Glutathione and Glutathione Transferase Levels in Mouse Granulocytes following Cyclophosphamide Administration

James Carmichael, David J. Adams, John Ansell, and C. Roland Wolf

ABSTRACT

Following an initial depletion, glutathione and glutathione transferase levels are transiently increased in mouse bone marrow following the administration of a low dose of cyclophosphamide. Similar effects are observed on subsequent administration of the drug. The separation of various bone marrow populations on a fluorescence-activated cell sorter has shown that increase in glutathione and glutathione transferase levels are restricted to the granulocytic fraction. This may well provide an explanation for the protective effect of a low "priming" dose of cyclophosphamide against a subsequent lethal dose. The changes in granulocytic glutathione and glutathione transferase levels can also be monitored in the peripheral circulation. The enhanced levels of glutathione in cells resulting from cytotoxic insult appear to be a general response of cells to cytotoxins and may be important in both antitumor therapy as well as the initiation of chemical toxicity and carcinogenicity.

INTRODUCTION

The response of normal cells to cytotoxins and chemical carcinogens is important in our understanding of the changes which result in malignant transformation (1). In addition, the response to cytotoxins is also an important aspect of current cancer chemotherapy in that toxicity to normal tissues is a dose-limiting factor. In this regard we are currently investigating the properties of bone marrow cells which in addition to being sites of malignant transformation are also sensitive targets for chemotherapeutic agents. In a previous communication we reported the response of mouse bone marrow to the cytotoxins cyclophosphamide, X-radiation, and 1-beta-d-arabinofuranosylcytosine (2). In particular, the effects of these reagents on marrow glutathione and glutathione transferases were studied as these components play a central role in the protection of cells from a wide variety of alkylating agents as well as radiation (3, 4). The data presented indicated that treatment of mice with a low cytotoxic dose of these reagents resulted in a significant increase in marrow GSH6 and GSH transferase content. These increases were paralleled by an increased resistance to a high dose of the cytotoxic compounds. These experiments raised the following questions: (a) Were the changes observed related to changes in marrow cell population? (b) Were the changes due to alterations in intracellular GSH and GSH transferase homeostasis and therefore did they represent a general response to cytotoxic compounds? If so, which cells were affected?

In this report we have investigated these possibilities following the administration of cyclophosphamide. In addition we have determined whether the changes in the marrow cells are reflected in the cells of the peripheral blood and therefore provide a potential method for monitoring the status of the bone marrow during chemotherapy.

MATERIALS AND METHODS

Male CBA mice (25 g) were used. Animals treated with cyclophosphamide received i.p. either 75 mg/kg body weight as a nonlethal "priming" dose (2) or 350 mg/kg in 0.9% saline. At various time points after drug administration, animals were killed by cervical dislocation. Blood samples were drawn from the posterior vena cava into a heparinized syringe. Bone marrow cells were obtained by passing cold isotonic saline buffered with 10 mM phosphate through the femurs as previously described (2).

Peritoneal granulocytes were prepared by giving mice i.p. injections of 2 ml of a solution of 2% calcium caseinate (5). At 3 h the animals were killed and 5 ml of 0.168 M NaCl was injected i.p. The peritoneal exudate was withdrawn after gentle peritoneal massage, and the cells were spun down at 650 x g for 5 min, washed three times with ice cold 0.168 M NaCl, and left to stand for 10 min to lyse erythrocytes. Cells were then suspended in 0.168 M phosphate buffered saline for GSH assay.

For cell sorter (FACS IV; Becton Dickinson) analysis and sorting, approximately 1 ml of blood was washed in 9 ml of cold phosphate buffered isotonic saline supplemented with 0.1% bovine serum albumen and 0.02% EDTA. After centrifugation at 450 x g for 7 min, the blood cell pellet was mixed with 18 ml of distilled water and left for 12 s to lye erythrocytes. Two ml of 10x concentrated Hanks' balanced salt solution (Grand Island Biological Co.) were then added and mixed. After a second centrifugation at 250 x g for 7 min, the cell pellet was resuspended in 1 ml of the supplemented saline. If erythrocytes were to be separated on the FACS, peripheral blood was used directly after appropriate dilution.) Bone marrow cells were obtained by flushing femurs with 1 ml of similarly supplemented saline.

Various cell types from bone marrow and peripheral blood suspensions were separated on the FACS on the basis of their differential forward and right angle light scattering properties (6, 7). The 488-nm line of an argon ion laser was used for the excitation of both the forward and right angle scatter signals and samples were run at approximately 1000 cells/s. Appropriate gates were set around granulocytes, lymphocytes, erythrocytes, and a fourth mixed cell population (mainly monocytes) from bone marrow preparations (see Fig. 4), and lymphocytes, granulocytes, and erythrocytes from peripheral blood (see Fig. 6), and these populations were sorted into cooled Eppendorf tubes. The purity of the cell fractions was tested cytologically by Leishman staining. The granulocytes were 89.9 ± 7.6% (SD) pure (n = 6) and the lymphocyte population was 70.1 ± 10.9% pure, the remainder of the cells being monocytes.

All procedures were carried out at 4°C. Cells were collected in aliquots

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* Present address: National Cancer Institute, Naval Medical Oncology Branch, Bethesda, MD.

* Present address: Department of Microbiology, University of Leeds, Leeds, LS2 9JT, United Kingdom.

* To whom requests for reprints should be addressed.

* The abbreviations used are: GSH, glutathione; FACS, fluorescence activated cell sorter.

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of $3 \times 10^6$ for further assays, frozen immediately after collection in solid CO$_2$ and assayed within 24 h. Control experiments showed that there was no difference in glutathione measurements between cells assayed immediately and cells stored in this manner.

Cellular glutathione content was determined from the deproteinated cell homogenates by the fluorimetric method of Hissin and Hilf (8) using o-phthalaldehyde. This assay was used for its simplicity, and the values obtained compared favorably with other published methods described previously (2). Glutathione transferase activity was measured in the cell samples lysed by addition of nonisotonic buffer, 0.1 M phosphate, pH 6.5, using 1-chloro-2,4-dinitrobenzene as substrate as described by Habig et al. (9).

All the reagents used in this study were obtained from commercial sources and were of the highest purity available.

RESULTS

The effect of nonlethal priming and a subsequent lethal dose of cyclophosphamide on bone marrow GSH and GSH transferase is shown in Fig. 1. In agreement with previous findings (2) after a dose of 75 mg/kg an initial depletion in glutathione and GSH transferase levels was followed by a significant increase over control levels on day 5, the concentrations being increased 1.8- and 2.5-fold for GSH and GSH transferase, respectively. On day 6 a normally lethal dose of cyclophosphamide (350 mg/kg) was administered which resulted in a second depletion in both GSH and GSH transferase levels to 32 and 40% of control values, respectively. This decrease was much less pronounced when compared with previously untreated animals where GSH content was reduced to 5% of controls (data not shown) (2). The GSH levels remained suppressed for 3 days but recovered and increased to much higher levels on day 11. In the case of the glutathione transferase activity, the activity was increased approximately 8-fold and GSH approximately 2.5-fold. The primed cells of the marrow which were more resistant to a lethal dose of cyclophosphamide therefore respond to the high dose in a manner very similar to their response to a priming dose. There are several possible explanations for measurement of increased GSH and GSH transferase levels in the marrow following cyclophosphamide administration, as whole marrow samples were taken for assay. Experiments were carried out to establish whether the GSH and GSH transferase activities were intracellular (Fig. 2). In these experiments marrow cells from control and primed animals were spun down. In both cases the glutathione content of the pelleted cells closely matched the theoretical content based on the measured cell viability of the original bone marrow sample, measured using trypan blue, and the GSH content of the original sample, demonstrating that the GSH measured in these assays was intracellular.

Administration of cyclophosphamide at either 75 or 350 mg/kg
Cyclophosphamide effects on GSH and glutathione transferase

Fig. 4. FACS IV dot plots of mouse bone marrow from untreated animals (a) and animals treated 5 days previously with cyclophosphamide (75 mg/kg) (b). The schematic diagram (c) indicates the position of the cell populations separated as described in “Materials and Methods.” I, erythrocytes; II, lymphocytes; III, granulocytes; IV, mixed cell population.

kg has a significant effect on bone marrow cellularity (Fig. 3). Glutathione levels bore an approximately inverse relationship to these changes (Fig. 1). Glutathione levels are expressed per 10^6 cells so that changes in cell number, i.e., in cell population, could explain the GSH overshoot observed as cell number decreased and GSH levels remained relatively constant. However, this would have the unlikely requirement that all the cells destroyed by cyclophosphamide had negligible GSH content. The changes in cell number cannot explain the increases in GSH transferase activity (Figs. 1b and 3). In order to investigate the changes in cellularity and the effects of cyclophosphamide on glutathione and GSH transferase in the different cell types, marrow cells from treated animals were separated using a FACS IV (Fig. 4).

No changes in the erythrocyte population could be observed following cyclophosphamide treatment. However, a significant reduction in the proportion of lymphocytes was observed in primed animals. In addition, there also appeared to be an increase in the proportion of granulocytes. The GSH and GSH transferase levels and activity of the various populations are shown in Fig. 5. Of the cell fractions tested the granulocytic fraction in control animals had approximately twice the glutathione content relative to the lymphocytes and four times the erythrocyte content. The cells contained in the mixed population had a slightly higher GSH content than did the erythrocytes. No change was observed in the GSH content of lymphocytes, erythrocytes, or in the mixed population from primed animals. However, a significant increase (1.8-fold) in the granulocytic GSH content was observed. Measurement of glutathione transferase activity in six separate determinations in these cells also showed it to be increased 2.2-fold from 7.35 to 16.2 nmol 1-chloro-2,4-dinitrobenzene conjugated/min/10^6 cells.

In order to determine whether the changes observed in the bone marrow could also be measured in the peripheral blood cells, cell fractions were separated on the FACS after the erythrocytes had been removed by lysis. The cell profiles are shown in Fig. 6, a and b. The changes observed following cyclophosphamide administration were very similar to those observed in the marrow with a dramatic reduction in the proportion of lymphocytes and an apparent increase in granulocytes. In this case there also appeared to be an increase in the number of cells in the mixed cell population. In these experiments the granulocyte glutathione content was increased from 5.49 ± 0.72 x 10^{-16} mol/cell (n = 4) in control animals to 8.78 ± 1.04 x 10^{-16} mol/cell (n = 4). In contrast, no changes were observed in the lymphocytes, the GSH contents being 1.94 ± 0.32 x 10^{-16} mol/cell (n = 4) and 1.93 ± 0.38 x 10^{-16} mol/cell (n = 4) for control and test samples, respectively. In another experiment to assess whether changes in the granulocytes could be assessed in circulating cells the granulocytes which accumulate in the peritoneal cavity following casein injection were also assayed. Both GSH and GSH transferase were increased, the contents being

Fig. 5. Glutathione content of various mouse bone marrow cell fractions separated on a FACS in untreated animals or animals treated 5 days previously with cyclophosphamide (75 mg/kg). Experimental details are as given in Fig. 4. Values, mean ± SD (bars) for five or six determinations.
Fig. 6. FACS IV dot plots of untreated (a) and cyclophosphamide-treated (b) mouse peripheral blood, prepared as described in "Materials and Methods." The schematic diagram (c) indicates the position of the populations separated. I, erythrocytes; II, lymphocytes; III, granulocytes.

approximately 2.0- and 1.7-fold higher than controls, respectively. The glutathione values were $5.30 \pm 1.09 \times 10^{-16}$ (n = 3) and $10.39 \pm 1.87 \times 10^{-16}$ (n = 3) mol glutathione/cell, and the glutathione transferase values were $10.42 \pm 1.87$ (n = 3) and $16.87 \pm 4.53$ (n = 3) nmol 1-chloro-2,4-dinitrobenzene conjugated/min/10^6 cells for control and primed animals, respectively.

DISCUSSION

In this study we have demonstrated that significant changes in intracellular marrow glutathione and glutathione transferase activity occur following the administration of a myelotoxic priming dose of cyclophosphamide (2, 10). In agreement with literature reports (11, 12), cyclophosphamide had significant effects on bone marrow cellularity as well as the relative proportions of the various cell types. The marked loss of lymphocytes at the dose used was of particular interest. On the basis of these changes our earlier observation of increased glutathione or GSH transferase activity in marrow fractions (2) could be partially explained by a changed marrow cell population. However, increases in intracellular granulocytic GSH and GSH transferase levels would also appear to be an important factor. Glutathione is known to protect against cyclophosphamide-induced bladder damage (13) and we have recently postulated that it is also protective against the myelotoxicity of this compound (2). The lethality of high doses of cyclophosphamide is associated with granulocytopenia. The protective effect of a priming dose could therefore be explained by increased resistance of the granulocytes to toxicity conferred by their higher glutathione content. The difference in granulocytic and lymphocytic GSH content could also explain the higher susceptibility of the latter cells to cyclophosphamide.

The changes in granulocyte GSH and GSH transferase content could also be measured in cells isolated from the peripheral circulation. This fact tends to rule out the possibility that the cells separated from the marrow by the FACS within the granulocytic fraction following priming represent an enrichment of precursor cells rather than granulocytes with higher GSH content. More importantly, the finding that changes associated with priming can be monitored in the peripheral blood could be used to optimize the timing of high dose chemotherapy regimens in which drug priming is used.

The changes in GSH and GSH transferase activities in the granulocytes were even more marked on a second administration of the cytotoxin (Fig. 1). This could also be of potential clinical value. The fact that the stimulatory response can be repeated and each time follows a suppression of enzyme or cofactor level may be indicative of a feedback control mechanism leading to overproduction. Such a mechanism has been identified in the case of GSH homeostasis (14). It has also been demonstrated that cyclophosphamide administration stimulates a burst of mitotic activity (11, 12). This may also be related to the changes observed. This finding together with other literature reports (15-17) provides an increasing body of evidence that changes in GSH and GSH transferase levels in cells represent a general response to cytotoxic damage caused by radiation or alkylating agents. Increases in GSH and GSH transferase levels have been related to the initiation of chemically induced neoplastic growth (1, 18). Such increases in the tumor would undoubtedly protect it from further therapy. Indeed there are certain examples of chemically induced drug resistance which have been explained by increased glutathione levels (19, 20).

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REFERENCES

pheric genotoxicants. For further information, contact: Dr. Shahbeg S. Sandhu, Scientific Coordinator, U. S. Environmental Protection Agency, Genetic Toxicology Division, Mail Drop 68, Research Triangle Park, NC 27711. Telephone: (919)541-3850.

CHEMOTHERAPY FOUNDATION SYMPOSIUM VII

The Chemotherapy Foundation Symposium VII, entitled "Innovative Cancer Chemotherapy for Tomorrow," will be held in New York City on November 12 to 14, 1986. This meeting is presented by the Division of Medical Oncology of the Department of Medicine, the Department of Neoplastic Diseases, and the Page and William Black Postgraduate School of Medicine of the Mount Sinai School of Medicine. Registration fees are $300 for physicians and $75 for staff, fellows, and other health care professionals. The symposium meets the criteria for 20 hours in Category 1 of the Physician's Recognition Award of the American Medical Association and for 20 cognates in Formal Learning by the American College of Obstetricians and Gynecologists. For more information, contact: Director, the Page and William Black Postgraduate School of Medicine, One Gustave L. Levy Place, New York, NY 10029. Telephone: (212)650-6737 or 650-6772.

THIRTY-FIFTH ANNUAL MEETING OF THE RADIATION RESEARCH SOCIETY

The Thirty-fifth Annual Meeting of the Radiation Research Society will take place in Atlanta, Georgia, on February 22 to 26, 1987. For more information, contact: Radiation Research Society, 925 Chestnut Street, Philadelphia, PA 19107. Telephone: (215)574-3153.

FIFTH INTERNATIONAL CONFERENCE ON THE ADJUVANT THERAPY OF CANCER

The Fifth International Conference on the Adjuvant Therapy of Cancer will be held from March 11 to 14, 1987, in Tucson, Arizona. The deadline for submission of abstracts is December 1, 1986. For abstract forms and further information, contact: Mary Humphrey, Conference Coordinator, Arizona Cancer Center, University of Arizona College of Medicine, Tucson, AZ 85724. Telephone: (602)626-6044.

FUTURE MEETINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

The dates and sites for future annual meetings of the American Association for Cancer Research are: May 20 to 23, 1987, Atlanta, Georgia; May 25 to 28, 1988, New Orleans, Louisiana; May 24 to 27, 1989, San Francisco, California; May 16 to 19, 1990, Washington, DC; May 15 to 18, 1991, San Antonio, Texas; and May 13 to 16, 1992, San Diego, California.

Errata

In the article by James Carmichael et al., entitled "Glutathione and Glutathione Transferase Levels in Mouse Granulocytes following Cyclophosphamide Administration," which appeared in the February issue (Cancer Res., 46: 735–739), it should be noted that part of the work was carried out at the Imperial Cancer Research Fund Medical Oncology Unit, Western General Hospital, Edinburgh, United Kingdom.

Due to an error in the computer program which the authors used to calculate intracellular polyamine levels from high performance liquid chromatography chromatograms, the data regarding the spermine content of HT-29 cells are incorrect in the article by Jerome Seidenfeld et al., entitled "Altered Cell Cycle Phase Distributions in Cultured Human Carcinoma Cells Partially Depleted of Polyamines by Treatment with Difluoromethylornithine," which appeared in the January issue (Cancer Res., 46: 47–53). For the same reason, all of the data regarding spermine content are incorrect in another article by the same authors, entitled "Reduced Cytocidal Efficacy for Adriamycin in Cultured Human Carcinoma Cells Depleted of Polyamines by Difluoromethylornithine Treatment," which appeared in the March issue (Cancer Res., 46: 1155–1159).

As a result of this error in the computer program, the HT-29 spermine content presented in Table 2 (final column, lines 5–8) on page 51 of the January article, and all of the data regarding spermine content in Table 2 (final column) on page 1157 of the March article, are only 50% of the true values.

These corrections do not change the relative decline in spermine content caused by difluoromethylornithine treatment in either of these studies, nor do they alter the authors' conclusions regarding the effect of difluoromethylornithine treatment and polyamine depletion on cell cycle phase distributions in the January article and on the efficacy of Adriamycin in the March article.

Recent Deaths

We regret to announce the deaths of the following members of the American Association for Cancer Research: Dr. Henry G. Kaplan, Mercer Island, Washington; Dr. Nathan O. Kaplan, Chemistry Department, University of California, La Jolla, California; Dr. Clara J. Lynch, Chevy Chase, Maryland; Dr. Pablo Mori-Chavez (emeritus), Laboratorio de Oncologia, Universidad Peruana Cayetano Heredia, Lima, Peru; and Dr. Edward A. Smuckler, Department of Pathology, University of California, School of Medicine, San Francisco, California.
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