

Myeloablation With Diaziquone: In Vitro Assessment

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The promising antineoplastic agent diaziquone is associated with prolonged aplasia and rare instances of bone marrow necrosis, but only mild extramedullary toxicity. To explore the drug's potential as a myeloablative agent prior to bone marrow transplantation, we compared its effects on hematopoietic versus marrow stromal cells. After short-term (one to six hours) or prolonged (three to seven days) exposure to the drug, marrow was assayed for hematopoietic (CFU-Mix, BFU-E, CFU-GM) and stromal (CFU-F) colony-forming cells and studied in long-term marrow culture (LTMC). One- and three-hour treatments produced little cytotoxicity, even at 5000 ng/mL. After six-hour treatments with this dose, marrow was depleted of CFU-Mix, BFU-E, and CFU-GM, but produced CFU-GM in LTMCs, indicating an ongoing input of CFU-GM from a surviving pre-CFU-Mix population. In contrast, elimination of the latter may be inferred from the absence of CFU-GM in LTMCs exposed for three to seven days to diaziquone at

DIAZQUONE (AZQ, NSC 182986) is a lipophilic quinone that was rationally synthesized to optimize blood-brain barrier penetration.¹ Its antineoplastic effect²⁻⁶ has been attributed to alkylating activity.⁷ Its slow uptake by leukemic cells⁸ but short plasma half-life^{1,3,9-11} led to phase I/II trials of prolonged infusions.⁶ Pancytopenia, not extramedullary toxicity, was the dose-limiting factor. This observation suggested that diaziquone may be best exploited in the context of bone marrow transplantation. Bone marrow necrosis, however, occurred in two of three patients given maximal doses, raising concern that diaziquone may damage the hematopoietic microenvironment and thereby jeopardize engraftment.¹² We investigated this issue by using clonal assays and long term marrow cultures (LTMCs) to correlate the drug's ablative capacity on hemopoietic cells with its effects on marrow stromal cells. Our studies underscore the usefulness of LTMCs for investigating drug effects on hematopoietic and nonhematopoietic components of marrow, demonstrate that diaziquone's cellular kinetics render LTMCs particularly appropriate for defining the effects on human marrow of prolonged exposure to this drug, and support the pretransplant use of diaziquone in myeloablative doses.

MATERIALS AND METHODS

Bone Marrow Cells

Heparinized marrow aspirated from the iliac crests of healthy volunteers who gave informed, written consent were centrifuged at 200 g for ten minutes. The buffy coat cells were collected and washed. For some experiments, marrow was fractionated by agglutination with soybean lectin and by rosetting with sheep erythrocytes to isolate a target population (SBA-E-cells) enriched for hematopoietic cells and 97% to 99+%-depleted of stromal cells¹³ and T cells.¹⁴

Diaziquone Preparation and Drug Exposure

Diaziquone was provided in 10-mg vials by the National Cancer Institute. It was reconstituted in *N,N*-dimethylacetamide and phosphate buffer saline immediately before use. Further drug dilutions

only 150 ng/mL. Under these conditions, CFU-F recovery was 40% and adherent stromal layers in LTMCs were similar to untreated controls regarding rate of development and cellular composition. Our in vitro pre-CFU-Mix-ablative regimen correlates with clinical data that show prolonged but reversible myelosuppression at steady-state diaziquone plasma levels of 101 ± 10 ng/mL (mean \pm standard error of mean) during 7-day constant infusions. In conclusion: (a) hematopoietic cells are more sensitive than marrow stromal cells to the dose- and highly time-dependent cytotoxicity of diaziquone, (b) a direct drug-induced noxious effect on the marrow microenvironment is an unlikely cause of the isolated episodes of marrow necrosis after the use of diaziquone in vivo, and (c) prolonged infusion of diaziquone represents an attractive means for achieving myeloablation in selected clinical situations.

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were prepared with the medium appropriate for the specific culture technique being used. When reconstituted in solution maintained at pH 7.0, diaziquone has a half-life of 17 days.¹⁵

Marrow buffy coat cells in alpha-medium (Flow Laboratories, Hamden, CT) with 10% heat-inactivated fetal calf serum (FCS) were incubated at 5×10^6 cells/mL with graded doses of diaziquone for 1, 3 or 6 hours at 37°C in a humidified 5% CO₂ atmosphere, and washed three times prior to use. In experiments examining the effects of prolonged drug exposure, marrow cells were resuspended and cultured in medium containing diaziquone in the amounts needed to achieve the desired drug concentration.

Culture Techniques

Multilineage and erythroid colony-forming cells. Mononuclear cells were recovered from untreated or diaziquone-treated marrow buffy coat cells by neutral density centrifugation in an isotonic sterile Percoll solution, 1.074 g/mL, 270 mosm (Pharmacia, Piscataway, NJ). These cells or SBA-E-cells were cultured¹⁶ in quadruplicate in 35-mm dishes (Lux Scientific Co, Newburg, CA) at 1.0×10^5 /mL of 1% methylcellulose in Iscove's modified Dulbecco medium with 30% human plasma, 10% phytohemagglutinin-stimulated leukocyte-conditioned medium, 5×10^{-5} mol/L 2-mercaptoethanol, and one unit of human recombinant erythropoietin (AMG-

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en, Thousand Oaks, CA). CFU-Mix and BFU-E were scored on day 14.

Granulocyte-macrophage colony-forming cells. Marrow cells were cultured as described¹⁷ in quadruplicate in 35-mm dishes (Corning Glass Works, Corning, NY) at 2×10^5 /mL of 0.3% agar in supplemented McCoy's 5A medium and 10% FCS. In each dish, 0.1 mL of medium conditioned by the 5637 bladder carcinoma cell line stimulated colony formation.¹⁸ CFU-GM (more than 50 cells/aggregate) were scored on day 7.

Fibroblast colony-forming cells. Marrow buffy coat cells (5×10^6) in 20 mL alpha-medium with 20% FCS were set up in T-75 flasks (Corning) that were gassed with 5% CO₂ in air and kept at 37°C. Medium was changed on day 4 in cultures of cells preincubated with diaziquone for 1, 3, or 6 hours. In cultures exposing cells to diaziquone for three or seven days, the initial medium contained graded amounts of diaziquone but was replaced by drug-free medium on day 3 in one set of experiments, and on day 7 in another set. Cultures were stopped on day 10, stained with Wright-Giemsa solution, and scored for CFU-F (more than 50 cells/aggregate).¹⁹

Long-term marrow cultures. Marrow buffy coat cells (20×10^6) suspended in 10 mL medium were inoculated into T-25 flasks (Corning) that were gassed with 5% CO₂ in air, and kept at 37°C. The medium consisted of supplemented McCoy's 5A medium,¹⁷ 12.5% horse serum, 12.5% FCS, and 10^{-6} mol/L hydrocortisone sodium succinate, with or without graded amounts of diaziquone, depending on the experimental design. On day 3 in one set of experiments and on day 7 in a separate set, supernatants were purged of erythrocytes and mature myeloid cells by Percoll separation; the mononuclear cells were returned to their flasks in fresh, drug-free medium. LTMCs were then maintained at 33°C. Half of the supernatant was removed weekly and the cells in suspension were assayed for CFU-GM. At week 3, adherent layers in some flasks were suspended¹⁷ using a nontoxic 0.01% (wt/vol) Ca⁺⁺- and Mg⁺⁺-free trypsin (GIBCO, Grand Island, NY) solution to determine their CFU-Mix, BFU-E, and CFU-GM content.

To monitor stromal layer development, the extent of flask coverage by an interlocking network of cells was estimated weekly by phase microscopy. Cellular composition was assessed in situ by immunofluorescence methods.¹⁷ Fibroblasts were identified by rabbit antibodies to fibronectin (Bethesda Research Lab, Gaithersburg, MD) and to collagen type III (Dr H Furthmayr, Yale University). Endothelial cells and macrophages were identified by mouse monoclonal antibodies (Australian Monoclonal Development, Antarmon, Australia) to factor VIII-related protein and to human monocytes/macrophages, respectively. Adipocytes were distinguished by their morphology.

Statistical Analysis

With the Minitab package program (Duxbury Press, Boston), regression analyses were performed for the clonogenic assays using the log of the percent of the control as the dependent variable and the diaziquone dose as the independent variable. Lines were forced through the value of the log of 100% at dose zero. Slopes of the fitted lines were compared based on F-tests computed from residual sums of squares.

RESULTS

Effect of Diaziquone on Marrow Colony-Forming Cells

Diaziquone exerted dose- and time-dependent killing of clonogenic hematopoietic and stromal cells (Fig 1). One-hour treatments did not significantly affect recovery of these

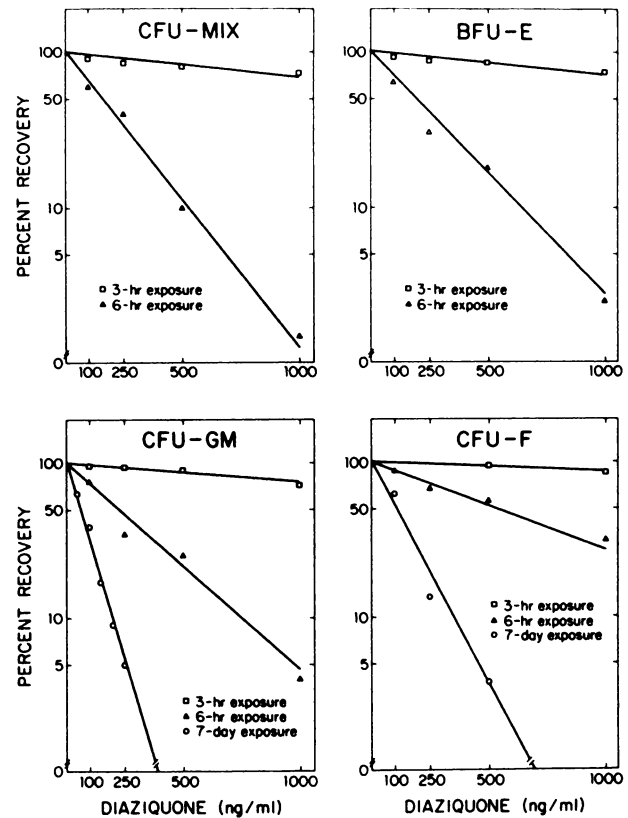


Fig 1. Survival curves of marrow hematopoietic and stromal progenitors. Results shown are the mean (SEM less than $\pm 10\%$) of three (CFU-Mix, BFU-E) and four (CFU-GM, CFU-F) experiments at each exposure time. Controls were: CFU-Mix, 19 ± 2 per 4×10^5 cells; BFU-E, 94 ± 9 per 2×10^6 cells; CFU-GM, 91 ± 7 per 10^5 cells; and CFU-F, 131 ± 15 per 5×10^5 cells. Note the marked time-dependency of diaziquone cytotoxicity on all four cell types.

cells, even at high drug concentrations (500 to 5,000 ng/mL, data not shown). With longer treatments, increasing doses caused an exponential decrease in progenitor recovery. Three-hour treatments with 1,000 ng/mL produced approximately 30% reductions in CFU-Mix, BFU-E, and CFU-GM survival, and 10% to 15% decrease in CFU-F. With 6-hour treatments, doses that inhibited 50% (ID₅₀) of CFU-Mix, BFU-E, and CFU-GM were 160 ng/mL, 200 ng/mL, and 245 ng/mL, respectively, which were significantly lower than the ID₅₀ of 530 ng/mL for CFU-F ($P = .001$). CFU-Mix and BFU-E did not differ significantly in sensitivity to diaziquone ($P = .15$), but these progenitors were significantly more sensitive than CFU-GM ($P = .005$). After 6-hour exposure to 5,000 ng/mL, hematopoietic progenitors were not detected, but CFU-F recovery was 10%.

When marrow buffy coat cells were plated with graded amounts of diaziquone in the culture medium, ie, 7-day constant exposure, the ID₅₀ of CFU-GM formation was 60 ng/mL, and CFU-GM recovery was 5% at 250 ng/mL (Fig 1). To ascertain whether this inhibition was a direct effect on hematopoietic cells or was mediated via effects on accessory "helper" cells, SBA-E-cells were cultured under the same constant-exposure conditions. Survival curves of CFU-GM

matched those observed using buffy coat cells (data not shown).

When marrow buffy coat cells were cultured for CFU-F with graded amounts of diaziquone in the medium for seven days, ie, 7-day constant exposure, the ID_{50} of CFU-F was 110 ng/mL (Fig 1). Similar results—CFU-F ID_{50} of 145 ng/mL—were obtained with 3-day constant exposures (data not shown).

Effect of Diaziquone on LTMCs

Diaziquone dosage and length of treatment significantly affected LTMCs regarding CFU-GM production, the rate of development, and the cellular composition of adherent layers. Marrow suspensions treated for one or three hours (Fig 2) with doses of diaziquone up to 5,000 ng/mL and then set up in LTMCs produced CFU-GM for five to six weeks and gave rise to adherent stromal layers that, similar to controls, reached confluence within three to four weeks and contained fibroblasts, macrophages, adipocytes, and rare endothelial cells (Table 1).

Treatments for six hours (Fig 3) caused more marked dose-dependent reductions in CFU-GM yields. In particular, following 6-hour treatments with very high doses of diaziquone, eg, 5,000 ng/mL, marrow suspensions that were depleted of CFU-Mix, BFU-E, and CFU-GM, as measured by direct assay in semisolid medium, generated CFU-GM for five weeks in LTMCs. Six-hour treatments with diaziquone up to 500 ng/mL did not adversely affect stromal layer development or composition (Table 1) as compared to controls; at 1,000 ng/mL, however, confluence was very delayed, and at 5,000 ng/mL confluence was not attained.

LTMCs exposed to diaziquone for three or seven days (Fig 4) at doses up to 100 ng/mL generated CFU-GM for five or more weeks. Doses of 150 ng/mL or greater abolished recovery of CFU-GM from either the nonadherent or the 3-week-old adherent cellular compartments of LTMCs. Prolonged exposure to doses of diaziquone up to 250 ng/mL retarded the development of confluent stromal layers but did not alter the heterogeneous cellular composition as compared to untreated controls (Table 1). After prolonged exposures to 500 ng/mL, confluence did not develop; only fibroblasts and

adipocytes were identified. Higher doses resulted in occasional-to-rare fibroblastic areas.

DISCUSSION

Our studies demonstrate differential sensitivities to diaziquone of hematopoietic as compared to marrow stromal cells, highlight the time-dependency of diaziquone's cytotoxic activity, and support using prolonged, myeloablative infusions of diaziquone in conjunction with bone marrow transplantation.

One- and three-hour treatments with up to 5,000 ng/mL diaziquone had little effect on hematopoietic progenitors, whereas 7-day exposure to doses as low as 50 ng/mL had a significant cytotoxic impact on these cells. Data from CFU-Mix, BFU-E, and CFU-GM, however, may not apply to the pluripotent stem cell that constitutes the target of definitive myeloablative procedures prior to bone marrow transplantation. Differences in drug effects on committed versus pluripotent hematopoietic cells are inferred from the *in vivo* hematologic regenerative capacity of harvested marrow depleted of CFU-Mix, BFU-E, and CFU-GM by purging with 4-hydroperoxycyclophosphamide (4-HC).^{20,21} Direct measurement of the pluripotent stem cell is not now feasible in humans, but LTMCs may provide an indirect means for detecting hematopoietic cells that are more primitive than CFU-Mix. We observed sustained production of CFU-GM in LTMCs initiated with marrow completely depleted of CFU-Mix, BFU-E, and CFU-GM by 6-hour treatments with 5,000 ng/mL diaziquone. These findings, similar to results with anti-Ia antibody,²² 4-HC,²³ and VP-16,^{24,25} indicate an ongoing input of CFU-GM from a surviving, pre-CFU-Mix population. In sharp contrast, CFU-GM were not recovered from LTMCs following three- or seven-day exposures to 150 ng/mL diaziquone. These data support an ablative effect on pre-CFU-Mix of prolonged exposure to low doses of this drug.

Although eliminated from LTMCs exposed for three to seven days to 150 ng/mL diaziquone, CFU-GM are detectable at seven days when marrow is cultured in semisolid medium containing doses up to 250 ng/mL. These seemingly inconsistent findings are not attributable to diaziquone

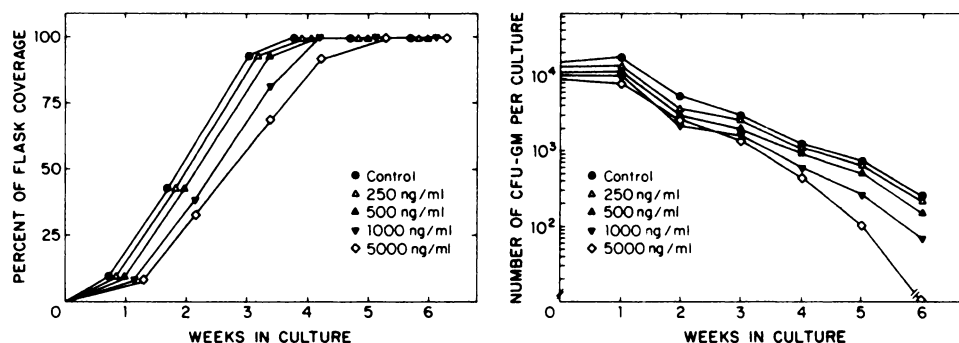


Fig 2. Effects of 3-hour treatments with diaziquone on LTMC stromal layer development (left) and CFU-GM generation (right). Note the relatively minor differences in these parameters over a wide range of diaziquone dosages. Results shown are the mean of three experiments.

Table 1. Composition of Stromal Layers in LTMCs Derived From Diaziquone-Treated Bone Marrow

Length of Exposure	250 ng/mL				500 ng/mL				1,000 ng/mL				5,000 ng/mL			
	F	M	Ad	EC	F	M	Ad	EC	F	M	Ad	EC	F	M	Ad	EC
3 hr	4+	2+	3+	1+	4+	2+	3+	1+	4+	2+	3+	1+	3-4+	1-2+	2+	±
6 hr	4+	2+	3+	1+	4+	2+	3+	1+	4+	1+	1-2+	0	2-3+	0	1+	0
3 days	4+	2+	3+	0-1+	2-3+	0	1-2+	0	1+	0	0	0	0-1+	0	0	0

The presence and relative amount of different types of adherent stromal cells was semiquantitatively assessed. Control stromal layers derived from 20×10^6 untreated marrow buffy coat cells exhibited the following composition: F, 4+; M, 2+; A, 3+; EC, ±-1+.

F, fibroblast; M, macrophage; Ad, adipocyte; EC, endothelial cell.

effects on accessory cells, since CFU-GM survival curves using buffy-coat cells or stromal-cell- and T-cell-depleted SBA-E-marrow fractions overlap. Diaziquone's cellular kinetics provide a more convincing explanation. Slow cellular uptake¹⁸ rather than cell-cycle-specificity²⁶ accounts for the time-dependency of diaziquone's cytotoxicity. When marrow is assayed for CFU-GM by direct plating in semisolid medium containing diaziquone (ie, constant 7-day drug exposure) progeny—appearing as colonies of cells—are generated before sufficient drug is accumulated to produce a cytotoxic effect on the progenitor cell. In contrast, when the effects of prolonged drug exposure are studied using LTMCs, drug uptake proceeds for several days, reaching cytotoxic intracellular levels, before hematopoietic progenitor cell assays are performed. In assessing the cytotoxicity of prolonged diaziquone exposure, therefore, we view the LTMC data as more reflective of the *in vivo* situation than the data from semisolid medium assays. In fact, the hematopoietic-stem-cell ablative diaziquone regimen defined in our system—3-day exposure to 150 ng/mL—correlates with clinical experience⁶: prolonged but reversible myelosuppression followed seven-day constant infusions of diaziquone that produced steady-state plasma concentrations of 101 ± 10 ng/mL (mean \pm SEM; range 54-150 ng/ml). Presumably, higher drug levels (as in our system) cause myelosuppression that is unacceptable in the absence of bone marrow rescue.

Bone marrow necrosis in two of three patients treated by prolonged infusions of maximal doses of diaziquone⁶ raises the issue of drug-induced damage to the marrow microenvironments.¹² Such an effect might block hematologic recovery posttransplantation of bone marrow and would make this method of diaziquone administration unsuitable for pretrans-

plant use. Our *in vitro* hematopoietic-stem-cell ablative diaziquone regimen is relatively sparing of marrow stromal progenitors, as shown by the 40% recovery of CFU-F. Moreover, adherent layers that formed in LTMCs after prolonged treatments with up to 250 ng/mL of diaziquone attained confluence and resembled untreated controls regarding heterogeneity of cellular composition. We interpret these data as supporting the analysis⁶ that other factors, eg, vessel occlusion secondary to rapid and extensive cell lysis within a markedly hypercellular marrow,²⁷ caused the necrosis. Additional support for this view comes from radiation data. *In vitro* radiation at 480 to 840 cGy (depending on the dose rate) abolishes human CFU-F recovery and inhibits LTMC stromal layer development,^{28,29} yet patients irradiated with up to 1,440 cGy routinely engraft with bone marrow transplants,³⁰ indicating an intact marrow microenvironment. It may be deduced that since (relative to their respective myeloablative dosages) diaziquone is much less toxic *in vitro* than radiation to marrow stromal cells, a similar situation prevails *in vivo*.

Total body irradiation and busulfan, the conventional methods of myeloablation prior to bone marrow transplantation, have overlapping pulmonary toxicities³¹ that make it risky to use both modalities in the same patient within a short period of time. Yet, repeat or intensified myeloablative measures may be indicated (a) to prepare patients for a second transplant,^{32,33} (b) to treat refractory acute leukemias, or (c) to ablate the expanded, abnormal primitive-stem-cell compartments of disorders such as juvenile chronic myeloid leukemia (JCML) or blastic-phase chronic myelogenous leukemia (Ph⁺ CML).³⁴⁻³⁶ Intensive diaziquone regimens—25 to 35 mg/m²/d \times 5 to 7 days by bolus

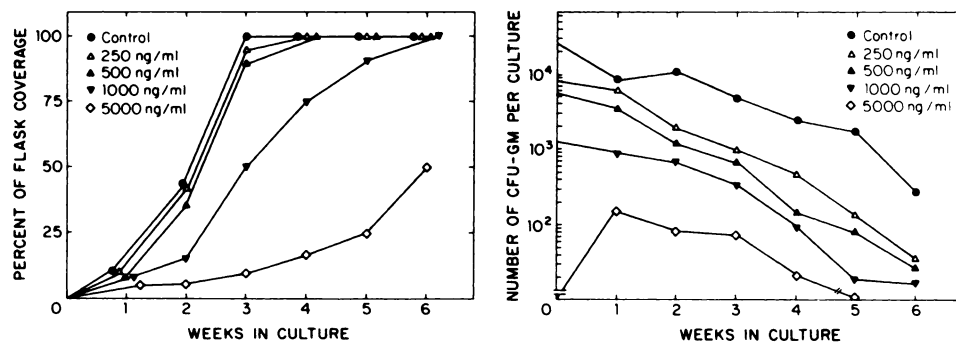


Fig 3. Effects of 6-hour treatments with diaziquone on LTMC stromal layer development (left) and CFU-GM generation (right). Results shown are the mean of three experiments. Note the prolonged generation of CFU-GM in LTMCs derived from marrow suspensions that at the time of LTMC initiation were depleted by treatments with 5,000 ng/mL of directly measurable hematopoietic progenitors.

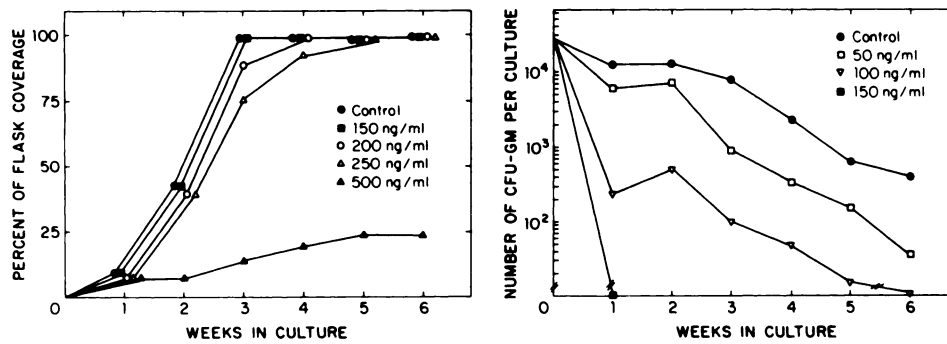


Fig 4. Effects of 3-day exposures to diaziqnone on LTMC stromal layer development (left) and CFU-GM generation (right). Results shown are the mean of four experiments and are similar to those obtained with 7-day treatments. Note that prolonged treatment with 150 ng/mL abolishes CFU-GM generation but does not impair stromal layer development.

injections^{4,5} or by constant infusion⁶—are effective in clearing blasts from patients with refractory or relapsed acute nonlymphocytic leukemias and from patients with panmyelopathies such as JCM, Ph⁺ CML, and secondary leukemia. These results have been clouded, however, by prolonged, life-threatening aplasia. No significant extramedullary toxicity, pulmonary in particular, has been seen at doses used thus far.^{6,37} Our data indicate that a diaziqnone regimen adjusted to maintain plasma levels of approximately 150 ng/mL for three to seven days is myeloablative without undue toxicity to the marrow microenvironment. Taken

together, these clinical and laboratory findings favor incorporation into transplant preparative protocols of this relatively nontoxic approach when, as in the clinical situations listed above, repeat or highly aggressive myelotoxic measures are desired. Since other studies demonstrate that prolonged exposure to suprapharmacologic doses of diaziqnone does not significantly suppress lymphocyte mitogenic responses significantly (Chin LJ, Kushner BH, Cunningham-Rundles S, Castro-Malaspina H: unpublished data), the use of diaziqnone in the allogeneic marrow transplant setting must be coupled with immunosuppressive measures.

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