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PRELIMINARY COMMUNICATIONS

REDUCTION IN GLUTATHIONE CONTENT OF L-PAM RESISTANT L1210 CELLS CONFERS DRUG SENSITIVITY

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L-Phenylalanine mustard (L-PAM) is an effective chemotherapeutic agent used alone or in combination with other agents in the treatment of a number of neoplastic diseases (1-3). However, repeated courses of therapy often result in a decreased therapeutic response which can be accompanied by the emergence of a drug-resistant cell population. The observation that the therapeutic response of selected tumors to the alkylating agent merophan inversely correlates with the ratio of cellular protein free:protein bound sulfhydryl groups (4) prompted this investigation into whether an L-PAM resistant tumor cell can be sensitized to the drug by reducing the intracellular concentration of the principal non-protein thiol of the cell, glutathione. For these studies we have utilized murine L1210 leukemia cells, highly sensitive to L-PAM, and an L-PAM resistant variant of this tumor developed *in vivo* by repeated treatment of mice bearing the L-PAM sensitive tumor with subcurative levels of the drug. This communication describes the results of this work as well as attempts to influence the intracellular glutathione content by nutritional deprivation of L-cysteine.

MATERIALS AND METHODS

Fetal calf serum was purchased from Flow Laboratories, Rockville, MD and RPMI 1630 medium (fully supplemented with amino acids or with glycine, L-glutamic acid or L-cystine omitted) and Dulbecco's phosphate buffered saline were supplied by the NIH Media Unit. Gentamicin (Schering, 50 mg/ml) was purchased from Microbiological Associates, Bethesda, MD. L-PAM was obtained from Burroughs Wellcome Co., Research Triangle Park, NC and was prepared as a 10 mM stock in 75% ethyl alcohol containing equimolar hydrochloric acid. Reduced glutathione and glutathione disulfide were obtained from Calbiochem-Behring Corp., Los Angeles, CA. NADPH, Type IV glutathione reductase and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from the Sigma Chemical Company, St. Louis, MO. 2-Vinylpyridine was obtained from Aldrich Chemical Company, Inc. Milwaukee, WI.

Tumor Transplantation and In Vitro Growth of Murine L1210 Leukemia Cells. The murine L1210 leukemia was obtained under contract from the Mason Research Institute, Boston, MA and was maintained in female DBA/2 mice by weekly intraperitoneal injection of 10^5 cells. Approximately 50% of mice bearing this tumor are cured following single injections of 13 mg/kg L-PAM 1 day following tumor inoculation (5). This tumor is designated L1210 in this communication. An L-PAM resistant tumor was developed at the Southern Research Institute, Birmingham, AL and was also maintained in female DBA/2 mice by weekly intraperitoneal injection of 10^6 cells. Mice bearing the tumor received an intraperitoneal injection of L-PAM (7.5 mg/kg) 2 days after tumor inoculation. This tumor is designated L1210/L-PAM$_1$ in this communication. One L-PAM resistant variant (L1210/L-PAM$_2$) was isolated from a single colony following clonal growth of L1210/L-PAM$_1$ in soft nutrient agar containing 50 μM β-mercaptoethanol and 2.5 μg/ml L-PAM for 2 weeks according to the procedure of Chu and Fischer (6). This L-PAM resistant variant has been maintained *in vitro* in RPMI 1630

medium supplemented with 16% heat-inactivated fetal calf serum containing 5.0 μg/ml L-PAM at a subculture interval of 2 days. The growth medium was supplemented with 50 μM β-mercaptoethanol.

Determination of Intracellular Reduced Glutathione and Glutathione Disulfide. Murine L1210 leukemia cells were harvested from experimental growth medium, washed twice in Dulbecco's phosphate buffered saline (pH 7.4) and lysed in 0.75 ml of distilled water. Cellular protein was precipitated by addition of 0.25 ml of 12% sulfosalicylic acid and removed by centrifugation at 12,000 x g for one minute. Total glutathione and glutathione disulfide in the supernatant were assayed by the method of Griffith (7) with minor modifications. The incubation mixture for determination of total glutathione consisted of 100 μl of the protein-free supernatant of the cell lysate, 100 μl of 1 M triethanolamine HCl buffer (pH 8.0), 700 μl of 0.3 mM NADPH, 100 μl of 6 mM DTNB and 0.48 units of glutathione reductase. All reagents were prepared in 125 mM sodium phosphate buffer containing 6.3 mM sodium EDTA (pH 7.5). The absorbance of 2-nitro-5-thiobenzoic acid at 412 nm was monitored on a Gilford Model 240 recording spectrophotometer. The concentration of glutathione disulfide was determined after derivatizing reduced glutathione with 2-vinylpyridine. Two hundred fifty microliters of the protein-free supernatant of the cell lysate, 250 μl of 1 M triethanolamine HCl buffer (pH 8.0) and 5 μl of 2 M 2-vinylpyridine were mixed vigorously for 1 minute (final pH 7.0-7.5). The mixture was incubated at 25°C for one hour, and then 400 μl of 0.5 mM NADPH, 100 μl of 6 mM DTNB and 0.48 units of glutathione reductase were added. The formation of 2-nitro-5-thiobenzoic acid was determined as described above.

RESULTS AND DISCUSSION

Sensitivity of Murine L1210 Leukemia Cells to L-Phenylalanine Mustard and the Content of Intracellular Glutathione. The L-PAM resistant tumor, L1210/L-PAM$_1$, and the L-PAM resistant variant, L1210/L-PAM$_2$, are 5-fold and 8-fold more resistant to the drug than is the L-PAM sensitive parent tumor (Table 1). This increased resistance to L-PAM is accompanied by 2-fold and 4-fold increases in the cellular content of reduced glutathione as well as substantial increases in the content of glutathione disulfide. These results suggest that resistance to L-PAM and the elevated glutathione levels may be causally related.

TABLE 1. Reduced glutathione and glutathione disulfide content of murine L1210 leukemia cells sensitive and resistant to L-phenylalanine mustard

L1210 Variant	LD_{37} of L-Phenylalanine Mustard μg per ml	Reduced Glutathione nmole per 10^6 cells	Glutathione Disulfide pmole per 10^6 cells
L1210	1.0*	2.9 ± 1.1**	74.6 ± 15.4***
L1210/L-PAM$_1$	4.8	5.8 ± 2.0	139.7 ± 34.6
L1210/L-PAM$_2$	8.0	11.2	208.3

*Cells, at a concentration of 2.25 x 10^5 cells/ml in RPMI 1630 medium supplemented with 16% heat-inactivated fetal calf serum and 50 μM β-mercaptoethanol, were exposed to L-PAM for 48 hours. Cells were then washed twice in fresh medium containing 20% heat-inactivated fetal calf serum, 50 μM β-mercaptoethanol and cell survival was assessed following growth of surviving cells for 2 weeks in nutrient soft agar (6).

**Mean ± standard deviation; n = 11.

***Mean ± standard deviation; n = 6.

Reduction of Intracellular Glutathione and Sensitization of the Resistant Tumor Cell to L-PAM. In order to determine whether the cytotoxic potency of L-PAM is determined by the intracellular concentration of glutathione an attempt was made to reduce the cellular glutathione content of L-PAM resistant cells and to determine whether such a reduction is accompanied by an increase in the cytotoxic potency of the drug. Cells were grown in RPMI 1630 medium with a reduced concentration† of glycine, L-glutamic acid or L-cystine, the latter amino acid serving as a source of cysteine for glutathione biosynthesis. Incubation of cells in growth medium with a reduced concentration of L-cystine resulted in a rapid decrease in the intracellular content of glutathione disulfide in both the L-PAM sensitive tumor and in the resistant tumor, L1210/ L-PAM$_1$ (Figure 1). After 6 hours of incubation the intracellular concentrations were equivalent. The cellular content of reduced glutathione also decreased, and after 18 hours incubation in medium with a reduced concentration of L-cystine, both the L-PAM sensitive tumor and L1210/L-PAM$_1$ had an identical intracellular content of reduced glutathione (Figure 1). Attempts to reduce the cellular glutathione content by incubation of cells in medium with a reduced concentration of glycine or L-glutamic acid were not as successful (data not shown).

L-PAM was equitoxic to L-PAM resistant cells incubated in medium with a reduced concentration of L-cystine for 24 hours to reduce the intracellular glutathione content and L1210 cells grown in medium with L-cystine (Figure 2).

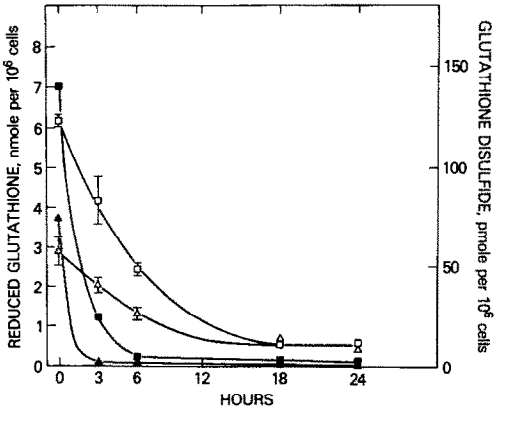

Figure 1. Lowering of intracellular reduced glutathione and glutathione disulfide. L1210 cells (triangles) and L1210/L-PAM$_1$ cells (squares) were washed 3 times in RPMI 1630 growth medium with a reduced concentration of L-cystine and suspended in it at a cell concentration of 2.5 x 10^5 cells/ml. Cells were harvested at the indicated time points and the intracellular content of reduced glutathione (△,□) and glutathione disulfide (▲,■) was determined as described in Materials and Methods.

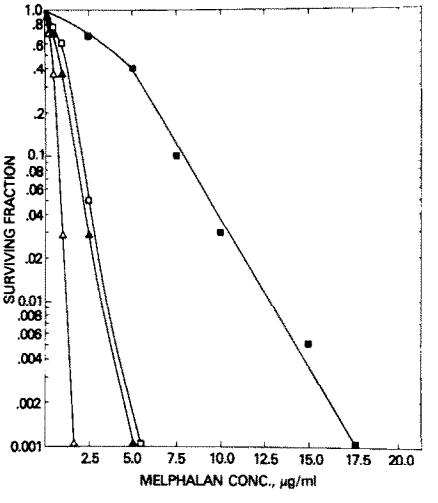

Figure 2. Identical intracellular glutathione content results in an equitoxic response to L-PAM. L1210 cells (triangles) and L1210/L-PAM$_1$ cells (squares) were washed 3 times in RPMI 1630 growth medium with 100 mg/L L-cystine (▲,■) or with a reduced concentration of L-cystine (△,□) and then incubated for 24 hrs in the same medium at a cell concentration of 2.25 x 10^5 cells/ml. Cells were then washed in fresh growth medium containing the respective concentration of L-cystine, the cell concentration adjusted to 2.25 x 10^5 cells/ml and the indicated concentrations of L-PAM added. An equal volume of 75% ethyl alcohol was added to control cultures. The cells were harvested 2 days following exposure to L-PAM and washed with fresh RPMI 1630 medium containing 100 mg/L L-cystine and 50 μM β-mercaptoethanol. The cytotoxicity of L-PAM was assessed following clonal growth of surviving cells in nutrient soft agar according to the procedure of Chu and Fischer (6).

The results described in this communication indicate that an L-PAM resistant tumor cell can be completely sensitized to L-PAM by reducing the intracellular concentration of glutathione. The possibility is raised that an improved therapeutic response to the drug can be achieved by limiting glutathione biosynthesis, either by reducing the supply of cysteine available to the tumor cell or by using specific inhibitors of its synthesis.

FOOTNOTE

†RPMI 1630, prepared without glycine, L-glutamic acid or L-cystine, was supplemented with 16% fetal calf serum. The serum contributes amino acids to the growth medium and therefore the medium is not entirely free of them.

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