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Development and Characterization of a Cyclophosphamide-Resistant Subline of Acute Myeloid Leukemia in the Lewis x Brown Norway Hybrid Rat

By Todd M. Koelling, Andrew M. Yeager, John Hilton, Darlene T. Haynie, and Joseph M. Wiley

Preclinical studies of resistance to alkylating agents in the Lewis x Brown Norway hybrid (LBN) rat model of acute myeloid leukemia (AML) have hitherto been limited by the sensitivity of LBN AML cells to cyclophosphamide (CY). We developed a CY-resistant subline of LBN AML by serial intravenous (IV) passage of AML cells followed by in vivo exposure to CY (100 mg/kg) 14 days later. After 18 and subsequent passages, CY-treated AML cells remained viable despite ex vivo incubation with 70 to 100 μmol/L 4-hydroperoxycyclophosphamide (4HC) or in vivo exposure to 100 to 300 mg/kg of CY. Once established, resistance to incubation with 4HC was stable in LBN AML cells after at least six serial in vivo passages without exposure to CY. Nevertheless, both control and CY-treated AML cells demonstrated similar dose-dependent sensitivity to 100 to 500 μmol/L phosphoramide mustard (PhM), the active alkylating end-product of CY activation in vivo. Levels of aldehyde dehydrogenase (ALDH), which inactivates CY by prevention of formation of PhM, were significantly elevated in these CY-resistant AML cells: cytosolic and particulate ALDH fractions from these cells were 11 to 13 times control with NAD cofactor and propanal substrate and three to four times control with NADP cofactor and benzaldehyde substrate. Further studies with this animal model of AML, in which resistance to CY is mediated by elevated ALDH activity, may elucidate mechanisms for effective elimination of drug-resistant leukemic cells ex vivo and in vivo.

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PRECLINICAL models of acute leukemia have been used to study the response of neoplastic cells to selected antileukemic agents in vitro and in vivo, and thus to predict for effective drug dosage and scheduling in clinical settings. In particular, the model of acute myeloid leukemia (AML) in the Brown Norway rat,[1-3] successfully introduced into the Lewis x Brown Norway F_1 hybrid (LBN),[4] has been studied extensively in this regard and is an authentic animal model of human AML.[1-6] The LBN AML model was used to demonstrate the efficacy of ex vivo chemotherapeutic treatment of leukemic bone marrow with the cyclophosphamide (CY) congener 4-hydroperoxycyclophosphamide (4HC),[7] and this chemopurging technique has been applied to clinical trials of autologous marrow transplantation in patients with AML.[8-10] However, the extrapolation of these studies to relapsed and potentially drug-resistant human AML is limited by the in vitro and in vivo sensitivity of LBN AML cells to CY.[11,12] To address this issue, and thus to develop alternative chemopurging strategies that may be applicable in clinical autologous marrow transplantation in relapsed and/or CY-resistant AML, we developed a subline of LBN AML made resistant to CY through chronic intermittent in vivo exposure to the drug and serial transfer of leukemic cells. In addition, we evaluated the mechanisms responsible for CY resistance in this experimental tumor system.

MATERIALS AND METHODS

Rats. Female LBN rats, age 6 to 8 weeks, were obtained from commercial sources (Harlan Sprague-Dawley, Walkersville, MD). Animals were housed in polycarbonate plastic cages and were given standard laboratory chow and tap water ad libitum.

AML cells. AML cells, originally developed in the Brown Norway rat,[1,3] were obtained from Dr Anton Hagenbeek, TNO Radiobiological Institute, Rijswijk, The Netherlands, and were successfully introduced into LBN hybrid rats.[4] The LBN AML was maintained by serial passage into healthy syngeneic animals. Briefly, each rat received an intravenous (IV) injection of 10^6 viable AML cells; florid leukemia with circulating myeloblasts and limb paralysis was apparent 17 to 20 days later. Leukemic rats were killed by CO_2 narcosis, and their femurs were removed under aseptic conditions; the marrow plugs were flushed into sterile medium (RPMI-1640; GIBCO, Grand Island, NY) with 5% fetal bovine serum (FBS; GIBCO). Single-cell suspensions of AML cells were obtained through repetitive gentle pipetting and passage through a series of sterile needles of decreasing lumen size. The hematocrit of AML suspensions thus prepared was consistently less than 1%, and morphologic examination of cytocentrifuge preparations consistently demonstrated at least 98% leukemic myeloblasts. Cell counts were made with an electronic particle counter (Model ZB I, Coulter Electronics, Hialeah, FL), and cell viability was assessed by the exclusion of trypan blue dye. Suspensions of AML cells were kept on ice until injection.

CY treatment. CY was obtained as lyophilized powder from commercial sources (Cytoxan; Mead Johnson Laboratories, Evansville, IN) and reconstituted with sterile water immediately before use. Fourteen days after IV injection of viable AML cells (10^6 cells per rat), LBN rats received a single intraperitoneal dose of 100 mg/kg of CY. Animals were observed for development of AML (eg, limb paralysis, presence of myeloblasts in peripheral blood) after injection of CY, at which time they were killed by CO_2 narcosis. Suspensions of AML cells from these CY-treated rats were then prepared as described above and injected IV into syngeneic animals. This cycle of leukemic passage, exposure to CY, and re-passage of

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AML cells appearing after CY treatment was repeated. At selected passages, suspensions of drug-exposed and control AML cells were used for studies of sensitivity to 4HC and phosphoramide mustard (PhM), and for determination of aldehyde dehydrogenase (ALDH) activity.

Ex vivo drug incubation. 4HC and PhM were obtained from Dr O. Michael Colvin, The Johns Hopkins Oncology Center, and stored in a dessicator at 4°C. Solutions of 4HC and PhM were prepared in RPMI-1640 immediately before use. Suspensions of CY-exposed or control LBN AML cells (1.5 to 2.0 × 10^6 cells/mL) were obtained from the bone marrow of leukemic rats, incubated for 30 minutes at 37°C with graded concentrations of 4HC or PhM, washed and resuspended in cold RPMI-1640, and injected into syngeneic rats (10^6 AML cells per rat IV). Recipients of AML incubated ex vivo with 4HC or PhM were then observed for development of leukemia or leukemia-free survival.

Release of Cy exposure. To determine whether resistance was stable in the absence of continued in vivo exposure to CY, a sample of CY-treated LBN AML cells obtained after the 50th passage was serially transferred in vivo for six additional passages without exposure to the drug. Leukemic cells obtained from the sixth passage were incubated for 30 minutes with 100 μmol/L of 4HC and injected into LBN rats (10^6 cells per rat), which were then observed for development of AML or for disease-free survival.

ALDH assay. Single-cell suspensions of AML cells obtained from the spleens of leukemic rats bearing either CY-treated or control AML were prepared in RPMI-1640 by teasing through a sterilized stainless-steel wire mesh (Newark Wire Cloth Co, Newark, NJ) and passage through a series of needles of decreasing lumen size. Leukemic cells were isolated by centrifugation of single-cell suspensions with Ficoll-diatrizoate (5.7 g of Ficoll 400 and 9.0 g of sodium diatrizoate/100 mL of distilled water) at 500 × *g* for 30 minutes.[13] The AML cells were collected at the medium/Ficoll-diatrizoate interface, recentrifuged, and resuspended in freezing mixture that consisted of 0.1 mol/L of sodium-phosphate buffer (pH 7.4), 1 mmol/L of EDTA, 5 mmol/L of 2-mercaptoethanol, 20 μg/mL of aprotinin, and 100 μg/mL of leupeptin (all from Sigma Chemical Co, St Louis, MO). Aliquots of AML cells in freezing mixture were placed in vials and frozen in a − 40°C freezer until time of ALDH assay. Cytosolic protein extracts of AML samples were made by repeated freeze-thawing in a mixture of dry ice and ethanol and centrifugation at 48,000 × *g* for 30 minutes; the resultant supernatants were then used as the ALDH source. Particulate fractions were prepared by resuspension of the AML cell pellet in a solution of 0.25 g/dL of Triton X-100 (Sigma), incubation for 30 minutes at 4°C, and centrifugation for 30 minutes at 48,000 × *g*; the supernatant was used as the particulate ALDH source. Enzyme activity was determined at room temperature (24°C) by a spectrofluorometric technique that measured the change in fluorescence (excitation 350 nm, emission 450 nm) as a result of substrate-dependent reduction of nicotinamide adenine dinucleotide (NAD) as previously described.[14] The reaction mixture contained 20 μL of cytosolic protein extract (10 to 20 mg protein/mL), 0.1 mol/L of sodium-phosphate buffer (pH 7.4), 1 mmol/L of EDTA, 5 mmol/L of 2-mercaptoethanol, and 100 umol/L of NAD (Sigma) with 1 mmol/L propanol as the substrate (total volume, 1.0 mL). Additional assays for ALDH were performed on freeze-thawed whole-cell extracts of LBN AML cells using 100 mmol/L of NAD phosphate (NADP) as the cofactor and a saturated solution of benzaldehyde as the substrate.[15] Protein content was measured by the technique of Lowry et al,[16] and ALDH activity was expressed as a nanomole of substrate cleaved per minute per milligram of protein.

Statistical analysis. Routine statistical calculations were performed with a hand-held scientific calculator (Hewlett-Packard, Corvallis, OR). Analysis of data from experimental and control groups was performed with nonparametric (eg, Wilcoxon rank-sum) tests. Graphic analysis of the data was done with a microcomputer (Model XT, IBM Corp, Armonk, NY) and a spread-sheet software package (Lotus Development Corp, Cambridge, MA).

RESULTS

Development of CY resistance. Syngeneic recipients of untreated LBN AML developed florid leukemia and died at a median of 21 days (range, 20 to 27; n = 10) after IV injection of 10^6 cells per rat. Initially, rats given 100 mg/kg of CY 14 days after AML cell injection had substantial prolongation of survival and died with leukemia at a median of 65 days (range, 62 to 66; n = 3) after tumor cell transfer. With successive passages, resistance to CY was manifested by decreases in median survival times because of the earlier appearance of AML. By the 18th passage and CY exposure, rats died of leukemia at a median of 24 days (range, 23 to 29; n = 4) after injection of AML cells ($P = .05$ when compared with survival of rats given control LBN AML cells). The resistance to CY was stable after more than 40 subsequent passages: a statistically significant decrease ($P < .001$) in survival was observed in passages 11 through 20 and remained so in passages 21 through 60 (Fig 1). The growth kinetics in successive passages of CY-treated LBN AML were similar: the mean survival (±SD) of AML-bearing rats was 30.4 ± 6.4 days in passages 11 through 20, 31.9 ± 3.4 days in passages 21 through 30, 28.6 ± 4.5 days in passages 31 through 40, 27.1 ± 4.0 days in passages 41 through 50, and 27.2 ± 3.8 days in passages 51 through 60 (Fig 1).

To determine whether these AML cells were also resistant to administration of high-dose CY in vivo,[17] groups of rats were given single intraperitoneal doses of CY (200 or 300 mg/kg) 14 days after injection of CY-treated or control CY-sensitive LBN AML (10^6 cells per rat). Because these

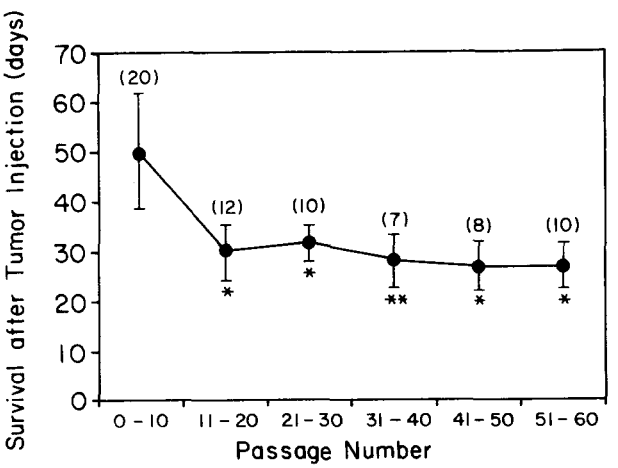


Fig 1. Survival of LBN rats after injection of AML cells from serially passaged CY-treated animals. Animals received 1 × 10^6 AML cells and then 100 mg/kg of CY intraperitoneally 14 days later. All animals died of leukemia. The mean survival (±SD) is shown; numbers in parentheses indicate the number of animals studied. *$P < .001$ and **$P < .002$ versus survival in passages 0 through 10 (Wilcoxon rank-sum test).

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doses of CY are curative in the original LBN AML but necessitate syngeneic marrow rescue,[12] these animals then received 5×10^7 syngeneic marrow cells IV, 24 hours after high-dose CY. Rats given no CY or syngeneic marrow transplant died with AML at a median of 25 days after tumor transfer, similar to the recipients of the parent LBN AML (Table 1). Ten of 11 animals injected with CY-treated AML cells and given 200 mg/kg of CY plus marrow rescue died of AML (one animal died of drug toxicity), whereas all 11 recipients of control LBN AML had long-term leukemia-free survival after 200 mg/kg of CY and syngeneic marrow infusion ($P < .001$). Similarly, three animals with drug-resistant LBN AML given 300 mg/kg of CY and syngeneic marrow transplants died of AML (one other animal died of CY toxicity), while recipients of the original CY-sensitive AML line did not develop leukemia after this dose of CY and syngeneic marrow rescue (Table 1) ($P < .01$).

Ex vivo incubation with 4HC and PhM. The original LBN AML was eradicated by incubation with 70 μmol/L 4HC, a concentration that spared the hematopoietic repopulating ability of normal rat marrow cells[7]; after incubation with higher concentrations of 4HC (100 μmol/L and above), both LBN AML cells and normal rat stem cells are killed.[18] In concordance with these observations, we found that LBN rats injected with control AML cells exposed for 30 minutes to 70 μmol/L or 100 μM of 4HC did not develop leukemia, even after observation for more than 100 days following tumor cell injection; animals injected with 4HC-treated AML cells obtained from the ninth passage after exposure to CY also had long-term leukemia-free survival. In contrast, CY-treated AML cells obtained from the 18th and subsequent passages remained viable after ex vivo incubation with 70 μmol/L or 100 μmol/L of 4HC; all rats given AML cells incubated with these concentrations of 4HC died of leukemia at a median of 25 days (range, 21 to 32) or 28 days (range, 23 to 36), respectively, after injection ($P < .01$ and <0.05, respectively, *v* control) (Table 2).

The observed resistance of LBN AML to 4HC was stable after in vivo passage without exposure to CY. Drug-resistant leukemic cells obtained after six serial passages in the absence of CY were incubated with 100 μmol/L of 4HC and then injected into LBN rats; these animals died of AML at a median of 25 days (range, 22 to 26; n = 4) after tumor cell transfer ($P < .01$ when compared with control LBN AML), similar to that observed in recipients of 4HC-treated leukemic cells obtained from rats exposed to CY in vivo.

Since PhM is the active alkylating end-product of CY metabolism, we also studied the effects of ex vivo incubation with PhM on the growth of CY-resistant and control LBN AML. In contrast to the resistance to 4HC observed after multiple passages and exposures to CY, both CY-resistant and control AML cells demonstrated similar dose-dependent sensitivity to PhM (Table 3). Exposure to 100 μmol/L of PhM did not greatly affect the survival of either drug-resistant or control LBN AML cells, whereas prolongation of survival was seen after incubation with higher concentrations of PhM. LBN rats given 10^6 CY-resistant or control leukemic cells incubated with 500 μmol/L of PhM died of AML at a median of 35 and 37 days, respectively, after injection, consistent with approximately a 4-log cell kill.[1,4,19]

ALDH activity. The levels of ALDH in erythrocyte-free cytosolic protein extracts of CY-resistant LBN AML cells were significantly elevated relative to control values (Table 4). When the assay was performed with benzaldehyde substrate and NADP cofactor, ALDH activity in drug-

Table 1. Effect of High-Dose CY Administration and Syngeneic Marrow Rescue on the In Vivo Growth of CY-Sensitive and CY-Resistant LBN Myeloid Leukemia

CY Dose (mg/kg)	CY-Sensitive Cells	CY-Resistant Cells
None	21 (20-27)[10]	25 (25-26)[6]
200	100*[11]	22†; 28 (22-39)[10]‡
300	100*[4]	22†; 39[3]§

Each animal received 10^6 CY-resistant or parent LBN AML cells IV. CY-treated rats were given CY 14 days later, intraperitoneally with injection of syngeneic marrow (5×10^6 cells/rat IV) 24 hours later. The median survival (with range in parentheses) after injection of AML cells is shown; numbers in brackets indicate number of animals studied. Unless otherwise indicated, animals died of leukemia.

*Rats killed after long-term survival without evidence of leukemia at necropsy.

†Toxic death; no evidence of leukemia at necropsy.

‡$P < .001$.

§$P < .01$ versus CY-sensitive animals (Wilcoxon rank-sum test).

Table 2. Comparative Sensitivity of CY-Resistant and Control LBN Myeloid Leukemia Cells to Ex Vivo Incubation With 4HC

Passage No.	Dose of 4HC (μmol/L) 0	70	100
None (control)	22 (21-27)[8]	35; 100*[3]	100*[4]
9		49; 100*[3]	100*[4]
18-50		25 (21-32)[8]†	28 (23-36)[6]‡

CY-resistant or control AML cells were incubated with 4HC for 30 minutes and injected into LBN rats (10^6 cells per rat IV). The median survival (with range in parentheses) is shown; numbers in brackets indicate the number of animals studied. For passages 18 through 50, data were pooled from three representative experiments with two or three rats in each experimental group. Unless otherwise indicated, rats died of leukemia.

*Rats killed after long-term survival without evidence of leukemia at necropsy.

†$P < .01$.

‡$P < .05$ versus control (Wilcoxon rank-sum test).

Table 3. Comparative Sensitivity of CY-Resistant and Control LBN Myeloid Leukemia Cells to Ex Vivo Incubation With PhM

AML Cells	Dose of PhM (μmol/L) None	100	200	300	500
CY-resistant	20 (18-26)[14]	22 (20-28)[6]	26 (24-27)[6]	31 (25-35)[6]	35 (21-39)[6]
Control	22 (21-27)[8]	23 (22-25)[4]	27 (26-28)[4]	30 (29-31)[4]	37 (36-44)[4]

Each animal received 10^6 CY-resistant or control AML cells incubated with no drug or with PhM for 30 minutes. The median survival (with range in parentheses) is shown; numbers in brackets indicate numbers of animals studied. All deaths were due to leukemia.

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Table 4. ALDH Activity in LBN Myeloid Leukemia Cells

Preparation	Cofactor	Activity (nmol/min/mg protein)
Cytosolic		
Cy-sensitive AML	NAD	0.13 ± 0.04 (11)
CY-resistant AML	NAD	1.45 ± 0.58* (27)
CY-sensitive AML	NADP	0.19 ± 0.09 (6)
CY-resistant AML	NADP	0.69 ± 0.24* (8)
Particulate		
CY-sensitive AML	NAD	0.50 ± 0.09 (4)
CY-resistant AML	NAD	6.68 ± 1.01* (8)
CY-sensitive AML	NADP	0.35 ± 0.02 (6)
CY-resistant AML	NADP	0.90 ± 0.03* (6)

ALDH activity was measured as nanomoles NAD(P) cleaved per minute per milligram of protein. Trials using NAD as cofactor were performed in the presence of 1 mmol/L of propanal; trials using NADP were performed in a saturated solution of benzaldehyde. The mean ±SD is shown; numbers in parentheses indicate the number of determinations.

*$P < .01$ versus control (Wilcoxon rank-sum test).

resistant LBN AML cells was elevated by nearly fourfold relative to control leukemic cells: 0.69 ± 0.24 versus 0.19 ± 0.09 (mean ± SD) nmol of NADP cleaved per minute per milligram of protein, respectively ($P < .01$). Using NAD and propanal substrate, cytosolic ALDH levels were elevated by approximately 11-fold in drug-resistant LBN AML cells (1.45 ± 0.58 nmol of NAD cleaved per minute per milligram of protein, *v* 0.13 ± 0.04 in control LBN AML cells; $P < .01$). Particulate fractions of the CY-resistant cells had threefold elevations in ALDH using the NADP-benzaldehyde assay (0.90 ± 0.11 nmol NADP cleaved per minute per milligram of protein, *v* 0.35 ± 0.02 in control cells; $P < .01$) and 13-fold increases in ALDH with the NAD-propanal assay (6.68 ± 1.01 nmol NAD cleaved per minute per milligram of protein, v 0.50 ± 0.09 in control cells; $P < .01$) (Table 4).

DISCUSSION

We have demonstrated that resistance to CY can be developed in a rat model of AML by chronic intermittent in vivo exposure to high doses (ca. LD_{50}) of the drug. Previous studies with the murine L1210 model of acute lymphocytic leukemia have shown that stable resistance to CY can be induced by in vivo exposure of leukemic cells to the drug.[20] To our knowledge, however, this is the first report of the establishment of a CY-resistant subline of a rat model of AML. Since the LBN AML model is extensively used in the preclinical evaluation of antineoplastic agents and of the efficacy of allogeneic or syngeneic bone marrow transplantation in AML, the availability of a CY-resistant subline should be useful for complementary studies in a model of relapsed and/or drug-resistant leukemia. Indeed, fresh human leukemic cells have shown individual variability in sensitivity to the CY congener mafosfamide and may be resistant to this agent, even before in vivo exposure to intensive combination chemotherapy.[21]

As described with murine L1210 leukemia,[20] resistance of LBN AML to CY was developed only after multiple passages and in vivo exposures to the drug; the resistant leukemia thus obtained survived exposure to a concentration of 4HC that is toxic to normal LBN rat hematopoietic stem cells.[18] Since the parent LBN AML line cannot be grown in vitro cultures, the CY-resistant subline was developed by chronic intermittent in vivo exposure to the drug. It is therefore possible that the CY-resistant LBN AML subline described herein is not entirely a clonal population but retains some heterogeneity. Nevertheless, the level of resistance to CY has been stable after more than 50 in vivo passages (a time span of more than 3 years) and persists in the absence of continued exposure to the agent. In addition, the CY-resistant LBN AML has unique karyotypic markers[22] distinct from the original chromosomal abnormalities described in the parent LBN AML line.[23]

In concordance with observations in CY-resistant L1210 leukemia,[24,25] we found that resistance of LBN AML to CY and 4HC correlated with significantly increased levels of ALDH, which inactivates the intermediate aldophosphamide to carboxyphosphamide. As expected with ALDH-mediated resistance, the sensitivity to PhM was similar in both CY-resistant and control LBN AML cells. Finally, coincubation with diethylaminobenzaldehyde (DEAB), an inhibitor of ALDH,[26,27] restores the sensitivity of CY-resistant LBN AML cells to 4HC.[28] Taken together, these observations strongly suggest that resistance to CY in these rodent models of leukemia is most likely conferred by elevated ALDH activity.[29]

The different magnitudes of increased ALDH activity observed with assays using either NAD or NADP as cofactors suggest that more than one ALDH system may have been affected by exposure to CY. Studies in rat hepatoma lines have showed multiple isozymes of ALDH,[30,31] suggesting that continual selection pressures by drug exposure may enhance expression of more than one isozyme system. Although previous studies indicate that CY resistance is conveyed primarily through elevations of ALDH in soluble cytosolic fractions,[32] we found that both particulate and cytosolic cellular fractions of drug-resistant AML also exhibit significantly increased enzyme activity. Nevertheless, the exact mechanisms that induce the observed increases in ALDH activity of CY-resistant LBN AML cells are not known at this time.

These studies of CY resistance of LBN AML currently require the use of syngeneic rats for serial in vivo transfer of drug-resistant cells. Recently, a subline of this rat leukemia has been described, which can be propagated in vitro in both suspension culture and clonogenic assay[19,33]; development of a clonable in vitro subline of CY-resistant LBN AML by exposure to 4HC may complement investigations with the in vivo drug-resistant subline. Further study with this rodent leukemia model may not only provide insight into the treatment of relapsed human AML but may also lead to strategies for eradication of occult and potentially drug-resistant leukemic cells by ex vivo chemopurging before clinical autologous marrow transplantation.

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