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HEPATIC-MEDIATED ELEVATION AND MAINTENANCE OF METASTATIC TUMOR CELL GLUTATHIONE*

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Abstract—Metastatic migration of murine L1210 leukemia cells, sensitive and resistant to the antitumor agent L-phenylalanine mustard, from the peritoneal cavity of mice to the liver resulted in a 2-fold elevation in their GSH content. This increase in GSH was accompanied by a corresponding increase in their resistance to the drug. Cell surface binding studies with the non-penetrating disulfide, 6,6′-dithiodinicotinic acid, indicated that both tumors isolated from the liver had a greater than 5-fold elevation in surface sulfhydryls when compared to their ascitic counterparts. These results indicate a role for the hepatic microenvironment in the maintenance of tumor cell GSH, drug responsiveness, and surface sulfhydryls.

An understanding of host: tumor interactions which affect chemotherapy is essential if selective eradication of tumor cells is to be achieved. Such successful treatment regimens depend not only on elimination of drug-sensitive tumor cells but also on those resistant tumor cells present prior to chemotherapy and tumor cells which develop resistance following the initial chemotherapeutic regimen.

Studies in this laboratory have addressed the latter problem of acquired resistance of tumor cells to selected alkylating agents including L-phenylalanine mustard (L-PAM†). These studies with murine L1210 leukemia cells [1, 2] and human ovarian cancer cell lines established from patients exhibiting clinical signs of drug resistance [3] indicate that resistance is not accompanied by decreases in drug uptake by, or increased efflux from, the resistant tumor cell but is related to conversion of L-PAM to its non-cytotoxic derivative 4-[bis(2-hydroxyethyl)amino]L-phenylalanine. Such conversion correlates with a 2- to 3-fold increase in the intracellular concentration of the tripeptide glutathione (GSH) in the resistant tumor cell. These observations prompted studies designed to reduce intracellular GSH by either nutritional deprivation of L-cysteine [1] or pharmacological intervention with DL-buthionine-*S*,*R*-sulfoximine (BSO) [4], an inhibitor of γ-glutamylcysteine synthetase, a key enzyme in the γ-glutamyl cycle.

In vivo studies with tumor-bearing animals indicated that sustained infusion of BSO results in sensitization of L-PAM resistant tumor cells within the peritoneal cavity to L-PAM but is accompanied by only minimal increases in the life span of animals bearing the tumor [5]. The failure to observe greater increases in the life span of these animals was due to metastatic infiltration into and growth within specific host organs, notably the liver. This marked insensitivity to chemotherapeutic eradication prompted the current study which was designed to examine the possibility that the hepatic microenvironment regulates the thiol status and drug sensitivity of tumor cells located within the liver.

MATERIALS AND METHODS

Fraction V bovine serum albumin (BSA) and its sulfhydryl modified counterpart were obtained from Miles Laboratories Inc., Elkhart, IN. Fetal bovine serum was purchased from Advanced Biotechnologies Inc., Silver Spring, MD. RPMI 1630 medium, Williams Medium E, Hanks' balanced salt solution without calcium and magnesium and Dulbecco's phosphate-buffered saline (PBS) with and without calcium and magnesium were supplied by Gibco Laboratories, Chagrin Falls, OH. L-Phenylalanine mustard (L-PAM) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NIH, Bethesda, MD. Glutathione (GSH) was obtained from the Calbiochem-Behring Corp., Los Angeles, CA. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), type IV glutathione reductase, and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from the Sigma Chemical Co., St. Louis, MO. Collagenase (CLS II) (125–150 units/mg) was purchased from Cooper Biomedical, Malvern, PA. Versilube F-50 silicone oil was obtained from the General Electric Co., Waterford, NY. 6,6′-Dithiodinicotinic acid was purchased from the Aldrich Chemical Co., Milwaukee, WI. [*carboxyl*-^{14}C]-6,6′-Dithiodinicotinic acid, sp. act. 51 mCi/mmole, was obtained from Research Products International, Mt. Prospect, IL.

Tumor transplantation. The murine L1210 leukemia was obtained from the Tumor Repository,

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† Abbreviations: L-PAM, L-phenylalanine mustard; BSA, bovine serum albumin; PBS, phosphate-buffered saline; GSH, glutathione; GSSG, glutathione disulfide; NADPH, nicotinamide adenine dinucleotide phosphate, reduced; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); and BSO, DL-buthionine-*S*,*R*-sulfoximine.

Frederick Cancer Research Center (FCRC), Frederick, MD, and was maintained in male CDF_1 mice by weekly intraperitoneal injection of 10^5 cells. An L-PAM resistant variant of this tumor was developed at the Southern Research Institute and obtained from FCRC. The resistant tumor was used for selected studies and was also maintained in male CDF_1 mice by weekly intraperitoneal injection of 10^6 cells. Mice bearing this tumor received an intraperitoneal injection of 7.5 mg/kg L-PAM 48 hr following tumor inoculation.

Isolation of murine L1210 leukemia cells. Male CDF_1 mice bearing 6-day tumors were anesthesized with chloral hydrate (0.5 g/kg). The ascitic fluid, containing tumor cells, was aspirated from the peritoneal cavity with two 5-ml aliquots of calcium- and magnesium-free Dulbecco's PBS containing 1 mg/ml sulfhydryl modified BSA and 10 mM glucose (pH 7.4). The peritoneal cavity of the mouse was then rinsed thoroughly with Dulbecco's PBS, and the liver was perfused with collagenase according to the two-step procedure of Maslansky and Williams [6]. Following the perfusion, the liver was removed and teased apart in calcium- and magnesium-free Dulbecco's PBS containing 1 mg/ml sulfhydryl modified BSA and 10 mM glucose. The cell suspension containing L1210 tumor cells and hepatocytes was filtered through gauze to remove any large aggregates of cells, harvested by centrifugation at 300 g, and washed twice in the same buffer system. The cell mixture was then resuspended in 50 ml of the same buffer system, transferred to a 50-ml Falcon conical centrifuge tube, and allowed to stand undisturbed at 20° for 90 min. The top 30 ml was removed, and a small aliquot was examined microscopically for tumor cell purity and viability using trypan blue dye exclusion. This procedure routinely resulted in obtaining cell preparations containing >99% tumor cells exhibiting viability of 99–100%. The yield of tumor cells using this procedure was approximately $3–5 \times 10^7$ cells/liver. Cells, isolated from the ascites and liver as described here, were used for GSH determination as well as cytotoxicity and radiolabeled studies described below.

Determination of cellular glutathione. Murine L1210 leukemia cells derived from the ascites and liver were centrifuged for 6 min at 300 g, and any red blood cells were removed by a 5-min hypertonic lysis of the cell suspension (2.0×10^6 tumor cells/ml) in 0.87% ammonium chloride. Cells (1×10^7) were lysed in 900 μl of distilled water by vigorous vortexing for 5 min. Cellular protein was precipitated by addition of 100 μl of 30% sulfosalicylic acid, and the mixture was allowed to stand at 4° for 15 min. Protein was removed by centrifugation at 12,000 g for 2 min, and the total glutathione (GSH + GSSG) content was determined by the method of Griffith [7] with minor modifications. The incubation mixture for the determination of total glutathione consisted of 100 μl of the protein-free supernatant fraction of the cell lysate, 100 μl of 125 mM sodium phosphate buffer containing 6.3 mM sodium EDTA (pH 7.5), 700 μl of 0.3 mM NADPH, 100 μl of DTNB and 0.5 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 Beckman model 34 recording spectrophotometer.

Binding of [^{14}C]-6,6′-dithiodinicotinic acid to murine L1210 leukemia cells isolated from the ascites and liver. L1210 cells from the ascites and liver were centrifuged at 300 g for 6 min, and any red blood cells were removed by hypertonic lysis of the cell suspension as described above. Cells were then washed twice in Dulbecco's PBS containing 1 mg/ml sulfhydryl modified BSA and 10 mM glucose. Cells (1.5×10^7 cells/ml) were exposed to 100 μM [^{14}C]-6,6′-dithiodinicotinic acid with gentle agitation for 15 min at 37° in Dulbecco's PBS containing 10 mM glucose (pH 7.2). Cells were then washed twice in Dulbecco's PBS containing 1 mg/ml sulfhydryl modified BSA and 10 mM glucose (pH 7.2) and suspended in the same medium with or without 1 mM GSH for 30 min at 37°.

Cells (1.0×10^7) were layered onto Versilube F-50 silicone oil and pelleted by centrifugation for 2 min at 12,000 g. The supernatant fraction, containing [^{14}C]-6-thiopyridone-3-carboxylic acid was counted by liquid scintillation spectrometry.

Comparative cytotoxicity of L-phenylalanine mustard toward murine L1210 leukemia cells isolated from the ascites and liver. Murine L1210 cells from the ascites and liver were washed twice in complete growth medium consisting of RPMI 1630, 20% heat-inactivated fetal bovine serum, 50 μg/ml gentamicin and 50 μM β-mercaptoethanol. The cytotoxicity of L-PAM was evaluated following a 2-day exposure to the drug in the same medium. Cells were harvested and washed in RPMI 1630 growth medium, and cell survival was assessed by growth of surviving cells in soft nutrient agar for 2 weeks according to the procedure of Chu and Fischer [8].

Influence of 6,6′-dithiodinicotinic acid on L-PAM cytotoxicity. Murine L1210 cells from the liver were washed twice in Dulbecco's PBS containing 1 mg/ml sulfhydryl modified BSA and 10 mM glucose and resuspended in the same medium with or without 250 μM 6,6′-dithiodinicotinic acid at a cell concentration of 3.5×10^5 cells/ml. All incubations were carried out for 30 min at 37°. L-PAM was added and the incubation continued for an additional 35 min. The cells were harvested and washed twice in complete growth medium, and the cytotoxicity of L-PAM was assessed as described above.

RESULTS

Differential GSH content and drug sensitivity of ascites and liver isolates of L1210 cells. Murine L1210 leukemia cells isolated from the liver had a 2-fold greater GSH content than tumor cells in the ascitic fluid of the peritoneal cavity (Table 1). The increased GSH content of the liver isolates was accompanied by a 2-fold increase in their resistance to L-PAM (Fig. 1A). A similar differential in GSH content existed in L-PAM resistant tumor cells isolated from the ascitic fluid and liver (Table 1) and was accompanied by increased resistance of these liver isolates to the drug (Fig. 1C).

To assess the role of the environment of the liver in the elevation of tumor cell GSH and increased

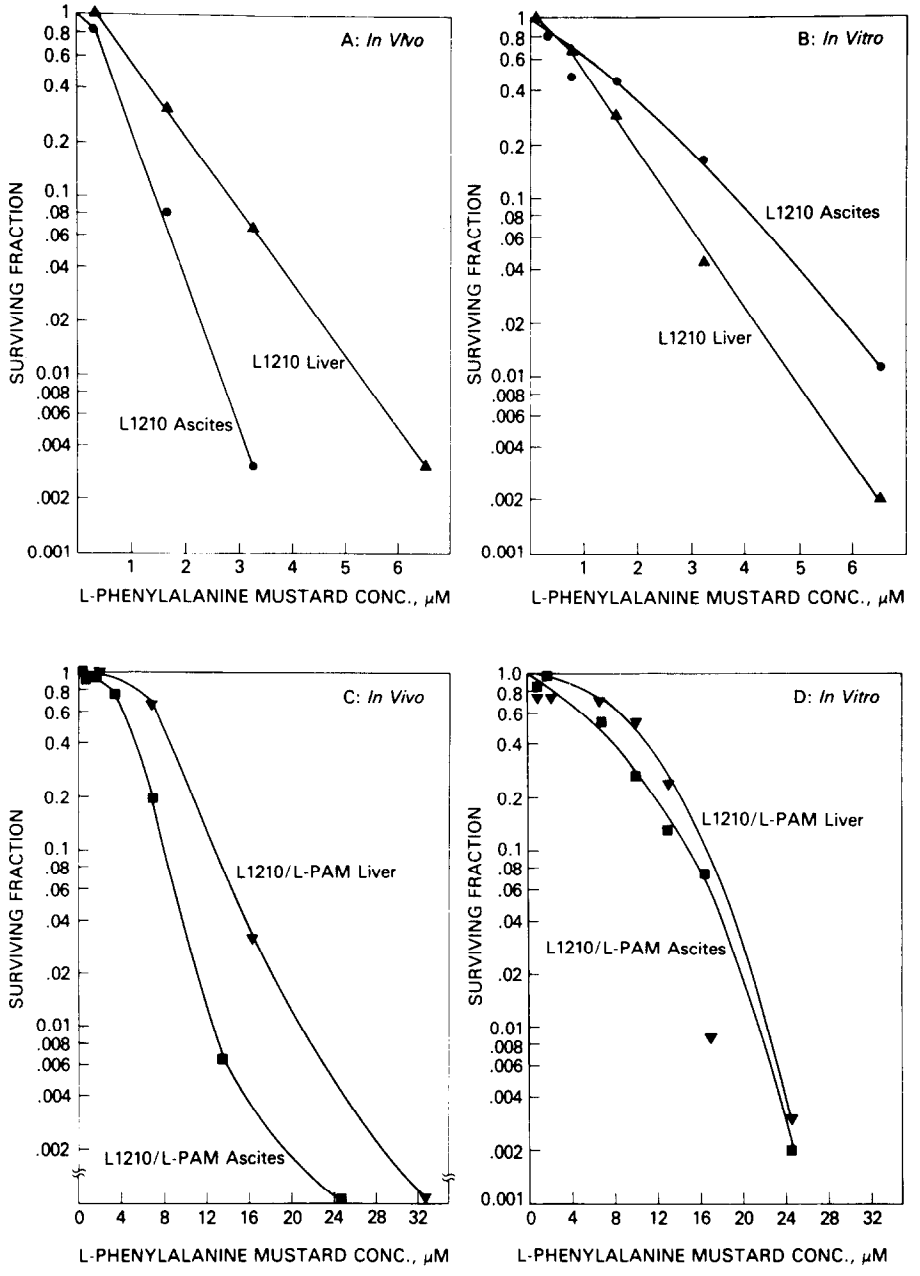

Fig. 1. L-Phenylalanine mustard sensitivity of murine L1210 and L1210/L-PAM leukemia cells isolated from the ascites and liver. Murine L1210 and L1210/L-PAM leukemia cells, isolated from the ascites and liver as described in Materials and Methods, were exposed to the indicated concentration of L-PAM at a cell concentration of 5.0×10^5 cells/ml for 2 days in RPMI 1630 medium containing 20% heat-inactivated fetal bovine serum and 50 μM β-mercaptoethanol. L-PAM cytotoxicity was determined following growth of surviving cells in nutrient-agar for 2 weeks according to the procedure of Chu and Fischer [8]. *In vitro* dose–response curves (panels B and D) were determined following growth of cells for 2 days prior to drug exposure in RPMI 1630 containing 20% heat-inactivated fetal bovine serum and 50 μM β-mercaptoethanol. Key: (●) L1210 ascites; (▲) 1210 liver; (■) L1210/L-PAM ascites; and (▼), L1210/L-PAM liver.

resistance to L-PAM, L1210 tumor cells were isolated from the ascites and liver and were then cultured for 48 hr *in vitro*; both their GSH content and sensitivity to L-PAM were determined. As can be seen in Table 1, the GSH content of L1210 cells isolated from the ascites increased 5-fold *in vitro* and was accompanied by a significant increase in their resistance to L-PAM (Fig. 1B). Similarly, the glutathione content of L1210/L-PAM cells from the ascites increased 2-fold *in vitro*, and this increase resulted in increased resistance of these tumor cells to L-PAM (Fig. 1D). These results contrasted with those obtained with L1210 and L1210/L-PAM cells isolated from the liver. Although increases in GSH were observed

Table 1. Glutathione content of murine L1210 and L1210/L-PAM leukemia cells isolated from ascites and liver

Tumor	GSH (nmoles/10^6 cells)	
	In vivo	*In vitro*
Sensitive		
L1210 ascites	1.27 ± 0.22[a]	6.8 ± 1.7[b]
L1210 liver	2.8 ± 0.3[c]	5.9 ± 0.8[d]
Resistant		
L1210/L-PAM ascites	3.6 ± 0.3[e]	6.6 ± 0.1[f]
L1210/L-PAM liver	5.2 ± 0.1[g]	5.8 ± 0.2

Male CDF_1 mice were inoculated with 1×10^5 cells (L1210) or 1×10^6 cells (L1210/L-PAM) on day 0. Tumor cells were isolated from the liver following perfusion with collagenase on day 7 and separated from hepatocytes as described in Materials and Methods. GSH was determined by the method of Griffith [7] following removal of red blood cells by hypertonic lysis with ammonium chloride as described in Materials and Methods. GSH determinations designated *in vivo* were performed immediately after isolation of cells from the host, whereas *in vitro* GSH was determined following growth of cells for 2 days in RPMI 1630 containing 20% heat-inactivated fetal bovine serum and 50 μM β-mercaptoethanol. Values are mean ± S.D. from three separate experiments. Data were analyzed for statistical significance by Student's *t*-test. Key: a—significantly different from b and c ($P < 0.01$); c—significantly different from d ($P < 0.01$); and e—significantly different from f and g ($P < 0.01$).

when these cells were cultured *in vitro* (Table 1), this was not accompanied by any increase in resistance of these cells to L-PAM (Fig. 1, panels B and D).

Cellular binding of 6,6′-dithiodinicotinic acid and its influence on L-PAM cytotoxicity. The results described above indicate that metastatic migration of tumor cells from the peritoneal cavity to the liver resulted in both an elevation in GSH and an increase in their resistance to L-PAM. In an attempt to establish a possible relationship between GSH and surface sulfhydryl groups, we examined the binding of 6,6′-dithiodinicotinic acid, a non-penetrating disulfide [9], to tumor cells isolated from the ascites and liver. As can be seen in Table 2, both L-PAM sensitive and resistant tumor cells isolated from the liver bound in excess of 5-fold more of the disulfide than did their ascitic counterparts. This increased binding of 6,6′-dithiodinicotinic acid by the sensitive tumor cells isolated from the liver was found only *in vivo* as evidenced by a 13-fold decrease in its binding following *in vitro* culture of the tumor cells (Table 2). Exposure of drug-sensitive tumor cells isolated from the liver to 6,6′-dithiodinicotinic acid both prior to and following L-PAM treatment resulted in a greater than 2-fold sensitization of the liver isolates (Fig. 2).

DISCUSSION

The results described in the present paper indicate that metastatic migration of tumor cells from the peritoneal cavity and infiltration into the liver resulted in increases in both the GSH and surface sulfhydryl content of L-PAM sensitive and resistant murine L1210 leukemia cells. These increases were accompanied by a 2-fold increase in the resistance of these tumor cells to L-PAM. The observation that the elevation in GSH, surface sulfhydryls, and L-PAM resistance occurred only in cells following their isolation from the liver strongly suggests that the hepatic microenvironment is responsible. The results described here do not identify the mechanism of the hepatic-mediated increase in tumor cell GSH. However, the large increase in GSH which occurred when ascitic tumor cells were cultured *in vitro* suggests that conditions for GSH biosynthesis are more favorable than *in vivo*. Concentrations of one or more of the amino acids utilized for synthesis of the tripeptide, i.e. cysteine, glycine or glutamic acid, may be limiting *in vivo*. A similar rationale may explain the observed difference in the GSH content of tumor cells isolated from the ascites and liver. The liver may supply precursor amino acids for GSH synthesis to the tumor cells following their infiltration into the liver. Indeed, it has been shown that rat liver GSH can serve as a reservoir for cysteine [10].

The present studies with sensitive and resistant tumor cells from the ascites suggest that acquisition of resistance to L-PAM is associated with a slight decrease in surface sulfhydryls. This result suggests

Table 2. Hepatic-mediated elevation of surface sulfhydryls in L1210 and L1210/L-PAM leukemia cells isolated from ascites and liver

Tumor	6,6′-Dithiodinicotinic acid (pmoles/10^7 cells)	
	In vivo	In vitro
Sensitive		
L1210 ascites	33	31
L1210 liver	179	14
Resistant		
L1210/L-PAM ascites	18	ND*
L1210/L-PAM liver	100	ND

Murine L1210 leukemia cells from the ascites and liver (1.5×10^7 cells/ml) were exposed to 100 μM [^{14}C]-6,6′-dithiodinicotinic acid for 15 min at 37° in Dulbecco's PBS containing 10 mM glucose. Cells were processed as outlined in Materials and Methods.

* Not determined.

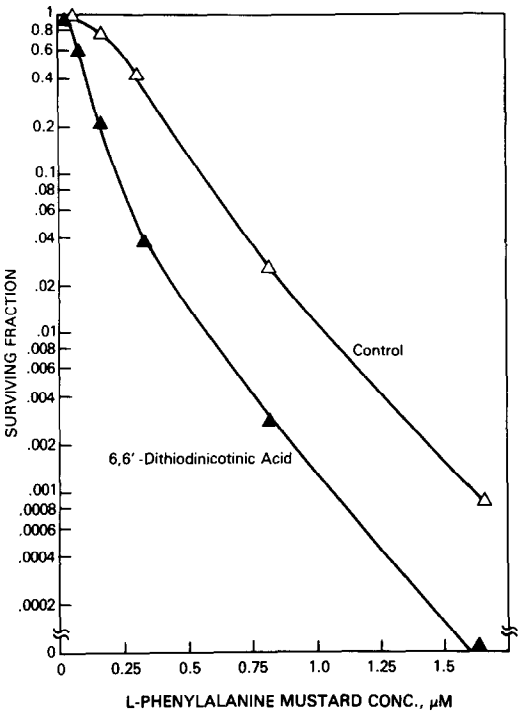

Fig. 2. 6,6′-Dithiodinicotinic acid sensitization of L1210 cells isolated from the liver to L-phenylalanine mustard. Murine L1210 leukemia cells, isolated from the liver as described in Materials and Methods, were washed twice in Dulbecco's PBS containing 1 mg/ml sulfhydryl modified BSA and 10 mM glucose. Cells (3.5×10^5 cells/ml) were exposed to 250 μM 6,6′-dithiodinicotinic acid in the same medium for 30 min, the indicated concentration of L-PAM was added, and the incubation was continued for an additional 35 min. The cells were then washed in RPMI 1630 growth medium containing 75 μM 6,6′-dithiodinicotinic acid and grown for 2 days in the same medium *containing* 6,6′-dithiodinicotinic acid. Cell survival was assessed by clonal growth of surviving cells for 2 weeks according to the procedure of Chu and Fischer [8]. Key: (△) L1210 liver, and (▲) L1210 liver plus continuous exposure to 6,6′-dithiodinicotinic acid. Removal of 6,6′-dithiodinicotinic acid following exposure of the cells to L-PAM resulted in no sensitization.

that blocking surface sulfhydryls with 6,6′-dithiodinicotinic acid should result in an increase in resistance to L-PAM. However, the present study clearly demonstrates that pretreatment of cells with the disulfide for a time period sufficient to allow reaction with surface sulfhydryls did not affect L-PAM sensitivity. The requirement for additional exposure of cells to 6,6′-dithiodinicotinic acid following drug treatment in order to achieve sensitization suggests that the ability of the disulfide to sensitize may not involve surface sulfhydryls at all or perhaps may require reaction with sulfhydryl groups located intracellularly or within the plasma membrane.

The role of the increased surface sulfhydryls within the liver in the response of tumor cells to L-PAM is unclear at the present time. The observation that a 13-fold reduction in their surface sulfhydryls, accomplished by *in vitro* growth of the cells, was not accompanied by a change in L-PAM resistance argues against a determinant role for them. Their role, if unrelated to drug cytotoxicity, may reside in increasing the cellular adhesiveness of tumor cells within the hepatic environment. It has been demonstrated that surface sulfhydryl groups play a critical role in the cellular adhesiveness of chick embryonic liver and kidney cells [11]. The possibility that the sulfhydryl groups in metastatic L1210 cells participate in increasing their adhesiveness and retention within the hepatic environment is under current investigation.

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REFERENCES

1. K. Suzukake, B. J. Petro and D. T. Vistica, *Biochem. Pharmac.* **31**, 121 (1982).
2. K. Suzukake, B. P. Vistica and D. T. Vistica, *Biochem. Pharmac.* **32**, 165 (1983).
3. J. A. Green, D. T. Vistica, R. C. Young, T. C. Hamilton, A. M. Rogan and R. F. Ozols, *Cancer Res.* **44**, 5427 (1984).
4. O. W. Griffith and A. Meister, *J. biol. Chem.* **254**, 7558 (1979).
5. S. Somfai-Relle, K. Suzukake, B. P. Vistica and D. T. Vistica, *Biochem. Pharmac.* **33**, 485 (1984).
6. C. J. Maslansky and G. M. Williams, *In Vitro* **18**, 683 (1982).
7. O. W. Griffith, *Analyt. Biochem.* **106**, 207 (1980).
8. M-Y. Chu and G. A. Fischer, *Biochem. Pharmac.* **17**, 753 (1968).
9. J. N. Mehrishi and D. R. Grassetti, *Nature, Lond.* **224**, 563 (1969).
10. N. Tateishi, T. Higashi, A. Naruse, K. Nakashima, H. Shiozaki and Y. Sakamoto, *J. Nutr.* **107**, 51 (1977).
11. J. V. George and K. V. Rao, *J. cell. Physiol.* **85**, 547 (1975).