only to sex steroid hormones. Normal T-CFC appeared less sensitive to steroid inhibition than did T-cells grown in liquid cultures (19) in our previous studies. It has been reported that corticosteroids inhibit T-lymphocyte proliferation by decreasing production of T-cell growth factor (30). Decreased inhibition of T-CFC growth observed in these studies may have been due in part to addition of an exogenous source of T-cell growth factor (PHA-TCM) to the cultures.

When the steroid hormone sensitivity of T-CFC was compared to that of CLL-CFC, differences were seen, but there was no consistent pattern. However, in contrast to normal T-CFC, the in vitro sensitivity of CLL-CFC to compounds, such as hydrocortisone, was extremely variable from patient to patient. This type of heterogeneity has been observed in our studies of acute myeloblastic leukemia blast CFC and may be a characteristic of malignant cell populations (31). At the highest concentrations studied (10^-5 and 10^-4 M), it appears that CFCs from most CLL patients were more sensitive than normal T-CFC to hydrocortisone. The range between individuals, however, appears very large, and the 50% inhibitory dose varied by up to 2 logs (Chart 2).

Normal T-CFCs were fairly resistant to achievable concentrations (<5 μg/ml) of most chemotherapeutic agents (3). Dividing cells are generally more sensitive to alkylating agents (chlorambucil and melphalan) and cisplatin, and to the cell cycle-specific antibiotic, bleomycin. PHA exposure, which stimulates >90% of
cells to synthesize DNA (5), did not affect in vitro sensitivity of T-CFC to these drugs, consistent with our observation that PHA does not alter the fraction of T-CFC in S phase. Lectin stimulation did, however, alter in vitro sensitivity to Adriamycin.

Recent studies have shown that Adriamycin need not enter cells to result in cytotoxicity (37), suggesting a critical action at the cell membrane. Adriamycin also inhibits lectin binding to sarcoma cells (22), again suggesting actions at a membrane level. Many of the actions of Adriamycin on cell membranes occur at or below concentrations affecting DNA function (41). Exposure to PHA, then, either prior to or during culture, could result in increased interaction between the activated T-cell membrane and Adriamycin resulting in cell death, or could interfere with a membrane function necessary for colony formation. This observation could account, in part, for the effectiveness of this agent in T-cell lymphomas and leukemias, in which T-cells are actively cycling.

Alterations in T-CFC sensitivity to Adriamycin occurred after lectin exposure, but not after inclusion of serum in the culture medium (Chart 4). In contrast, the presence of protein as either FCS or BSA altered in vitro results with melphan by decreasing T-CFC and CLL-CFC kill. These studies again illustrate the importance of defining in vitro conditions for drug exposure for each drug under study and against each target cell. Thus, continuous exposure to Adriamycin altered the sensitivity of T-CFC to this drug, but did not affect the sensitivity of CLL-CFC. In contrast, inclusion of a protein source, either by continuous exposure or 1-hr induction, had similar effects on sensitivity of CLL-CFC and T-CFC to melphan.

When the in vitro sensitivity of CLL-CFC to a panel of antineoplastic drugs was assessed, the surprising finding was the sensitivity of these cells to short-term exposure to cell cycle-specific agents. For reasons which are unclear at present, the CLL-CFCs were much more sensitive to a 1-hr, serum-free exposure to antimetabolites and bleomycin than when exposed continuously. Unlike the T-CFC exposed to methotrexate, CLL-CFCs were also not “rescued” by subsequent culture in PCS containing nucleosides. Differences in ability of preformed purines to “rescue” normal and malignant cells from methotrexate have been described in other experimental systems (12, 34) and would be consistent with this observation.

One is tempted to attribute the excess sensitivity of CLL-CFC to S-phase drugs to some artifact of the in vitro system. This remains a distinct possibility; however, this phenomenon was observed not only with antimetabolites, but with the relative S-phase-specific antibiotic, bleomycin, indicating similar effects by multiple classes of drugs. Previous studies by others (35) have shown cytotoxicity by the mitotic inhibitor, colchicine, for CLL cells at concentrations 5 logs less than those for normal lymphocytes. We have previously shown that nearly all CLL-CFCs are in cycle, as determined by the dThd suicide “technique” (32), so that sensitivity of these cells to S-phase agents is not without rationale. Our previous studies indicated that CLL patients with high WBCs, and presumably higher-stage disease, had fewer CFCs in cycle, suggesting that advanced cases might be less sensitive to these S-phase-specific drugs. The reasons for the profound differences between 1-hr and continuous exposure for drugs other than methotrexate, however, remain unclear.

The significance of this observation is unknown. Reported experience in the treatment of CLL with S-phase-specific agents is extremely sparse (13) but, by modern standards, many of the drugs tested in vitro in these studies have not received adequate clinical trials. If clinical trials demonstrate activity for agents such as 5-fluorouracil or bleomycin, drug testing with the CLL assay system would be partially validated.

We have found these studies to be helpful in design of prospective in vitro-in vivo drug trials in CLL, and would suggest guidelines which may be applicable to structure and analysis of other studies. The number of drugs examined should probably be limited. Varying effects of in vitro drug exposure on CFC cell kill should be known for each drug against each cell type under study. Analysis of in vitro effects must take into account the possibility that some drugs may act by mechanisms other than cytotoxicity (Adriamycin), and effects of others may be changed by culture reagents (methotrexate). Inclusion of protein during drug exposure, or use of continuous exposure, may also alter effects of some drugs but not others. However, variations in in vitro effects of these drugs on lymphocyte CFC demonstrated here may not be relevant to systems such as those for solid tumors, and the relevant drug concentrations for in vitro study of lymphocyte CFC may, therefore, differ from those for solid tumors.

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