(7) KONING F, STINGL G, YOKOYAMA WM, ET AL: Identification of a T3-associated gamma delta T cell receptor on Thy-1+ dendritic epidermal cell lines. Science 236:834–837, 1987
(8) MORIO T, NAGASAWA M, NONOYAMA S, ET AL: Phenotypic profile and functions of T cell receptor-gamma delta-bearing cells from patients with primary immunodeficiency syndrome. J Immunol 144:1270–1275, 1990
(9) REMÉ T, PORTIER M, FRAYSSINOUX F, ET AL: T cell receptor expression and activation of synovial lymphocyte subsets in patients with rheumatoid arthritis. Phenotyping of multiple synovial sites. Arthritis Rheum 33:485–492, 1990
(10) BALBI B, MOLLER DR, KIRBY M, ET AL: Increased numbers of T lymphocytes with gamma delta-positive antigen receptors in a subgroup of individuals with pulmonary sarcoidosis. J Clin Invest 85:1353–1361, 1990
(11) JANIS EM, KAUFMANN SH, SCHWARTZ RH, ET AL: Activation of gamma delta T cells in the primary immune response to Mycobacterium tuberculosis. Science 244:713–716, 1989
(12) CARBONARI M, CHERCHI M, PAGANELLI R, ET AL: Relative increase of T cells expressing the gamma/delta rather than the alpha/beta receptor in ataxia-telangiectasia. N Engl J Med 322:73–76, 1990
(13) MICHIE SA, ABEL EA, HOPPE RT, ET AL: Expression of T-cell receptor antigens in mycosis fungoides and inflammatory skin lesions. J Invest Dermatol 93:116–120, 1989
(14) HORN TD, FARMER ER: Distribution of lymphocytes bearing TCR gamma/delta in cutaneous lymphocytic infiltrates. J Cutan Pathol 17:165–170, 1990
(15) KAYE FJ, BUNN PA JR, STEINBERG SM, ET AL: A randomized trial comparing combination electron-beam radiation and chemotherapy with topical therapy in the initial treatment of mycosis fungoides. N Engl J Med 321:1784–1790, 1989
(16) KNOWLES DM: Immunophenotypic and antigen receptor gene rearrangement analysis in T cell neoplasia. Am J Pathol 134:761–785, 1989
(17) BUECHNER SA, WINKELMANN RK, BANKS PM: T cells and T-cell subsets in mycosis fungoides and parapsoriasis. A study of 18 cases with anti-human T-cell monoclonal antibodies and histochemical techniques. Arch Dermatol 120:897–905, 1984
(18) HAYNES BF, HENSLEY LL, JEGASOTHY BV: Phenotypic characterization of skin-infiltrating T cells in cutaneous T-cell lymphoma: Comparison with benign cutaneous T-cell infiltrates. Blood 60:463–473, 1982
(19) NASU K, SAID J, VONDERHEID E, ET AL: Immunopathology of cutaneous T-cell lymphomas. Am J Pathol 119:436–447, 1985
(20) WEISS LM, WOOD GS, WARNKE RA: Immunophenotypic differences between dermatopathic lymphadenopathy and lymph node involvement in mycosis fungoides. Am J Pathol 120:179–185, 1985
(21) PICKER LJ, WEISS LM, MEDEIROS LJ, ET AL: Immunophenotypic criteria for the diagnosis of non-Hodgkin's lymphoma. Am J Pathol 128:181–201, 1987
(22) LINDAE ML, ABEL EA, HOPPE RT, ET AL: Poikilodermatous mycosis fungoides and atrophic large-plaque parapsoriasis exhibit similar abnormalities of T-cell antigen expression. Arch Dermatol 124:366–372, 1988
(23) HAYNES BF, METZGAR RS, MINNA JD, ET AL: Phenotypic characterization of cutaneous T-cell lymphoma. Use of monoclonal antibodies to compare with other malignant T cells. N Engl J Med 304:1319–1323, 1981
(24) VAN DER PUTTE SC, TOONSTRA J, VAN WICHEN DF, ET AL: Aberrant immunophenotypes in mycosis fungoides. Arch Dermatol 124:373–380, 1988
(25) TAKAHASHI K, TANAKA T, FUJITA M, ET AL: Cutaneous-type adult T-cell leukemia/lymphoma. A unique clinical feature with monoclonal T-cell proliferation detected by Southern blot analysis. Arch Dermatol 124:399–404, 1988
(26) SUGIMOTO H, NAKAYAMA F, YAMAUCHI T, ET AL: Ki-1+ Cutaneous lymphoma. Gene rearrangement analysis of tumor cells in tissue and short-term culture of a patient. Arch Dermatol 124:405–408, 1988
(27) CHU AC, ROBINSON D, HAWK JL, ET AL: Immunologic differentiation of the Sézary syndrome due to cutaneous T-cell lymphoma and chronic actinic dermatitis. J Invest Dermatol 86:134–137, 1986
(28) FOSTER CA, YOKOZEKI H, RAPPERSBERGER K, ET AL: Human epidermal T cells predominantly belong to the lineage expressing alpha/beta T cell receptor. J Exp Med 171:997–1013, 1990
(29) DUPUY P, HESLAN M, FRAITAG S, ET AL: T-cell receptor-gamma/delta bearing lymphocytes in normal and inflammatory human skin. J Invest Dermatol 94:764–768, 1990
(30) BONNEVILLE M, ITOHARA S, KRECKO EG, ET AL: Transgenic mice demonstrate that epithelial homing of gamma/delta T cells is determined by cell lineages independent of T cell receptor specificity. J Exp Med 171:1015–1026, 1990
(31) FIVENSON DP, NICKOLOFF BJ, KRULL EA: Thy-1 cells are dermal dendrocytes in mycosis fungoides. Clin Res 38:835A, 1990
(32) CERIO R, GRIFFITHS CE, COOPER KD, ET AL: Characterization of factor XIIIa positive dermal dendritic cells in normal and inflamed skin. Br J Dermatol 121:421–431, 1989
(33) NICKOLOFF BJ, GRIFFITHS CE: Isolation and characterization of dermal dendrocytes (DD) from psoriatic plaques. Clin Res 37:969A, 1989

Glutathione Content in Human Bone Marrow and Circadian Stage Relation to DNA Synthesis

Rune Smaaland, Asbjørn M. Svardal, Knut Lote, Per M. Ueland, Ole D. Laerum*

DNA synthesis and contents of reduced glutathione (GSH) and oxidized glutathione were determined every 4 hours during a 24-hour period in 70 human bone marrow samples from 10 healthy males. The mean GSH contents during the sampling periods were low, varying from 1.94 to 3.27 nmol/mg protein between the subjects; the mean values for all samples were 2.54 ± 0.06 nmol/mg protein. The GSH content varied markedly within the individual according to circadian stage (31.0% to 90.2%; mean, 51.4%). Between individuals the mean percentage of cells in DNA synthesis varied from 10.6% to 14.5%, but there was an intraindividual circadian stage-dependent variation, ranging from 48.9% to 274.0% (mean, 126.6%), relative to the lowest value. After adjustment for a slight phase difference between GSH content and DNA synthesis observed for some of the subjects, a statistically significant correlation was found between the GSH content and the fraction of cells in DNA synthesis. The myelosuppressive effect of many chemotherapeutic agents assumed to be detoxified by GSH-dependent mechanism(s) should be considered in the light of the low GSH content in human bone marrow, the circadian variation of DNA synthesis, and the circadian stage-dependent relationship of the GSH content and DNA synthesis. [J Natl Cancer Inst 83:1092–1098, 1991]

Reduced glutathione (GSH), a cysteine-containing tripeptide, has been assigned an important role in the cellular defense against free radicals and oxidative injury, in detoxification processes, and in the protection of the cell against

Received October 3, 1990; revised April 18, 1991; accepted May 2, 1991.
Supported by the Norwegian Cancer Society. R. Smaaland is a Fellow of the Norwegian Cancer Society.
R. Smaaland, O. D. Laerum, The Gade Institute, Department of Pathology, University of Bergen, Haukeland Hospital, Bergen, Norway.
A. M. Svardal, P. M. Ueland, Department of Pharmacology and Toxicology, University of Bergen, Haukeland Hospital.
K. Lote, Department of Oncology, University of Bergen, Haukeland Hospital.
We thank the volunteer subjects in this study and are grateful to Robert B. Sothern, Rhythmometry Laboratory, Department of Surgery, University of Minnesota, for help with the rhythmometric analysis of the data. We acknowledge the skillful technical assistance of Mr. Jan Solsvik and Mr. Mohammad A. Mansoor (both of the University of Bergen).
Correspondence to: Rune Smaaland, M.D., The Gade Institute, Department of Pathology, University of Bergen, N-5021 Haukeland Hospital, Bergen, Norway.

radiation damage (*1-6*). It is the most abundant intracellular nonprotein thiol, and the average cellular content amounts to 0.5-10 mmol/L (*7,8*).

It has been demonstrated that depletion of intracellular GSH in vitro enhances the cytotoxicity of several chemotherapeutic agents, e.g., melphalan (*6,9-11*), doxorubicin (*4,12*), cisplatin (*13,14*), mitomycin (*15*), and cyclophosphamide (*15-17*). It has also been shown that tumor cells obtained from a patient with ovarian adenocarcinoma after the onset of resistance have significantly higher levels of GSH than those obtained before onset of resistance (*18*). In vitro studies have further demonstrated that resistant cells can be sensitized by depleting GSH using the metabolic inhibitor buthionine sulfoximine (*8,10,13,19*).

These findings show that pharmacological modulation of cellular GSH content may alter the sensitivity toward several cytotoxic drugs, pointing to the possibility that physiological changes in cellular GSH content may be an important determinant for the susceptibility of both normal and malignant cells to such agents. This situation is exemplified by the findings of Behrens et al. (*20*), who demonstrated that the cellular GSH content was higher in monolayer cultures of cisplatin-resistant cells having a greater growth rate than in the nonresistant parent cell line and that the difference in GSH content was greatest at the earliest time after plating.

Cell-cycle, phase-dependent differences in the effects of cytotoxic drugs are well documented (*18*). Rapidly proliferating cells are usually more sensitive to cytotoxic drugs than quiescent cells (*21,22*), although dividing hematopoietic cells have been reported to develop resistance to cytotoxic agents and carcinogens (*23,24*). In these last two studies, the role of GSH was not evaluated.

In current cancer treatment, bone marrow suppression represents the main dose-limiting factor (*25*) and is increasingly seen because of the more frequent use of dose-intensive and combination therapy. Several ways of circumventing this problem are being explored, such as bone marrow transplantation (autografting or allografting) (*26*), use of recombinant human hematopoietic growth factors (granulocyte-macrophage colony-stimulating factor/granulocyte colony-stimulating factor) (*27,28*), and retroviral transfer of the MDR1 gene (also known as PGY1) to primary hematopoietic progenitor cells (murine studies) (*29*). Information is lacking, however, about GSH homeostasis in bone marrow as a critical normal tissue in humans with regard to cytotoxic chemotherapy. It is therefore important to measure the level of glutathione in human bone marrow to evaluate its detoxifying capacity and to relate it to proliferative activity, i.e., DNA synthesis.

Subjects and Methods

Chemicals

N-Ethylmaleimide, *N*-ethylmorpholine, dithioerythritol, GSH, and oxidized glutathione were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium borohydride was from Fluka Chemie A.G., Buchs, Switzerland. Dimethyl sulfoxide, hydrogen bromide, 5-sulfosalicylic acid (dihydrate), perchloric acid, acetic acid, and methanol (for chromatography) were purchased from Merck A.G., Darmstadt, Federal Republic of Germany. Monobromobimane was from Calbiochem-Behring Corp., La Jolla, Calif.

Subjects

From February 1988 to August 1988, we investigated the GSH content and the fraction of cells in DNA synthesis in the bone marrow of 10 healthy male volunteers (mean age = 30.7 years; range, 19-43 years). The volunteers had given their informed written consent to enter the study. The study was approved and performed in accordance with the guidelines of the regional medical ethics committee.

All the individuals had followed a regular diurnal rhythm for at least 3 weeks before the experiment. The experiment started at 1200 or 1600 hours. The subjects continued their usual activities during the study period, apart from the sampling periods. They went to sleep after the 2400-hour sample was taken but were awakened for the 0400-hour sample. Their regular diurnal rhythm was validated by the determination of cortisol levels at every sampling time point. The cortisol levels showed the usual circadian pattern for all individuals, i.e., high levels in the morning and low levels in the evening.

Protocol

Bone marrow was sampled every 4 hours during one 24-hour period (total of seven samples) and was drawn by puncturing the sternum and the anterior iliac crests. The sequence of sampling from different sites was randomized to reduce possible site-related variations in the parameters measured. To exclude that variations could be attributed to dilution of the samples caused by local bleeding at the puncture site, we performed differential counts on smears from all samples. No sample had to be discarded because of an unacceptably large peripheral blood admixture; i.e., all smears were characteristic of bone marrow (results not shown).

Procedure for Bone Marrow Sampling and Handling of Samples

No medication was given before samples were taken. The area of the puncture site was infiltrated with a local anesthetic (lidocaine, 20 mg/mL; Astra Pharmaceutical Products, Södertälje, Sweden). Bone marrow (0.2 mL) was aspirated into a 5-mL syringe, and two droplets of the aspirated sample were immediately put into liquid nitrogen for determination of GSH levels. One droplet was stained for flow cytometry by adding it to 2 mL ice-cold staining solution consisting of ethidium bromide, detergent, and RNase (direct staining), according to the method described by Vindeløv (*30*). The tube was sealed, and the solution was shaken before being placed in an ice bath for at least 10 minutes. Another droplet was placed onto each of two microscope slides that were tilted in order to let the blood run down and thereby increase the fraction of marrow elements. The marrow elements were then immediately removed from the slides and stained in the same way (indirect staining).

Flow Cytometry

The cytogram and histogram of the two single-cell suspensions were analyzed on a Cytofluorograf 50H, interfaced to a Model 2150 Computer (Ortho Diagnostic Systems, Inc., Westwood, Mass.), as previously described (*31-33*). We evaluated

the fraction of cells in DNA synthesis (S phase) by taking the mean value of the S phase of the two differently stained samples at each time point. The mean coefficient of variation of the DNA histograms was 3.3%.

Determination of GSH and Oxidized Glutathione Plus Soluble Glutathione-Mixed Disulfide

Two droplets of bone marrow that had been frozen in liquid nitrogen were extracted within 3 days after sampling with 1 mL of ice-cold 5% 5-sulfosalicylic acid containing 50 μ*M* dithioerythritol, and the precipitated protein was removed by centrifugation. This protein precipitate was prepared for subsequent protein determination as described below. GSH and oxidized glutathione plus soluble glutathione-mixed disulfide were determined in the acid extract by a previously published method (*34*).

Determination of Protein

The acid-precipitated protein (*See* above) was dissolved in 500 μL concentrated dimethyl sulfoxide followed by the addition of 500 μL distilled water. After thorough blending, aliquots were withdrawn and further diluted (1:15) with distilled water. This mixture was analyzed for protein according to the method of Bradford (*35*) using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, Calif.). Bovine γ-globulin was used as the protein standard. We verified that dimethyl sulfoxide in the concentration used did not interfere with the protein assay. The results (not shown) indicated that concentrations of dimethyl sulfoxide less than 10% in the standard solutions (≤0.1% in the final solution) do not interfere with this protein assay. This concentration is well above the one used (3.3% in the standard solution, 0.033% in the final solution).

Statistical Analysis

Data were analyzed by Student's *t* test (paired, two-tailed) and one-way analysis of variance (ANOVA). Spearman rank correlation coefficients were computed, and tests were done to determine whether the *r* value differed from zero. In addition, circadian rhythm data obtained for each individual and for each group were analyzed by a computerized inferential statistical method involving the fitting of a 24-hour cosine curve by the method of least squares (*36*). The primary disadvantage of the cosinor method is that the fitting is sensitive to an asymmetrical shape of the time series data (*37*). For this reason, the cosinor procedure may not accurately represent the characteristics of the actual time-dependent variation. However, the procedure is useful for assessing the presence of periodicities.

Results

Glutathione Content and DNA Synthesis

We measured both reduced and oxidized glutathione in the bone marrow samples. The oxidized form accounted for less than 10% of the total glutathione, i.e., reduced plus oxidized form. The critical step in the determination of glutathione and its different forms is the time between tissue sampling and analysis or freezing in liquid nitrogen. Analysis of a bone marrow aspirate, which has been instantly frozen in liquid nitrogen and stored either in this medium or at –80 °C, gives practically the same values as an immediate analysis of a fresh aspirate. We have found that this is also the case with other tissues, such as liver and kidney, as well as with cells grown in culture (e.g., C3H/10T½ Cl 8 and Cl 16 mouse embryo fibroblasts). In addition, the GSH-to-oxidized glutathione ratio is preserved.

The average GSH content from seven consecutive bone marrow aspirations sampled at 4-hour intervals from the same individual during a 24-hour period varied between 1.94 and 3.27 nmol/mg protein, i.e., a difference of 68.6% between the lowest and highest individual average GSH contents. The single lowest and highest GSH contents measured were 1.53 nmol/mg protein and 3.75 nmol/mg protein, respectively, i.e., a difference of 145.1%. The individual data are given in Table 1. The mean GSH content (± SE) for all 10 subjects was 2.54 ± 0.06 nmol/mg protein, representing 70 measurements. The average value for the fraction of cells in the DNA synthesis phase at the seven time points during the 24-hour sampling period measured by flow cytometry varied between the subjects from 10.6% to 14.5%.

No statistically significant difference was found for GSH content, DNA synthesis phase, or cortisol level measured 24 hours apart.

Circadian Variation in GSH Content

For each subject, we observed a circadian stage-dependent variation in GSH content from 31.0% to 90.2% (mean, 51.4%) compared with the lowest value. However, only one subject (BS) had a significant circadian rhythm detected by a single cosinor analysis (P = .03), while another individual (MJ) had a marginally significant rhythm (P = .09) (Table 1). When the data from all subjects were pooled, there was a trend toward increasing GSH contents from midnight until 1200 hours. By single cosinor analysis of the population mean, this circadian variation was marginally statistically significant (P = .055) with the time of highest concentration (acrophase) found to be at 0835 hours, and the time of lowest concentration (trough) at 2035 hours (Table 1).

Circadian Variation in DNA Synthesis

Each subject also demonstrated a circadian stage-dependent variation of the DNA synthesis phase, with a mean variation of 126.6% (range, 48.9%-274.0%) relative to the lowest value. The DNA synthesis-phase estimation was based on the mean values obtained from the directly and indirectly stained bone marrow cells, with both methods giving corresponding results (i.e., both demonstrated the same circadian variation for each individual and for the pooled data). Thus, in addition to the analysis of two bone marrow samples by flow cytometry at each time point, the demonstration of the same circadian variation by both methods of analyzing the samples corroborates the validity of the flow cytometric bone marrow circadian analysis. A one-way ANOVA between time points for the DNA synthesis resulted in $P<.05$ when variations were analyzed as percentages of the mean, with highest values found during the day compared with the night. The largest difference was observed between 1600 hours and 2400 hours ($P<.01$). Single cosinor analysis resulted in a circadian rhythm detection, with an acrophase at

Table 1. Circadian variation in GSH content in human bone marrow and results of cosinor analysis

Subject	Age, y	N*	Range†		ROC,‡	Results of fit of 24-h cosine (single cosinor)				
			Lowest	Highest	%	*P*	Mesor§ ± SE	Amplitude†	Acrophase‖	Trough‖
AA	19	7	1.94	2.80	44.3	.51	2.63 ± 0.12			
BS	33	7	1.55	2.40	54.8	.03	1.94 ± 0.04	0.27	0738	1938
GW	39	7	2.75	3.75	36.4	.80	3.27 ± 0.13			
IK	31	7	2.13	3.28	54.0	.44	2.75 ± 0.16			
KL	43	7	1.80	2.38	32.2	.71	2.06 ± 0.09			
MJ	24	7	1.53	2.80	83.0	.09	2.26 ± 0.10	0.46	1238	0038
OH	35	7	2.03	2.66	31.0	.25	2.23 ± 0.07			
RK	25	7	2.58	3.63	40.7	.53	2.93 ± 0.17			
EK	28	7	1.74	3.31	90.2	.28	2.78 ± 0.19			
SF	30	7	2.23	3.28	47.1	.59	2.68 ± 0.13			

Group rhythm summary by single cosinor analysis of the population mean according to circadian stage

No. of subjects	*P*	Mesor§ ± SE	Amplitude†	Acrophase‖	Trough‖
10	.055	2.54 ± 0.01	0.07	0835	2035

*N = No. of sampling procedures during the 24-h time span.
†nmol/mg protein.
‡ROC = range of change from lowest to highest value.
§Mesor = rhythm-adjusted mean (average).
‖An hour and minute specification.

1317 hours and a trough at 0117 hours (P = .055), i.e., the variation in GSH content preceding that of DNA synthesis by about 4.5 hours.

Relationship Between Glutathione Content and DNA Synthesis

For all 10 subjects, there seemed to be a covariation (i.e., a similar circadian rhythm) between GSH concentration and DNA synthesis phase according to circadian stage, either demonstrating a near-identical covariation or a slightly phase-shifted near-identical covariation. This covariation is shown in Fig. 1 for all the single individuals; both parameters demonstrated either the same phasing at most time points for five of 10 subjects or a different phasing in time with a difference of ± 4-8 hours. When adjusting for the phase difference for five of the 10 subjects, we found a close correlation between each subject's GSH content and DNA synthesis phase (Table 2), with a statistically significant correlation for the pooled data (r = .40; P = .001; n = 61). An even closer relationship was demonstrated when one highly deviating pair of values (GSH/DNA) at one time point during the 24-hour period for seven of 10 individuals was not taken into consideration. These values were not considered because in these cases *all but one* time point demonstrated a near-identical circadian stage-dependent covariation or a near-identical slightly phase-shifted circadian stage-dependent covariation between GSH content and DNA synthesis phase. The individual correlation coefficients in these cases are also shown in Table 2. The mean and median r values for all 10 individuals were then .77 and .82, respectively. As can be seen from Fig. 2, the pooled GSH values showed a highly significant correlation with the DNA synthesis phase when the few above-mentioned time points were discarded (r = .63; P<.0001; n = 54).

The curves for the pooled raw data are shown in Fig. 3 to indicate the close covariation for the average GSH content and DNA synthesis phase when the DNA synthesis phase was shifted 4 hours backward in time. A peak value for the GSH content was observed at 1200 hours for two registrations and was followed by a decrease in GSH content during the afternoon and evening.

Discussion

After repeated measurements during a 24-hour period, the mean GSH content in human bone marrow was found to be low. This finding is in accordance with murine data reported by others (*4,17,38*). The highest values of GSH content in human bone marrow were still lower than the average GSH content in other normal and tumor tissues in rodents and humans. In rodents, five to more than 10 times higher levels of GSH have been measured in many normal organs compared with the levels in the bone marrow (*4,17,19,39*), the difference being even greater when compared with the levels in malignant tumors (*38,40*). In addition, some human tumor cells, and especially cells with acquired or de novo multidrug resistance, contain high concentrations of GSH (*3,8,41,42*). The low GSH content in the bone marrow may, therefore, suggest a limited capacity of the GSH-dependent detoxification mechanisms in this tissue, which could be related to the high sensitivity of human bone marrow to many cytotoxic drugs.

Less than 10% of the glutathione content in bone marrow was oxidized. This finding implies that the circadian variations do not reflect different degrees of oxidation of glutathione in vivo. It also excludes improper sample handling causing glutathione oxidation as a source of erratic results. We avoided the problem of oxidation, which could contribute substantially to variations in GSH during a 24-hour period, by analyzing samples of total bone marrow immediately placed in liquid nitrogen. The circadian changes in total glutathione content are, therefore, probably due to variations in glutathione biosynthesis and utilization.

Possibly, an admixture of red blood cells (RBCs) could contribute to the GSH content measured, as may be indicated by preliminary results (data not shown). These results correspond with data from

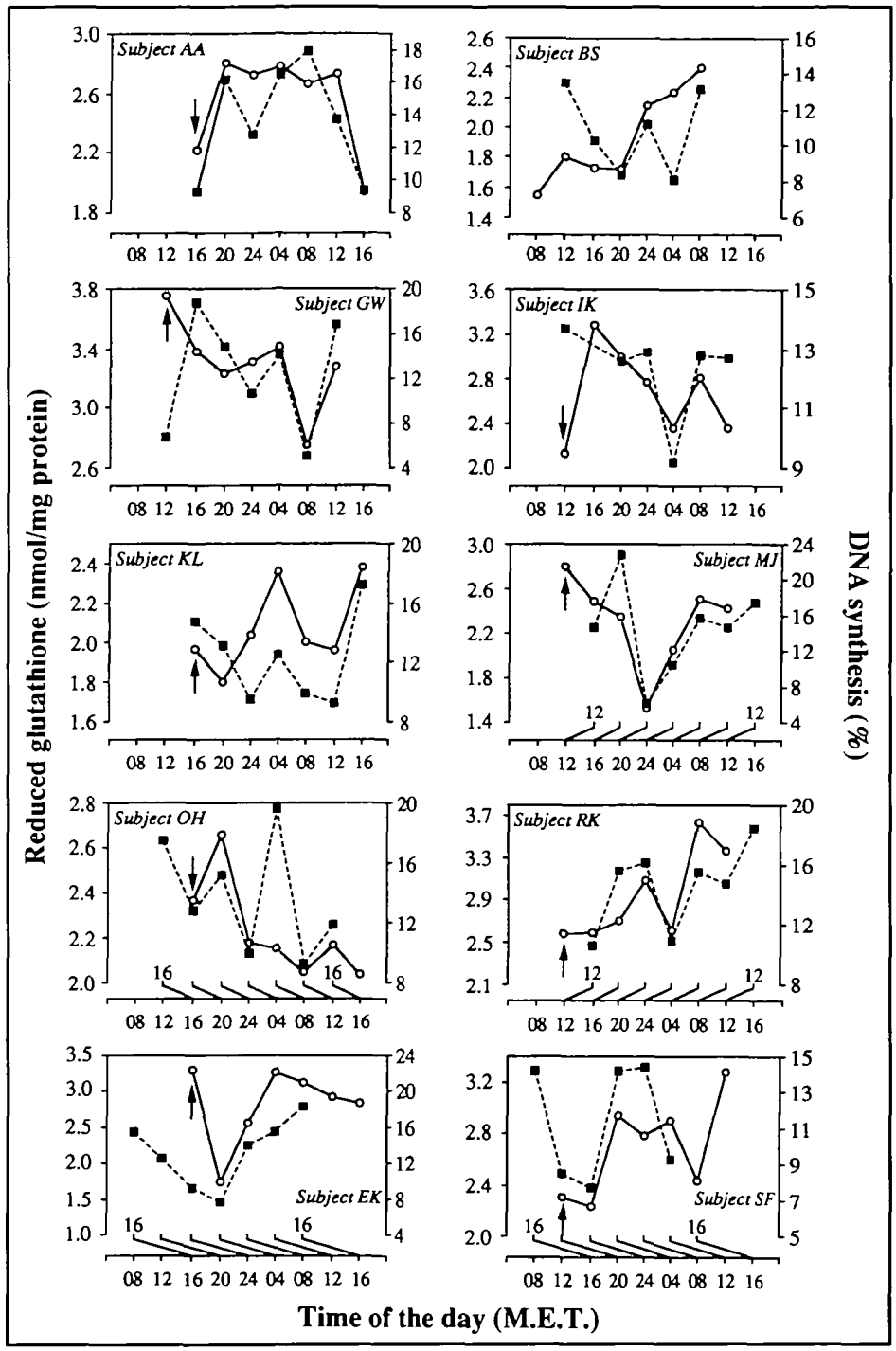

Fig. 1. Circadian stage-dependent covariation between DNA synthesis and GSH content in 10 individuals. DNA synthesis values of subjects MJ, OH, and RK are phase shifted ± 4 hours in time, while those for DNA subjects EK and SF are phase shifted 8 hours backward in time. Arrow indicates the start time for the experiment. The upper x axis denotes time when DNA synthesis is phase shifted. ○ = reduced glutathione (nmol/mg protein); ■ = DNA synthesis (%). M.E.T. = mean European time.

murine studies in which the GSH content in RBCs was reported to be somewhat lower than that of nucleated bone marrow cells (*4*). The measured GSH content in our study (including RBCs) is a little higher than the reported GSH content of nucleated bone marrow cells in mice, but it is in accordance with other reports of GSH content in bone marrow (*See* earlier). Finally, all the bone marrow samples used for GSH determination in our study were harvested in the same way by immediately putting two droplets of the initial sample of 0.2 mL bone marrow into liquid nitrogen. This technique also minimized variations resulting from different handling of varying amounts of RBCs in the samples. In agreement with

Table 2. Linear correlation coefficients between GSH and DNA synthesis for 10 subjects when difference in phasing is taken into consideration

Subject	r_1*	r_2†	Difference in phasing between GSH versus DNA synthesis, h
AA	.82		0
BS	.15	.52	0
GW	.21	.82	0
IK	.12	.58	0
KL	.45		0
MJ	.76	1.00	+4
OH	.30	.95	−4
RK	.64		+4
EK	.54	.93	−8
SF	.73	.97	−8

*Linear correlation coefficient for all time points during a 24-h period for the actual subject.

†Linear correlation coefficient for all time points but one during a 24-h period for the actual subject.

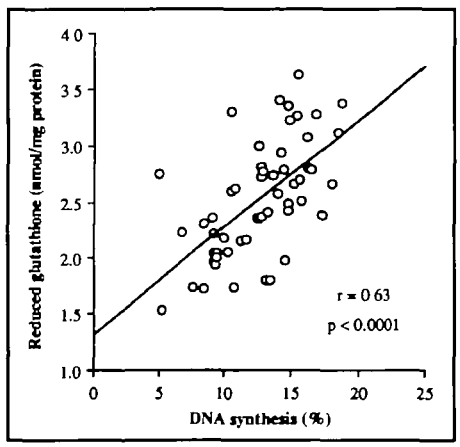

Fig. 2. DNA synthesis related to GSH content when data at various time points from all subjects were pooled and adjusted for difference in phasing and when the few time points were discarded as explained in the text.

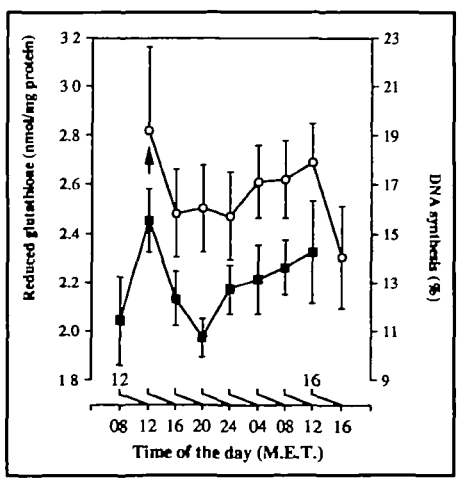

Fig. 3. Circadian stage-dependent covariation between mean values of DNA synthesis and GSH for pooled data of 10 individuals when DNA synthesis values were phase shifted 4 hours backward in time. ○ = reduced glutathione (nmol/mg protein); ■ = DNA synthesis (%). M.E.T. = mean European time.

the data of Bellamy et al. (*43*), our data indicated a higher level of glutathione in the morning than in the evening.

When data for all time points were pooled, a slightly higher DNA synthesis was found in samples taken from the sternum than in those taken from the iliac crests. This finding was most likely due to more blood in the samples obtained from the iliac crests. However, the mean contents of GSH sampled from the sternum and iliac crests showed no difference.

The GSH-depleting agent buthionine sulfoximine enhances the hepatotoxic and nephrotoxic effects of some alkylating agents (*44*). The lower GSH content in total bone marrow cells than in other normal and malignant tissues may indicate a unique response of the bone marrow to GSH-depleting agents, suggesting that depletion of GSH in tumors may not lead to an increased toxicity to the bone marrow, due to this tissue's basically low level of GSH. This possibility is supported by the observation that buthionine sulfoximine induces only a moderate reduction of GSH in mouse bone marrow and no sensitization toward cyclophosphamide (*17*). Likewise, buthionine sulfoximine has been shown not to sensitize bone marrow cells toward melphalan, as assessed by the granulocyte-macrophage colony-forming unit assay (*38,45*) and had no effect on stem cell colony-forming unit survival and peripheral white blood cell counts following melphalan treatment (*46*).

Shaw and Chou (*47*) have earlier demonstrated a positive relationship between increased proliferation and increased GSH content in 3T3 fibroblasts, as has also been shown in another study of monolayer cultures of cisplatin-resistant and nonresistant cells (*20*). In the first study, it was found that an elevation in GSH content was required for maximum induction of DNA synthesis. Recently, it was further demonstrated that tumor cells grown as in vitro cultures in the exponential growth phase have a considerably higher GSH content than tumors found in situ (*40*). In accordance with these data, our study demonstrates within the same subjects a relatively close circadian stage-dependent covariation of GSH content and fraction of cells in DNA synthesis phase in human bone marrow, either demonstrating the same phasing or being slightly phase shifted. This finding is reflected in a statistically significant correlation between GSH content and fraction of cells in DNA synthesis when half of the cases are adjusted for the phase difference observed. This covariation is even more pronounced when one out of five to seven data points in seven of the individuals is not considered. This procedure may of course be discussed. However, because blood contamination might contribute to a deviation of one single pair of GSH/DNA values from an otherwise close relationship, the procedure might be justified. The very high correlation coefficient for these seven individuals when this procedure is done is intriguing. Moreover, the correlation was also statistically significant when none of the data points were left out ($P = .001$).

In addition, evaluation of the pooled data according to circadian stage showed a close covariation between the mean values for GSH content and DNA synthesis when the DNA synthesis phase was shifted 4 hours backward in time; i.e., the variation in GSH content precedes that in DNA synthesis by about 4 hours. The observed circadian variation in DNA synthesis is in accordance with earlier preliminary reports (*48-50*). The absolute changes in mean values were smaller due to different phasing of each parameter between individual subjects, the circadian variation being only marginally significant for GSH by single cosinor analysis.

Our findings agree with previous reports demonstrating a relation between GSH content and DNA synthesis when cells are activated into cell-cycle progression or are in exponential growth (*20,40, 47*). These observations may support the theory that an elevation in the intracellular GSH content is a signal by which quiescent cells are activated into cell-cycle progression (*5*). However, since the timing was not always identical for the two parameters, this relationship may also be indirect and not causal.

It is possible that an increased GSH content may offer some protection against some agents in hematopoietic cells. Consequently, an interindividual difference between the lowest and highest mean GSH contents of almost 70% and an observed difference of 145.1% between the single lowest and highest GSH contents, could imply a predictive role of GSH content for the individual with regard to bone marrow toxicity.

The relatively large individual circadian stage-dependent variation observed in bone marrow GSH content and its relationship to cell proliferation could thus be considered a possible determinant of bone marrow suppression induced by cancer chemotherapy in humans. However, the near-identical circadian stage-dependent covariation between GSH content and cells in the DNA synthesis phase might theoretically neutralize each other. Which one of these parameters will be the most important may depend on the actual cytotoxic drug used. The larger and more consistent circadian stage-dependent variation seen in the DNA synthesis indicates that the proliferative status of the bone marrow may be the most important single factor of these two parameters to be taken into account for drugs having a major effect on DNA synthesis.

References

(*1*) Dethmers JK, Meister A: Glutathione export by human lymphoid cells: Depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation. Proc Natl Acad Sci USA 78:7492–7496, 1981

(*2*) Bump EA, Yu NY, Brown JM: The use of drugs which deplete intracellular glutathione in hypoxic cell radiosensitization. Int J Radiat Oncol Biol Phys 8:439–442, 1982

(*3*) Biaglow JE, Varnes ME, Clark EP, et al: The role of thiols in cellular response to radiation and drugs. Radiat Res 94:437–455, 1983

(*4*) Lee FY, Allalunis-Turner MJ, Siemann DW: Depletion of tumor versus normal tissue glutathione by buthionine sulfoximine. Br J Cancer 56:33–38, 1987

(*5*) Lee FYF, Siemann DW, Allalunis-Turner MJ, et al: Glutathione contents in human and rodent tumor cells in various phases of the cell cycle. Cancer Res 48:3661–3665, 1988

(*6*) Friedman HS, Colvin OM, Griffith OW, et al: Increased melphalan activity in intracranial human medulloblastoma and glioma xenografts following buthionine sulfoximine-mediated gluthathione depletion. J Natl Cancer Inst 81:524–527, 1989

(*7*) Meister A, Anderson ME: Glutathione. Annu Rev Biochem 52:711–760, 1983

(*8*) Dusre L, Mimnaugh EG, Myers CE, et al: Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumor cells. Cancer Res 49:511–515, 1989

(*9*) Suzukake K, Petro BJ, Vistica DT: Reduction in glutathione content of L-PAM-resistant L1210 cells confers drug sensitivity. Biochem Pharmacol 31:121–124, 1982

(*10*) Green JA, Vistica DT, Young RC, et al: Potentiation of melphalan cytotoxicity in

human ovarian cancer cell lines by glutathione depletion. Cancer Res 44:5427–5431, 1984
(11) KRAMER RA, GREENE K, AHMED S, ET AL: Chemosensitization of *L*-phenylalanine mustard by the thiol-modulating agent buthionine sulfoximine. Cancer Res 47:1593–1597, 1987
(12) RUSSO A, MITCHELL JB: Potentiation and protection of doxorubicin cytotoxicity by cellular glutathione modulation. Cancer Treat Rep 69:1293–1296, 1985
(13) HAMILTON TC, WINKER MA, LOUIE KG, ET AL: Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. Biochem Pharmacol 34:2583–2586, 1985
(14) ANDREWS PA, SCHIEFER MA, MURPHY MP, ET AL: Enhanced potentiation of cisplatin cytotoxicity in human ovarian carcinoma cells by prolonged glutathione depletion. Chem Biol Interact 65:51–58, 1988
(15) ONO K, SHRIEVE DC: Enhancement of EMT6/SF tumor cell killing by mitomycin C and cyclophosphamide following in vivo administration of buthionine sulfoximine. Int J Radiat Oncol Biol Phys 12:1175–1178, 1986
(16) CROOK TR, SOUHAMI RI, WHYMAN GD, ET AL: Glutathione depletion as a determinant of sensitivity of human leukemia cells to cyclophosphamide. Cancer Res 46:5035–5038, 1986
(17) TSUTSUI K, KOMURO C, ONO K, ET AL: Chemosensitization by buthionine sulfoximine in vivo. Int J Radiat Oncol Biol Phys 12:1183–1186, 1986
(18) LEWIS AD, HAYES JD, WOLF CR: Glutathione and glutathione-dependent enzymes in ovarian adenocarcinoma cell lines derived from a patient before and after the onset of drug resistance: Intrinsic differences and cell cycle effects. Carcinogenesis 9:1283–1287, 1988
(19) SOMFAI-RELLE S, SUZUKAKE K, VISTICA BP, ET AL: Reduction in cellular glutathione by buthionine sulfoximine and sensitization of murine tumor cells resistant to L-phenylalanine mustard. Biochem Pharmacol 33:485–490, 1984
(20) BEHRENS BC, HAMILTON TC, MASUDA H, ET AL: Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Cancer Res 47:414–418, 1987
(21) LOHRMAN H-P, SCHREML W: Granulopoietic toxicity of cytotoxic agents: Pathogenesis, pathophysiology, methods of modulation, and clinical aspects. *In* Cytotoxic Drugs and the Granulopoietic System (Lohrman H-P, Schreml W, eds). Berlin, Heidelberg, and New York: Springer-Verlag, 1982, pp 155–182
(22) POLLAK MN, BRENNAN LV, ANTMAN K, ET AL: Recombinant GM-CSF in myelosuppression of chemotherapy. N Engl J Med 320:253–254, 1989
(23) DUNN CD: Cell proliferation and drug resistance in the response of colony forming units of rat bone marrow to repeated doses of cytotoxic agents. J Natl Cancer Inst 48:639–649, 1972
(24) FOHLMEISTER I, HOHENTANNER O, PORR A: S-phase resistance of rat hematopoietic stem cells to 7,12- dimethylbenz[*a*]anthracene cytocide and its implications for leukemia development. Cancer Res 44:3661–3664, 1984
(25) GALE RP: Myelosuppressive effects of antineoplastic chemotherapy. *In* Hematopoiesis. Long-Term Effects of Chemotherapy and Radiation (Gale RP, Testa NG, eds). New York: Marcel Dekker, 1988, pp 63–73
(26) CHESON BD, LACERNA L, LEYLAND-JONES B, ET AL: Autologous bone marrow transplantation. Current status and future directions. Ann Intern Med 110:51–65, 1989
(27) ANTMAN KS, GRIFFIN JD, ELIAS A, ET AL: Effect of recombinant human granulocyte-macrophage colony-stimulating factor on chemotherapy-induced myelosuppression. N Engl J Med 319:593–598, 1988
(28) BRONCHUD MH, SCARFFE JH, THATCHER N, ET AL: Phase I/II study of recombinant human granulocyte colony-stimulating factor in patients receiving intensive chemotherapy for small-cell lung cancer. Br J Cancer 56:809–813, 1987
(29) MCLACHLIN JR, EGLITIS MA, UEDA K, ET AL: Expression of a human complementary DNA for the multidrug resistance gene in murine hematopoietic precursor cells with the use of retroviral gene transfer. J Natl Cancer Inst 82:1260–1263, 1990
(30) VINDELØV LL: Flow microfluorometric analysis of nuclear DNA in cells from solid tumors and cell suspensions. A new method for rapid isolation and straining nuclei. Virchows Arch B 24:227–242,1977
(31) SLETVOLD O, LAERUM OD: Alterations of cell cycle distribution in the bone marrow of aging mice measured by flow cytometry. Exp Gerontol 23:43–58, 1988
(32) DEAN PN, JETT JH: Mathematical analysis of DNA distributions derived from flow microfluorometry. J Cell Biol 60:523–527, 1974
(33) GRAY JW, DEAN PN: Display and analysis of flow cytometric data. Annu Rev Biophys Bioeng 9:509–539, 1980
(34) SVARDAL AM, MANSOOR MA, UELAND PM: Determination of reduced, oxidized, and protein-bound glutathione in human plasma with precolumn derivatization with monobromobimane and liquid chromatography. Anal Biochem 184:338–346, 1990
(35) BRADFORD M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254, 1976
(36) NELSON W, TONG YL, LEE JK, ET AL: Methods for cosinor-rhythmometry. Chronobiologia 6:305–323, 1979
(37) KLEVECZ RR, SHYMKO RM, BLUMENFELD D, ET AL: Circadian gating of S phase in human ovarian cancer. Cancer Res 47:6267–6271, 1987
(38) SOMFAI-RELLE S, SUZUKAKE K, VISTICA BP, ET AL: Glutathione-conferred resistance to antineoplastics: Approaches toward its reduction. Cancer Treat Rev 11:43–54, 1984
(39) JAESCHKE H, WENDEL A: Diurnal fluctuation and pharmacological alteration of mouse organ glutathione content. Biochem Pharmacol 34:1029–1033, 1985
(40) LEE FY, VESSEY A, ROFSTAD E, ET AL: Heterogeneity of glutathione content in human ovarian cancer. Cancer Res 49:5244–5248, 1989
(41) LEE FY, VESSEY AR, SIEMANN DW: Glutathione as a determinant of cellular response to doxorubicin. Natl Cancer Inst Monogr 6:211–215, 1988
(42) KRAMER RA, ZAKHER J, KIM G: Role of glutathione redox cycle in acquired and de novo multidrug resistance. Science 241:694–697, 1988
(43) BELLAMY WT, ALBERTS DS, DORR RT: Daily variation in non-protein sulfhydryl levels of human bone marrow. Eur J Cancer Clin Oncol 11:1759–1762, 1988
(44) KRAMER RA, SCHULLER HM, SMITH AC, ET AL: Effects of buthionine sulfoximine on the nephrotoxicity of 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosurea (MeCCNU). J Pharmacol Exp Ther 234:498–506, 1985
(45) OZOLS RF, LOUIE KG, PLOWMAN J, ET AL: Enhanced melphalan cytotoxicity in human ovarian cancer in vitro and in tumor-bearing nude mice by buthionine sulfoximine depletion of glutathione. Biochem Pharmacol 36:147–153, 1987
(46) RUSSO A, TOCHNER Z, PHILLIPS T, ET AL: In vivo modulation of glutathione by buthionine sulfoximine: Effect on marrow response to melphalan. Int J Radiat Oncol Biol Phys 12:1187–1189, 1986
(47) SHAW JP, CHOU IN: Elevation of intracellular glutathione content associated with mitogenic stimulation of quiescent fibroblasts. J Cell Physiol 129:193–198, 1986
(48) MAUER AM: Diurnal variation of proliferative activity in the human bone marrow. Blood 26:1–7, 1965
(49) SMAALAND R, LOTE K, SLETVOLD O, ET AL: Circadian stage dependent variations in the DNA synthesis-phase and G2/M-phase of human bone marrow. Proc Am Assoc Cancer Res 30:35, 1989
(50) SMAALAND R, LOTE K, SLETVOLD O, ET AL: Rhythms in the bone marrow and blood: Differences as to day and night. Dtsch Med Wochenschr 114:845–849, 1989

Involvement of Vacuolar H^+-Adenosine Triphosphatase Activity in Multidrug Resistance in HL60 Cells

*David Marquardt, Melvin S. Center**

HL60 cells isolated for resistance to vincristine (HL60/Vinc cells) or doxorubicin (HL60/Adr cells) contain enhanced levels of an energy-dependent drug efflux pump. HL60/Vinc cells contain the drug transporter P-glycoprotein, whereas the HL60/Adr isolate does not. In the present study, we examined the possible involvement of vacuolar H^+-adenosine triphosphatase (H^+-ATPase) activity in drug resistance in HL60 cells. We utilized bafilomycin A_1, an agent which selectively inhibits vacuolar H^+-ATPase activity at low concentrations. The results showed that bafilomycin A_1 induced a major increase in drug accumulation and inhibited drug efflux in both HL60/Adr cells and HL60/Vinc cells. Similar results were obtained with 7-