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Assessment of Methylprednisolone Purging Efficacy on Daudi Burkitt Lymphoma Cells from Normal Bone Marrow

Nalini Janakiraman, MD,* and Loreen M. Niewenhuis, BSMT*

Studies on normal bone marrow and Daudi Burkitt lymphoma cells were performed to determine the efficacy of selective, in vitro chemopurging with methylprednisolone (MP). We found that MP reduces the number of lymphoma cells without significant damage to bone marrow cells. This information is important because we need to improve the existing in vitro purging regimens used to cleanse autologous marrows of metastatic disease before transplantation into cancer patients who have received high-dose chemotherapy. Normal human bone marrow (NBM) and Daudi lymphoma cells were treated in parallel with various purging regimens. NBM death was evaluated using soft-agar culture, while Daudi cell death was evaluated using one-week liquid culture. A protocol of 2.0 mg/mL of MP for four hours demonstrated optimal selectivity. When treatment was followed by cryopreservation, a 1.7 log purge of Daudi cells was increased to 2.3 logs while preserving 36% of committed NBM precursors. We repeated these experiments on a simulated contaminated marrow to model closely the mixture of normal and malignant cells found in advanced, metastatic disease. We evaluated this mixed system by flow cytometric immunoanalysis using the two-color CD10/CD20 markers to detect residual, viable Daudi cells. Our initial results were reproducible in this mixed-cell system, further supporting the evidence for effective in vitro purging of bone marrow using MP.
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Autologous bone marrow transplant (ABMT) employed to circumvent the hematopoietic toxicities associated with high-dose chemotherapy offers hope to many cancer patients with advanced or relapsed disease. The presence of metastases to the marrow, however, excludes patients from undergoing this procedure. While most attention has been paid to purging leukemic blasts from normal bone marrow (NBM) (1-3), much less work has been done with lymphoma purging. Over 40,000 new non-Hodgkin's lymphoma cases are diagnosed annually (4) and a majority of these patients will have bone marrow metastases (5-7). Bone marrow contamination excludes these patients from undergoing ABMT after high-dose chemotherapy—a potentially curative treatment (8). An effective in vitro purging regimen is needed to make this therapy available to patients whose best other hope for cure is allogeneic marrow transplant with its limitations of matched-donor availability and graft versus host disease (9). Many approaches have been investigated to purge the marrow of contaminating tumor cells, including immunologic (10-12), pharmacologic (1), physical separation (13,14), and combination techniques (15,16), among others (17-19).

We chose to evaluate in vitro purging using methylprednisolone (MP). Steroids act on cells in several ways: they control the rate of protein synthesis in lymphoid cells (20), impair antigen recognition and cell-to-cell cytotoxicity by lymphocytes (21), suppress interleukin-2 and γ-interferon production, and suppress T-cell proliferation and the generation of cytotoxic T-cells (22). Steroid hormones and their derivatives are used in virtually all combination chemotherapy regimens for both Hodgkin's and non-Hodgkin's lymphomas (23,24). MP possesses antilymphocytotoxic activity in vivo with minimal toxicity to NBM (25).

In the initial study treating NBM and Daudi cells in parallel, soft-agar cultures were used to assess NBM toxicity, and one-week liquid cultures were used to assess the effects on clonogenic Daudi cells. For the liquid cultures, both control and treated cells were put into Daudi cell growth media and growth was monitored over a one-week period by cell counts and trypan blue percent viability. These cultures permitted assessing the clonogenic potential of the malignant cells as compared to the control cultures. These assays were carried out after MP treatment alone or after MP treatment followed by cryopreservation

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Editor's Note: Some technical details have been omitted in the interest of brevity. Those interested in further technical information should communicate with the authors.

and thawing. The cryopreservation step allowed us to evaluate any additional kill of the MP-weakened Daudi cells from the stress of the freezing/thawing process. Once an optimal treatment protocol was formulated, we proceeded to test the treatment without cryopreservation on a simulated contaminated marrow (SCM). Our analysis at this stage was via determination of colony-forming units–granulocyte macrophage (CFUs-GM) for NBM precursors and semi-quantitation by flow cytometric immunoanalysis for residual Daudi cells utilizing dual CD10/CD20 antigens.

Methods

Normal Bone Marrow: Bone marrow was obtained after informed written consent from various oncology patients undergoing the procedure as part of their staging workup. Bone marrow was collected onto tissue culture media with preservative-free heparin, diluted with RPMI 1640, and passed over ficoll (1.077 g/cm^3) to isolate the mononuclear cells for treatment.

Cell Line: Daudi Burkitt lymphoma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 with 10% fetal calf serum (FCS) with twice weekly passages. The characteristics of this cell line have been described previously (26). Cells were used in log phase of division, two to three days after cutting back and feeding.

Drugs: MP was obtained from the Upjohn Company (Kalamazoo, MI), and all dilutions were made with RPMI using 5% FCS immediately prior to treatment of cells.

Treatment: MP was used at a concentration of 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/mL and for treatment times of 2, 4, 8, and 24 hours. All cellular concentrations during treatment were 5×10^6 cells/mL. Specimens were incubated for the treatment period at 37° C with 5% CO_2 and no shaking after the initial mix. After treatment, specimens were centrifuged, diluted, and plated in soft-agar cultures. Daudi cells were processed similarly, then placed in 10 mL of RPMI plus 10% FCS for 7-day liquid culture analysis.

NBM committed progenitor assays

CFU-GM: Using an inverse microscope, colonies of 40 or more cells were scored on day 14 of the plated cultures of NBM cells employing a modification of the technique previously described (27).

Mixed Progenitor Assay: CFU-GM, colony-forming unit–granulocyte, erythroid, macrophage, megakaryocyte, and burst-forming unit–erythroid were assessed simultaneously in a methylcellulose matrix system by an established method (28).

Liquid Culture: Daudi clonogenicity or death was assessed over 7 days while the liquid cultures were maintained at 37° C with 5% CO_2. Cell counts and percent viability by trypan blue dye exclusion test were determined manually at time points 1 hour and days 1, 3, and 7 posttreatment. Cultures were considered negative for viable cells when 1,000 scanned cells were nonviable on day 7.

Cryopreservation: Some Daudi samples were treated as described, then cryopreserved, quickly thawed, and placed in a liquid culture for 7-day analysis as described above.

SCM Treatment: NBM mononuclear cells were isolated over ficoll. After counting, 30% Daudi cells were added to simulate a contaminated marrow. Treatment with 2.0 mg/mL MP for 4 hours was carried out at 37° C/5% CO_2 and processed as before. A sample was taken for cell count and soft-agar culture plating, and the remaining cells were put into liquid culture for 24 hours before further analysis. After 24 hours, cell counts for viability were performed on a portion of this specimen, and the remainder was prepared for flow cytometric immunoanalysis.

Labeling for Flow: The dual tags CD10 with fluorescein isothiocyanate and CD20 with phycoerythrin were used to label the Daudi cells in the SCM. These antigenic markers are found on more mature cells and therefore produce little marking on the NBM cells. The treated SCM was washed twice with phosphate-buffered saline and subsequently passed over ficoll to remove all dead cells. Cells at the interface were labeled according to the manufacturer's instructions (Becton-Dickinson, Mountainview, CA).

Flow Cytometric Analysis: The samples were analyzed on a Coulter Epics V system flow cytometer (Coulter Electronics, Inc, Hialeah, FL) with a Coherent Innova 90-5 Ion Argon laser. The photo multipliers were calibrated using dual-stained beads; red and green fluorescence peaks were put into consistent channels before each run. Two-dimensional graphs of log red fluorescence versus log green fluorescence were used to analyze the specimens for residual Daudi cells, which were dual-stained.

Statistical Analysis: The parallel studies (NBM and Daudi) were individually analyzed using the Bonferroni multiple comparison analysis method of two-sided paired *t* tests. Flow cytometric data were used to calculate log reduction but were not statistically analyzed due to its semi-quantitative nature. Normal progenitor recovery, calculated as mean ± SD from all plates for each treatment point, served as control values.

Results

Parallel studies

Effect on NBM suspension—Dose, time-course response curves of MP were established for NBM cells using the soft-agar culture assay CFU-GM as the gauge for the system (Fig 1). Duration of treatment was more critical than drug concentration as represented by the three curves. A 4-hour exposure to MP did not produce significant CFU-GM depletion at any drug concentration. The 8-hour treatment produced significant depletion of colonies at the 1.5 mg/mL concentration; the 24-hour treatment data are more variable due to a decrease in the number of colonies in the 24-hour control specimen.

Effect on Daudi Burkitt's lymphoma cell line—Identical treatments were performed on the Daudi cells, and cell counts with trypan blue dye exclusion viability done periodically during one-week liquid culture. Survival was compared to that of control cells at day 7 after treatment. A statistically significant depletion in Daudi cells was seen at all levels and time courses *except* 4 hours with 0.25 or 0.50 mg/mL MP (Fig 2).

The best differentiation between Daudi cell death and CFU-GM preservation was achieved with a drug concentration of 2.0 mg/mL MP with treatment for 4 hours. This drug regimen fol-

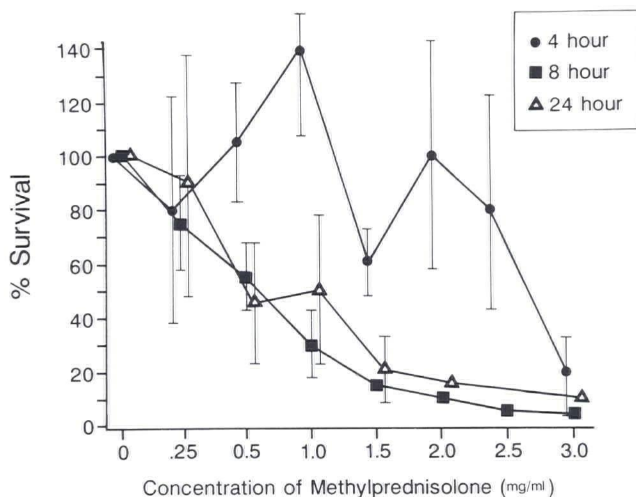

Fig 1—Percent survival of CFU-GM colonies in bone marrow specimens treated with different concentrations and for different time courses with methylprednisolone. Bars represent standard deviation (n = 3). Where there are no bars the SD was less than or equal to 10%.

Table
Effect of Methylprednisolone Treatment With and Without Cryopreservation on Normal Human Progenitors*

Specimen	CFU-GM	BFU-E	CFU-GEMM
Non-cryopreserved control†	23 ± 4 (100%)	162 ± 58 (100%)	5 ± 2 (100%)
Non-cryopreserved MP-treated‡	24 ± 5 (104%)	74 ± 61 (46%)	4 ± 2 (80%)
Cryopreserved control†	18 ± 3 (78%)	107 ± 40 (66%)	5 ± 1 (100%)
Cryopreserved MP-treated‡	16 ± 4 (70%)	58 ± 36 (36%)	4 ± 1 (80%)

*Methylprednisolone treatment = 4.0 mg/mL for 4 hours.
†N = 3.
‡N = 7.
Note: CFU per 2 x 10^5 cells.
Numbers in parentheses indicate percent of non-cryopreserved control.
CFU-GM = colony-forming unit–granulocyte macrophage; BFU-E = burst-forming unit–erythroid; CFU-GEMM = colony-forming unit–granulocyte, erythroid, macrophage, megakaryocyte; MP = methylprednisolone.

lowed by cryopreservation was then used in the Daudi treatment and the SCM treatment experiments.

Treatment plus cryopreservation—Cryopreservation following treatment with 2.0 mg/mL of MP for 4 hours was found to increase the log kill of Daudi cells from an average of 1.7 (range 1.5-1.9) to 2.3 logs (range 2.0-2.6). This effect is thought to be due to the increased stress on MP-weakened cells. NBM progenitor recovery was also determined postcryopreservation (Table). We performed the mixed-progenitor assay in methylcellulose to ascertain any selective depletion of one type of progenitor.

SCM studies—In the SCM experiment we were again able to recover CFU-GM colonies posttreatment. Flow cytometric immunoanalysis yielded a distinct population of CD10/CD20-labeled Daudi cells. Normal marrows had less than 1.5% positive dual-staining cells, and this background amount was subtracted from each SCM control (SCMC) and SCM treatment (SCMTX) specimen (Fig 3). These background-corrected percentages were multiplied by the 10,000 cells counted per sample to compute the actual number of Daudi cells per 10,000 in both the SCMC and SCMTX specimens (Fig 4). Log reduction was calculated by the equation:

$$\log \left\{ \frac{\#\text{Daudi cells in SCMC} - \#\text{background}}{\#\text{Daudi cells in SCMTX} - \#\text{background}} \right\}$$

The average log reduction in these samples assayed 24 hours posttreatment was 1.4 logs. We allowed an intervening 24-hour period between treatment and flow cytometry due to the slow nature of cell death following treatment with MP. The largest decline in cellular viability occurs in the first 24 hours following treatment.

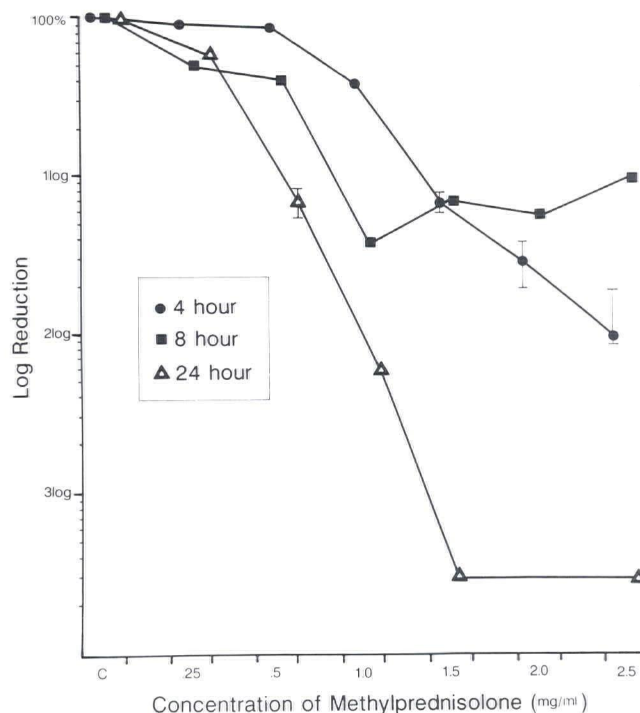

Fig 2—Daudi cell death as compared to the control after seven days in culture following the specified treatment regimens with methylprednisolone.

Discussion

There is clearly a need for a selective and effective method of purging contaminating tumor cells from bone marrow while pre-

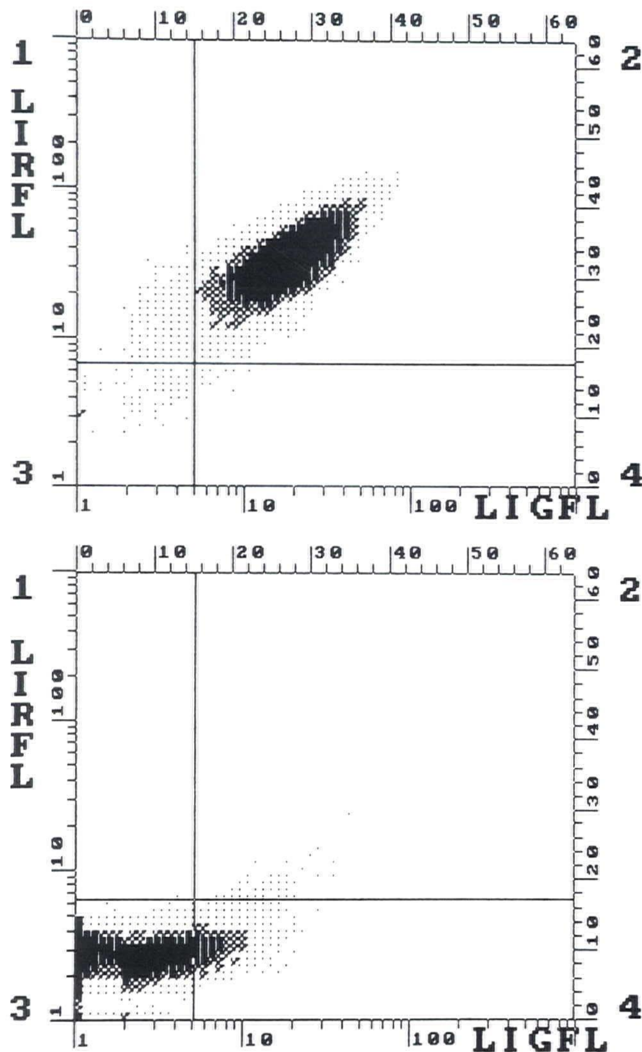

Fig 3—CD10/CD20 staining of Daudi cells and normal bone marrow. LIRFL = log integrated red fluorescence of the phycoerythrin-labeled CD20 monoclonal antibody; LIGFL = log integrated green fluorescence of the fluorescein isothiocyanate-labeled CD10 monoclonal antibody. Quadrant #2 is where any dual-stained, CD10 and CD20 dual-positive, cells will be detected. Top: Daudi cells dual-staining over 95%. Bottom: Normal bone marrow stained with same reagents showing less than 1.5% background dual-staining population.

serving the capability for engraftment. Chemopurging agents without myelosuppressive activity are the most promising means of in vitro purging. Many steroid hormones and their derivatives known for lymphocytotoxicity and lack of myelosuppression are used in combination chemotherapy for lymphoma (24).

Our study entailed two phases. First, parallel studies on NBM and Daudi Burkitt lymphoma cells were performed to determine the efficacy of MP to kill the tumor cell line selectively. The studies demonstrated the effect of treatment alone and treatment coupled with subsequent cryopreservation. Marrow mononuclear cells and Daudi cells were treated in parallel with concentrations of the drug ranging from 0.25 to 3.0 mg/mL for a duration of 4, 8, or 24 hours. Daudi cell survival was evaluated by periodic cell counts with trypan blue viability using one-week liquid cultures. MP demonstrated optimal selectivity for purging Daudi lymphoma cells without causing significant damage to NBM precursors using a treatment protocol of 2.0 mg/mL for 4 hours. This protocol eliminates an average 1.7 logs of Daudi cells when used alone and an average 2.3 log reduction when followed immediately by cryopreservation and thawing, while there is little detectable damage to NBM precursors as measured by CFU-GM assays. Unlike the murine model, there is no "stem cell" assay for human bone marrow. Therefore, it is difficult to assess the engraftment potential of any purged marrow short of actual in vivo hematopoietic reconstitution. We used in vitro soft-agar culture assays for committed hematopoietic progenitors to appraise the NBM cell status. CFU-GM assay results have correlated with engraftment potential in some studies (29), while no correlation was found in others (30). For example, using in vitro, high-dose (80 μg/mL or higher) 4-hydroperoxycyclophosphamide purging, hematopoietic reconstitution was produced in vivo, although no CFU-GM colonies were recovered (31).

The second phase of this study involved using our protocol on a SCM, a mixture of 70% marrow mononuclear cells and 30% Daudi cells. This mixed-cell system was evaluated by the CFU-GM assay and semi-quantitation by flow cytometric immunoanalysis for the dual markers CD10 and CD20, which are found on the Daudi cells but rarely together on NBM cells. The CD10 and CD20 markers are found together only on the pre-B lymphocyte stage of normal cells. Once the cell differentiates into a resting B-cell, it loses the CD10 cluster antigen, and when activated it is negative for both CD10 and CD20. The Daudi cells, however, stain uniformly CD10+/CD20+. This unusual combination of antigens makes flow cytometry an attractive method for evaluating purging efficiency. Our initial results were reproducible in the more realistic setting of SCM. The minimum average reduction of Daudi cells in these SCM samples 24 hours posttreatment (to allow for slow cell death due to the treatment) was 1.4 logs as detected by flow cytometry, with no significant loss of NBM committed precursors as detected by CFU-GM. A longer time course could not be studied with this system due to the demise of NBM in liquid culture. The sensitivity of flow cytometric immunoanalysis is approximately 1% of a population of cells. The percentages become firmer as we increase the number of cells counted, but the sensitivity of the method does not increase. Using flow cytometry, we were able to utilize an authentic model of tumor-involved marrow; the malignant cells in SCM were not altered in any way prior to admixing with NBM.

More aggressive regimens should be explored, including combination treatments of MP with other synergistic drugs. Trials with actual patient bone marrow with contaminating tumor are anticipated. Such marrow can be evaluated for residual tumor cells using other sensitive methods, e.g., prelabeling with Hoestch 33342 stain (32,33) or limiting dilution analysis (34). Polymerase chain reaction could be applied as a very sensitive

method to detect residual occult malignant cells (35,36). This application, however, would be restricted to detecting those tumors with a known, consistent genetic marker.

Acknowledgment

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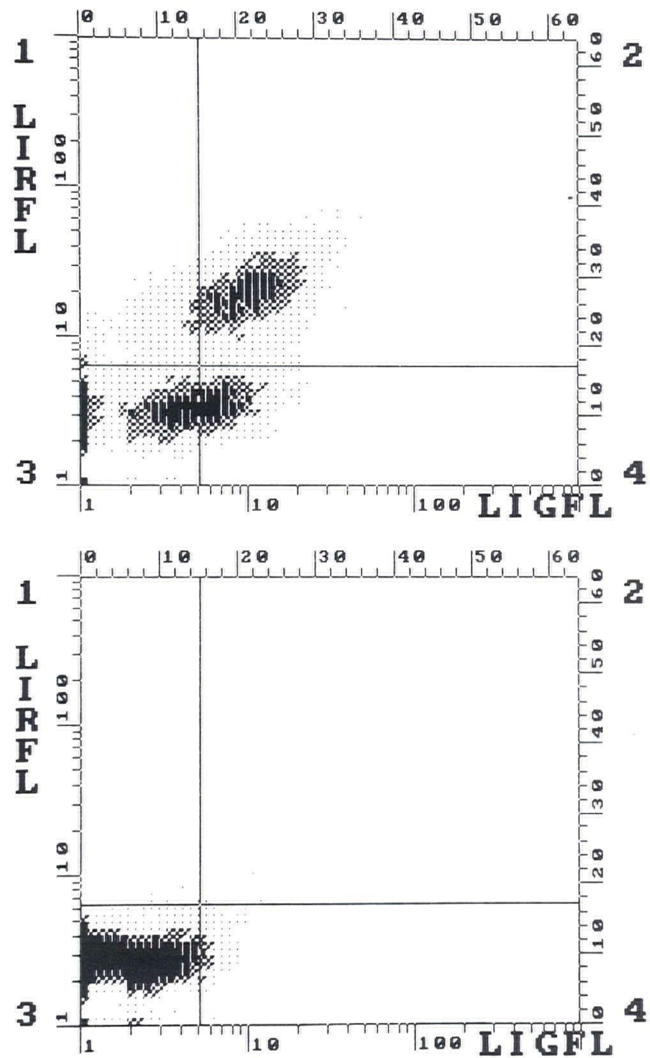

Fig 4—Flow cytometric analysis of methylprednisolone purge of a simulated contaminated marrow (SCM). Each SCM was split: one half serving as the control (SCMC) and the other half as the treated specimen (SCMTX). After treatment and processing, the specimens were put into liquid culture for 24 hours to allow cells affected by the treatment to begin to die. Top: This untreated SCMC has 34% Daudi cells as detected in quadrant #2. Bottom: The other half of this specimen after exposure to methylprednisolone has less than 1% Daudi cells (which is negligible in this sample due to the 1% background dual-staining cells in this marrow).

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