Depletion of tumour glutathione *in vivo* by buthionine sulfoximine: modulation by the rate of cellular proliferation and inhibition of cancer growth

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We have investigated in Ehrlich-ascites-tumour-bearing mice the effect of buthionine sulfoximine (BSO), a selective inhibitor of GSH synthesis, on the rate of GSH depletion of tumour versus normal tissues and its relation to tumour cell proliferation. In normal tissues, GSH and GSSG remain unchanged or close to normal values during tumour growth, even at the last stage of growth when the animal is close to death. After administration of a single dose of BSO (4 mmol/kg), the rates of GSH depletion and recovery in the tumour and in several normal tissues are very different. BSO depletes GSH in cancer cells to a level of

0.3–0.4 $\mu\text{mol/g}$. The fall in GSH levels is faster when tumour cells do not proliferate actively. Four treatments of 4 mmol of BSO/kg at 48 h intervals induce a significant decrease (about 44 %) in tumour growth. Our data show that the rate of BSO-induced GSH depletion in cancer cells depends on the stage of tumour growth, and that BSO administration also inhibits cancer-cell proliferation. A mechanism involving changes in protein kinase C activity and intracellular pH is proposed to explain the inhibition of cancer growth elicited by BSO.

INTRODUCTION

GSH, the major non-protein thiol in mammalian cells, is involved in many cellular functions, i.e. bioreductive reactions, maintenance of enzyme activity, amino acid transport, protection against oxidative stress, and detoxification of xenobiotics (Meister, 1983; Sies, 1986; Deneke and Fanburg, 1989). The intracellular concentration of this tripeptide plays an important role in determining the sensitivity of cells to radiation and drug-induced cytotoxicity (Arrick and Nathan, 1984; Mitchell and Russo, 1987). In fact, induction of glutathione deficiency in tumours has been postulated as potentially useful in cancer therapy (Meister, 1983).

GSH depletion to very low levels can be achieved by using L-buthionine (S,R)-sulfoximine (BSO), a selective inhibitor of γ -glutamylcysteine synthetase (Meister, 1983). Some studies *in vitro* using human tumour cell lines have demonstrated that depletion of cellular GSH by BSO can indeed increase the sensitivity to cytotoxic chemotherapy and radiation (Crook et al., 1986; Mitchell et al., 1989). Furthermore, after BSO administration *in vivo*, significant variations in the rate and degree of GSH depletion and recovery among cancer versus normal tissues have been shown (Lee et al., 1987). The results shown by Lee et al. (1987), using three different tumour models in mice, suggested that the optimal use of BSO as an adjuvant to selected cancer treatment modalities requires a detailed knowledge of the GSH kinetics in normal tissues and tumours. However, these studies did not report whether GSH kinetics or the rate of tumour cell proliferation influence the effect of BSO *in vivo* on normal or cancer cells. Recently, we have studied the glutathione metabolism in cancer cells during the progression of growth of an Ehrlich ascites tumour *in vivo* (Estrela et al., 1992). GSH, but

not GSSG, content decreases when cell proliferation and the rate of protein synthesis in the tumour decrease (Estrela et al., 1992). Indeed, elevation of intracellular GSH has been associated with mitogenic stimulation (Shaw and Chou, 1986). In the present paper, we have used Ehrlich-ascites-tumour-bearing mice to investigate the rate of depletion of glutathione *in vivo* by BSO in tumour versus normal tissues, and its relation to tumour growth and cell proliferation.

MATERIALS AND METHODS

Animals and tumour inoculation

Adult male mice were purchased from IFFA CREDO (Madrid, Spain) and placed in individual cages. The animals, fed *ad libitum* on a stock laboratory diet (Letica, Barcelona, Spain), were kept on a 12 h/12 h light/dark cycle with the temperature maintained at 22 °C. All experiments were carried out or started between 10:00 and 12:00 h to minimize the effects of diurnal variation. The Ehrlich ascites tumour was kindly provided by the Department of Pathology (Universidad de Valencia) and inoculated intraperitoneally as previously described (Estrela et al., 1992). Approx. 25×10^6 viable cells were injected into each mouse. At the moment of inoculation, mouse weight was 28.1 ± 0.3 g ($n = 12$). All injected mice developed an ascites tumour. The animals died 15.3 ± 0.5 days ($n = 12$) after inoculation of the tumour.

Control data related to growth of the Ehrlich ascites tumour *in vivo*, i.e. body weight, carcass weight, tumour volume, tumour cell density, were similar to those previously published (Lopes et al., 1989; Estrela et al., 1992). For these measurements, animals were killed by cervical dislocation and all the ascitic fluid was collected into a known volume of ice-cold Krebs–Henseleit

Abbreviations used: BSO, L-buthionine (S,R)-sulfoximine; GSH ester, glutathione monoisopropyl(glycyl) ester; PKC, protein kinase C; pH_i, intracellular pH; [¹⁴C]DMO, [¹⁴C]dimethylloxazolidine-2,4-dione.

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bicarbonate medium (pH 7.4) without added Ca^{2+} or Mg^{2+} and containing 0.5 mM EGTA (4 ml of buffer/ml of ascitic fluid containing the tumour cells). This washing procedure avoided intercellular adherence (Estrela et al., 1992). Residual cells were washed out of the peritoneum with 5–10 ml of buffer and added to the cell suspension. The volume of the tumour was easily obtained by volume difference. After removal of the tumour, the remaining carcass was weighed. Cell number was estimated by using a Bürker chamber.

Drug administration

BSO, obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), was dissolved in physiological saline solution, and injected intraperitoneally. The volume injected intraperitoneally either into controls or into BSO-treated animals was never higher than 0.008 ml/g body wt.

Glutathione monoisopropyl(glycyl) ester (GSH ester) was prepared as previously described (Mårtensson and Meister, 1989) and injected intraperitoneally in an iso-osmolar solution (pH 6.7–6.8).

Isolation and incubation of tumour cells

Tumour cells were collected from tumour-bearing mice, and then isolated and incubated at 37 °C in Krebs–Henseleit bicarbonate medium (pH 7.4) as previously described (Estrela et al., 1992). Under our experimental conditions we determined cell membrane integrity by measuring Trypan Blue exclusion (cell viability was always > 95%) and leakage of lactate dehydrogenase (always less than 3% of the total activity measured in the incubation flask) from incubated cells.

To separate intracellular and extracellular compartments we used a variation (Estrela et al., 1992) of the technique originally described by Zuurendonk and Tager (1974).

Sample preparation, metabolite and enzyme assays

Mice were killed by cervical dislocation at various times after BSO treatment. Tumour cells were rapidly washed twice at 4 °C in Krebs–Henseleit bicarbonate medium (pH 7.4) without added Ca^{2+} or Mg^{2+} and containing 0.5 mM EGTA, and finally 6% HClO_4 was added to the pellet. Organs or tissues were quickly dissected and removed, washed in the same medium, dried on tissue paper, frozen in liquid nitrogen, powdered in a mortar and homogenized in 6% HClO_4 for glutathione determination. GSH was determined as described by Akerboom and Sies (1981). GSSG was measured by h.p.l.c. as described by Fariss and Reed (1987). For GSSG determination in blood we used a modification of this h.p.l.c. method using *N*-ethylmaleimide (Sigma) to prevent GSH oxidation (M. Asensi, J. Sastre, F. V. Pallardó, J. M. Estrela and J. Viña, unpublished work). Blood samples treated with *N*-ethylmaleimide and, as described by Fariss and Reed (1987), with HClO_4 and bathophenanthrolinedisulphonic acid (Sigma), were modified by adding 50 μl of 1 mM γ -glutamylglutamate (Sigma) prepared in 0.3% HClO_4 to 500 μl of the acidic supernatant. Then samples were taken to pH 8.0 with KOH (2 M) + Mops (0.3 M), centrifuged, and then a 25 μl portion of the supernatant was mixed with 50 μl of 1% 1-fluoro-2,4-dinitrobenzene (Sigma). After this, derivative formation was completed in 45 min, and desiccated samples remained stable at –20 °C for several weeks until injection. This procedure

decreases GSH oxidation in blood to about 1%. Values for GSH and GSSG in experiments with incubated tumour cells refer to the total values found in the incubation flasks. All other GSH and GSSG measurements reported for tumour cells are intracellular values.

Lactate dehydrogenase activity was measured as described by Bergmeyer (1974).

The method for determination of protein kinase C (PKC) activity was adapted for Ehrlich ascites cells from those described previously for isolated rat hepatocytes (Kikkawa et al., 1983; Cooper et al., 1987; Kass et al., 1989). Samples of tumour cells [approx. $(3\text{--}4) \times 10^6$ cells] were centrifuged for 20 s at 500 *g*, and the cell pellets were resuspended in 1 ml of ice-cold buffer A (20 mM Tris/HCl, 200 mM sucrose, 0.5 mM EGTA, 0.5 mM EDTA, pH 7.4 at 4 °C) supplemented with 0.1 mM phenylmethanesulphonyl fluoride (Boehringer, Mannheim, Germany) and 100 μg of leupeptin/ml (Sigma). The cell suspensions were then sonicated on ice and the resulting homogenates were centrifuged at 100 000 *g* for 60 min. The supernatants were used for determining 'cytosolic' PKC activity. The pellets were resuspended in buffer A containing 0.2% (v/v) Triton X-100 by using a glass-glass homogenizer, incubated for 30 min at 4 °C, and then centrifuged at 100 000 *g* for 30 min. The supernatant was used for determining 'particulate-associated' PKC activity. Protein content in these fractions was determined by the method of Lowry et al. (1951). All PKC samples were kept at –70 °C until determination, which was carried out at 30 °C in assays containing (in a final volume of 200 μl) 10 μM [γ - ^{32}P]ATP (0.5 μCi ; Amersham International, Amersham, Bucks., U.K.), 200 μg of histone type III-S (Sigma)/ml, 20 μg of *L*- α -phosphatidyl-L-serine (Sigma)/ml, 1 μg /ml 1,2-dioleoyl-*sn*-glycerol (Sigma), 5 mM CaCl_2 , 20 mM Tris/HCl, pH 7.5 at 30 °C, and 25 μg of protein (in 50 μl of buffer A) from tumour cell fractions. Concentrations of ATP, histone and cofactors were not rate-limiting; therefore the PKC assay was run under apparent V_{max} conditions. Assays were stopped after 10 min by addition of 0.5 ml of 50% (w/v) trichloroacetic acid at 4 °C. Samples were kept on ice for 20 min and then centrifuged. Radioactivity was counted in the acid-precipitated material after several washings with 10% trichloroacetic acid. PKC activity was defined as described previously (Kass et al., 1989). One unit of activity catalyses the incorporation of 1 nmol of [^{32}P]P_i from [γ - ^{32}P]ATP into histone type III-S/min at 30 °C.

For calculations we used a wet-weight/dry-weight ratio of 3.4 ± 0.3 ($n = 6$) obtained previously for Ehrlich ascites-tumour cells (Estrela et al., 1992).

Measurement of intracellular pH (pH_i)

pH_i in tumour cells was determined by the [^{14}C]dimethyl-oxazolidine-2,4-dione ([^{14}C]DMO) (New England Nuclear, Boston, MA, U.S.A.) distribution ratio method (Rottenberg, 1979). Cells were incubated as indicated above in the presence of 1 μCi of [^{14}C]DMO. Samples (0.5 ml) from the incubation flasks were centrifuged through silicone oil (Estrela et al., 1992) for measurement of [^{14}C]DMO accumulation within the cells. The pH of the external medium was determined immediately after.

Expression of results and statistical significance

The results are expressed as means $\pm$ S.D. for the indicated numbers of different experiments. The statistical significance of differences was assessed by Student's *t* test.

RESULTS

GSH content in cancer versus normal tissues during tumour growth

We studied growth and GSH content in the tumour and in several normal tissues over a period of 2 weeks between inoculation of the cells and severe cachexia in the host (Lopes et al., 1989; Tisdale, 1991; Estrela et al., 1992). Until day 4 after inoculation, the amount of ascitic tumour was very small and variable. As shown in Figure 1, the rate of cellular proliferation *in vivo* was very rapid between days 4 and 9 approximately, and then decreased rapidly, reaching a plateau on days 12–13 after inoculation of the tumour. We followed the evolution of the intracellular content of GSH in the cancer cells during tumour growth *in vivo* (Figure 1), and found that GSH levels increase in the tumour cells when they proliferate actively. In contrast, GSSG levels did not change significantly as the tumour grew [i.e. $0.04 \pm 0.02 \mu\text{mol/g}$ ($n = 7$) at day 7 after tumour inoculation]. GSH content reaches a maximum on day 7, but then gradually decreases over the next days as the total cell number increases

Figure 1 Effect of tumour growth *in vivo* on the intracellular GSH content of cancer cells

Determinations were as described in the Materials and methods section. Results are means $\pm$ S.D. for 7–8 different animals.

Table 1 GSH content in several tissues from normal and tumour-bearing mice at different stages of tumour growth

For details see the text. The results are means $\pm$ S.D. for 5–6 different observations: *significantly different from non-tumour-bearing group ($P < 0.05$).

Tissue	GSH ($\mu\text{mol/g}$)			
	Mice ...	Non-tumour-bearing	Tumour-bearing	
			Day 7	Day 14
Brain		1.5 ± 0.2	1.7 ± 0.2	1.5 ± 0.2
Lung		1.5 ± 0.3	1.4 ± 0.2	1.1 ± 0.2
Heart		0.7 ± 0.2	0.7 ± 0.1	0.5 ± 0.1
Liver		6.7 ± 0.8	6.1 ± 1.0	$4.8 \pm 0.7^*$
Kidney		2.6 ± 0.7	2.4 ± 0.4	$1.7 \pm 0.3^*$
Skeletal muscle		0.4 ± 0.05	0.3 ± 0.03	0.3 ± 0.1
Blood (mM)		0.4 ± 0.04	$0.2 \pm 0.1^*$	$0.1 \pm 0.04^*$

and the growth rate declines. Thus apparently, there is a correlation *in vivo* between high rates of cellular proliferation and higher levels of intracellular GSH in the tumour cells.

We also studied the GSH content of several normal tissues comparing non-tumour-bearing mice and mice bearing the Ehrlich ascites tumour for 7 and 14 days (Table 1), two situations where GSH contents in the tumour are very different (Figure 1). Among the tissues studied, only liver, kidney and total blood GSH decreased significantly (Table 1). The highest decrease in GSH was found for total blood; at day 14 only 25 % of the initial content remained (Table 1). Thus apparently, with the exception of blood GSH, tumour growth does not affect the integrity of normal tissues by lowering their GSH contents. For blood, it has been reported that the haematocrit value can be decreased to 60 % of its control value in mice bearing a Lewis lung carcinoma for 15 days (Rivera et al., 1988). We measured the haematocrit and found that it decreased to $51.9 \pm 5.5\%$ ($n = 6$) of its control value in mice bearing the tumour for 14 days ($P < 0.05$ compared with controls). Therefore a decrease in this value explains, at least in part, the decrease in GSH in blood.

Previously we reported that GSSG concentration in liver and skeletal muscle of mice bearing the Ehrlich ascites tumour does not change significantly at any stage of tumour growth (Estrela et al., 1992). Indeed, as compared with controls, GSSG contents did not change significantly in any of the tissues displayed in Table 1 (results not shown).

Effect of BSO on GSH synthesis in tumour cells

Before treating animals with BSO, we tested its efficiency to deplete isolated tumour cells of GSH. As shown in Figure 2, when cancer cells (from mice bearing the Ehrlich tumour for 7 days) were incubated in the presence of a mixture of amino acid precursors at concentrations (see the legend to Figure 2) that ensure maximal rates of GSH synthesis in these cells (Estrela et al., 1992), we found a linear rate of synthesis of approx. 35.0 ± 3.7 ($n = 4$) nmol/min per g. When BSO (5 mM) was present in the incubation medium, after a lag period of about 20 min, which is apparently required by the inhibitor to enter the cancer cell and block the enzyme, GSH synthesis in the presence of amino acid precursors was inhibited completely (Figure 2). From the values obtained for Figure 2, and leaving out the first 20 min, we calculated an approximate rate of GSH disappearance of 10.1 ± 1.5 nmol/min per g ($n = 4$).

Dose–response relationship of GSH depletion in tumour cells by BSO

The BSO dose–response relationship (Figure 3) was measured on day 7 after inoculation of the tumour, when cancer cells show the highest GSH content (Figure 1). The maximum GSH depletion that could be achieved with a single dose of BSO was obtained by administering 4 or more mmol/kg (Figure 3), which led to values of about 15 % of the controls.

Time course of GSH depletion in tumour versus normal tissues by administration of BSO *in vivo*

As shown in Figure 4, after a single dose of BSO (4 mmol/kg), GSH content of the tumour and of several normal tissues was depleted in a time-dependent manner. In addition to the tissue-dependent kinetics, it is important to emphasize that the degree of depletion varied among tissues (Figure 4). Recovery of the GSH after a single dose of BSO also differed among the different tissues. As shown in Figure 4, GSH recovery started in the

Figure 2 Effect of BSO on GSH synthesis in isolated cancer cells

The initial GSH concentration was $3.1 \pm 0.2 \mu\text{mol/g}$. Mice bearing the tumour for 7 days were used. Cells were isolated as described in the Materials and methods section, and incubated in the presence of 5 mM glucose, 5 mM glutamine, 2 mM glycine, 1 mM serine and 1 mM *N*-acetylcysteine. In this experiment only L-amino acids were used. BSO, when present (○), was at 5 mM. All values in the Figure are means $\pm$ S.D. for 4 different experiments: *significantly different from the rate of synthesis in the absence (●) of BSO ($P < 0.05$).

Figure 3 Dose-response relationship of GSH depletion by BSO

BSO was administered on day 7 after inoculation of the tumour. Intracellular GSH content was measured, in each case, 24 h after BSO administration. Each value is the mean $\pm$ S.D. for 4 independent experiments.

kidney and the liver 12 h after BSO administration, 24 h after in the lung and the skeletal muscle, and 48 h after in the brain, the heart and the tumour. Moreover, the rates of GSH recovery were also different among tissues (Figure 4). For instance, at 96 h after BSO administration, only the brain, the lung, the liver and the kidney had more than 50 % of their initial GSH contents (Figure 4).

Administration of BSO and GSH depletion at different stages of tumour growth

We have tested the effect of BSO with different rates of cellular proliferation by administering BSO *in vivo* at different stages of tumour growth (Table 2). We found that BSO induces GSH depletion in tumour cells to a level of about $0.3\text{--}0.4 \mu\text{mol/g}$ (Table 2), which is reached at any stage of tumour growth. However, the rate of GSH depletion may differ, depending on the GSH content of the tumour cells before BSO administration. This fact is of special relevance, since, independently of the stage

Figure 4 GSH content in the tumour versus normal tissues after BSO administration

Mice bearing the tumour for 7 days were used. A value of 100% was given to each control before BSO administration. The first value shown in the Figure for every tissue was that obtained 3 h after BSO injection. Each datum point represents the average of 5–6 mice. S.D. values were 5–16% of the absolute value for all points represented in this Figure. The initial GSH concentration for each normal tissue is given in Table 1. Key: □, brain; ■, lung; ○, heart; ●, liver; △, kidney; ▲, skeletal muscle; ×, tumour.

Table 2 Effect of administration of BSO *in vivo* on GSH content in Ehrlich ascites cells at different stages of tumour growth

For details see the text. A single dose of BSO was administered as described in the Materials and methods section. The results are means $\pm$ S.D. for 4–5 different observations: *significantly different from controls (0 h for 'Time after administration of BSO') ($P < 0.05$).

Time after inoculation of the tumour (days)	Time after administration of BSO (h) ...	GSH ($\mu\text{mol/g}$)			
		0	3	12	24
4		1.7 ± 0.2	$0.8 \pm 0.2^*$	$0.4 \pm 0.1^*$	$0.4 \pm 0.1^*$
7		3.0 ± 0.3	$1.7 \pm 0.3^*$	$1.1 \pm 0.2^*$	$0.4 \pm 0.05^*$
10		1.5 ± 0.1	$0.7 \pm 0.2^*$	$0.5 \pm 0.1^*$	$0.3 \pm 0.05^*$
13		0.9 ± 0.05	$0.5 \pm 0.2^*$	$0.3 \pm 0.05^*$	$0.3 \pm 0.1^*$

of tumour growth, GSH levels in normal tissues remain at or close to those levels found in non-tumour-bearing mice (Table 1).

Inhibition of cellular proliferation by multiple doses of BSO

Furthermore, we studied whether the BSO-induced GSH depletion *in vivo* is also accompanied by changes in the rate of tumour cell proliferation. A single dose of BSO induces a strong GSH depletion in the tumour, to approx. 15% of its control value (Figure 3). However, although this low GSH level was

Table 3 Effect of BSO and GSH ester on GSH levels, PKC activity, pH_i and growth of Ehrlich tumour cells

Numbers in parentheses under 'Treatment' refer to mmol/kg. BSO was administered at 48 h intervals, starting 2 h after inoculation of the tumour. GSH content, PKC activities, pH_i and tumour growth were measured on day 7 after inoculation. GSH ester was injected intraperitoneally daily, for 7 days, starting 30 min after the first dose of BSO. GSH and propan-2-ol were injected in amounts equimolar to the highest dose of ester administered (5 mmol/kg). Mice injected with GSH ester or GSH and propan-2-ol were killed 6 h after the last administration. Data are means $\pm$ S.D. ($n = 4$): * $P < 0.05$ compared with controls (no treatment); † $P < 0.05$ compared with those mice treated with BSO alone.

Treatment	GSH (μ mol/g)	PKC activity (m-units/g)		pH _i	10 ⁻⁸ $\times$ Tumour cell number
		Cytosolic	Particulate		
None	3.1 $\pm$ 0.5	352 $\pm$ 27	190 $\pm$ 16	7.33 $\pm$ 0.03	41.2 $\pm$ 6.5
BSO (4)	0.5 $\pm$ 0.1*	127 $\pm$ 14*	133 $\pm$ 25*	6.90 $\pm$ 0.06*	23.0 $\pm$ 4.4*
BSO (4) + GSH ester (2.5)	1.1 $\pm$ 0.3*†	166 $\pm$ 13*†	150 $\pm$ 7*	6.98 $\pm$ 0.05*	29.1 $\pm$ 4.7*
BSO (4) + GSH ester (5)	2.7 $\pm$ 0.5†	320 $\pm$ 21†	206 $\pm$ 15†	7.30 $\pm$ 0.03†	39.3 $\pm$ 5.0†
BSO (4) + GSH (5) + propan-2-ol (5)	0.6 $\pm$ 0.2*	138 $\pm$ 9*	127 $\pm$ 14*	6.90 $\pm$ 0.02*	25.4 $\pm$ 4.0*

maintained until 48 h after BSO administration, a single dose of BSO did not promote any significant change in the rate of tumour cell proliferation (results not shown). To investigate if maintenance of low GSH levels for a longer period could affect cell proliferation, we used a multiple BSO-dosing (see legend to Table 3). By this procedure, GSH content in the tumour was kept very low (approx. 15% of its control; Table 3) in those mice treated with BSO. We found that tumour-bearing mice treated with BSO had approx. 44% less cancer cells than the controls, a significant decrease in tumour growth (Table 3). To prove further that GSH can directly modulate tumour cell proliferation, we tested whether tumour growth can be enhanced again in the presence of BSO by restoring GSH. As shown in Table 3, administration of GSH ester *in vivo* increases GSH content in tumour cells and restores tumour growth to control values. Simultaneous administration of BSO, GSH and propan-2-ol did not alter significantly the degree of GSH depletion induced by BSO (Table 3).

Role of GSH in regulating the onset of cell proliferation

GSH has been suggested as a possible regulator of DNA synthesis in human T lymphocytes and, consequently, of cell division (Suthanthiran et al., 1990). However, the possible involvement of GSH in the control of cell proliferation and the underlying mechanism remain unclear. The regulation of cellular growth can be triggered by many different extracellular signals which act on control elements within the cell. One of the major signal-transducing pathways involves the Ca²⁺- and phospholipid-dependent, *sn*-1,2-diacylglycerol-stimulated, protein kinase (PKC) (Nishizuka, 1984). Despite its close association with the plasma membrane, PKC phosphorylates specific cellular proteins that then contribute to various physiological processes, particularly secretion and proliferation (Berridge, 1984). PKC is a cysteine-rich enzyme (Nishizuka, 1988), and modification of the thiol redox status of these cysteine residues could affect its activity. In fact, Kass et al. (1989) have shown in isolated hepatocytes that treatment with thiol-binding reagents (i.e. *N*-ethylmaleimide) inhibit PKC activity. To explore this possibility, we measured PKC activity in our experimental conditions and found that maintenance of low GSH levels *in vivo* inhibits PKC activities, both cytosolic and particulate, in Ehrlich ascites tumour cells (Table 3). Moreover, in the presence of BSO, administration of GSH ester restores GSH levels and PKC activity (Table 3). Further, it is important to point out two experimental facts: (i) PKC activities are significantly higher in

tumour cells 7 days after inoculation of the tumour (see control values in Table 3) than in cells from mice bearing the tumour for 14 days (177 $\pm$ 14 m-units/g for the cytosolic PKC activity and 110 $\pm$ 32 m-units/g for the particulate PKC activity; $n = 5$; $P < 0.05$ compared with controls); (ii) PKC activities are not significantly changed by a single dose of 4 mmol of BSO/kg (337 $\pm$ 41 m-units/g for the cytosolic PKC activity and 202 $\pm$ 28 m-units/g for the particulate PKC activity; $n = 4$; BSO was administered on day 6 after inoculation of the tumour and PKC activities were measured 24 h after, on day 7, which allows comparison with control data in Table 3).

One of the major ionic events that contribute, next to Ca²⁺ mobilization, to the onset of proliferation is the activation of an electroneutral Na⁺/H⁺ exchange carrier (for a review see Berridge, 1984). Interestingly, when fibroblasts are stimulated with mitogens, the extrusion of protons causes a marked alkalization of the cytoplasm (Moolenaar et al., 1983). Thus we also measured whether changes in GSH content in the tumour cells also correlate with changes in their pH_i. As shown in Table 3, pH_i in cancer cells from mice bearing the tumour for 7 days is higher than that in similar cells from tumour-bearing mice treated with BSO. As occurs with PKC activity, in the presence of BSO, when GSH levels are restored, pH_i values also return to control values (Table 3). Finally, it is important to emphasize that: (i) pH_i in cancer cells from mice bearing the tumour for 14 days is 6.95 $\pm$ 0.03 ($n = 5$; $P < 0.05$ compared with the value found for tumour cells 7 days after the inoculation of the tumour; see Table 3). Also, as stated above for PKC activities, a single dose of BSO does not change significantly the pH_i value of cancer cells (results not shown). Therefore, since key factors such as PKC activity and pH_i are not changed by a single dose of BSO, our results suggest that short-term effects of GSH depletion on regulating the onset of cellular proliferation *in vivo* can be ruled out.

DISCUSSION

The present results show large differences in the response of cancer cells and normal tissues to treatment with BSO (Figure 4). These variations, as well as the evolution of GSH content in the tumour (Figure 1), suggesting that cell populations under active proliferative states may have higher GSH levels, support the idea that approaches to cancer treatment based on modulation of GSH levels must take into consideration the glutathione status and the rate of GSH synthesis in the tumour. Indeed, it was reported that drug-resistant tumours have increased cellular

levels of GSH (Meister, 1989). Moreover, knowing that each particular tissue has a specific response to BSO, our results may be useful when the anticancer therapy must be applied to specific locations in the body. In addition, we demonstrate that GSH depletion in cancer cells promotes a significant inhibition of proliferation, which correlates with an inhibition of PKC activity and a decrease in pH_i (Table 3).

The discovery of BSO provided a method to test the effect of GSH depletion in the cellular response to different anti-cancer treatments. BSO is superior to other GSH depletors, because it inhibits selectively the γ -glutamylcysteine synthetase and because it lacks toxic effects (Meister, 1983). This is not the case for other agents, i.e. the $\alpha\beta$ -unsaturated carbonyl compounds diethylmaleate or phorone, which have been widely used in toxicological investigations, but show many side-effects (see, e.g., Estrela and Pallardó, 1990; Sáez et al., 1985). Nevertheless, in isolated tumour cells, low concentrations of diethylmaleate (0.1 mM) may induce a more important and quicker GSH depletion than BSO (5 mM) (results not shown). Perhaps, in some cases or for some tissues, a combination of diethylmaleate (at very low doses) and BSO could be useful to induce a rapid GSH depletion *in vivo*.

Previous studies have indicated that GSH levels fluctuate during the cell cycle (Harris and Patt, 1969; Dethmers and Meister, 1981; Lee et al., 1988). However, it remained unclear whether these variations are involved in the regulation of cell proliferation. GSH has been suggested as a possible regulator of protein synthesis (Kosower and Kosower, 1978), DNA synthesis and cell proliferation (Suthanthiran et al., 1990); however, there is no strong evidence showing a direct relationship between changes in cellular GSH contents and regulation of cell growth. Shaw and Chou (1986) evaluated cellular GSH levels during serum stimulation of quiescent Swiss 3T3 fibroblasts, and found that GSH of actively growing cultures gradually decreases as fibroblasts become quiescent by either serum deprivation or high cell density. Kang and Enger (1990), using A549 human lung carcinoma cells, showed that BSO may decrease both cellular GSH and growth. More recently, Kang and Enger (1991) reported experiments in normal rat kidney fibroblasts (NRK-49F) showing that the increase in cellular GSH that occurs in quiescent serum-stimulated cells is a result of nutrient repletion rather than mitogenic stimulation, and increased GSH levels do not necessarily precede DNA synthesis and mitosis. Obviously, an important question regards the possible relevance of these studies *in vitro* to the situation *in vivo*. In a previous paper, we studied glutathione metabolism in Ehrlich ascites tumour cells, and reported that GSH content and GSH synthesis decrease in cancer cells when cell proliferation and the rate of protein synthesis in the tumour decrease (Estrela et al., 1992). However, changes in the rate of GSH synthesis are not due to limitations in the supply of blood cysteine (the rate-limiting amino acid for GSH synthesis) or to changes in the intracellular amino acid pool of the cancer cells (Estrela et al., 1992).

Kang and Enger (1992), using normal rat kidney fibroblasts (NRK-49F), suggested that nutrient limitation, rather than cellular GSH depletion, may be a mechanism by which BSO exerts its effect on cell proliferation. Previously, we have shown that cysteine is the rate-limiting factor for GSH synthesis in tumour cells and that a defect in amino acid transport can not be argued to explain changes in the rate of GSH synthesis (Estrela et al., 1992). We found that cyst(e)ine concentration in whole blood was $23 \pm 7 \mu\text{M}$ ($n = 15$) in mice bearing the Ehrlich tumour for 7 days, and $32 \pm 4 \mu\text{M}$ ($n = 6$; $P < 0.05$) in tumour-bearing mice treated with multiple doses of BSO. This may be in agreement with the inhibition of cysteine and cystine uptake by BSO reported by Kang and Enger (1992) in cultured fibroblasts,

and could be one of the mechanisms involved in growth inhibition. To support their conclusions further, Kang and Enger (1992) also showed that addition of exogenous GSH or cysteine overcame BSO inhibition of epidermal-growth-factor-induced DNA synthesis, but not BSO-induced depletion of cellular GSH levels.

Our results also show that GSH is involved in the control of PKC activity and pH_i , and therefore in regulating the onset of tumour cell proliferation (Berridge, 1984). Ehrlich tumour cells that proliferate actively (day 7 after tumour inoculation) show higher GSH, PKC and pH_i values than for those showing lower rates of proliferation (Table 3). Values of pH_i obtained for many cancer cells and by using different techniques have been recently reviewed (Griffiths, 1991). As opposed to what has been generally believed for many years (pH in tumours was preferentially acid; Warburg, 1930), different tumours may show broad differences in pH (values of 5.6–7.6 have been reported for solid human tumours; see, e.g., Griffiths, 1991).

In conclusion, the relevant facts reported in this paper are: (1) GSH, but not GSSG, content in tumour cells changes as the tumour grows, being higher when there is active cellular proliferation; (2) GSH and GSSG contents in most normal tissues remain unchanged or close to normal values during the active phase of tumour cell proliferation; (3) after administration of a single dose of BSO, the rates of GSH depletion and recovery among tissues are different; (4) the efficiency of BSO to deplete tumour GSH depends on the stage of tumour growth; (5) multiple doses of BSO promote a significant inhibition of tumour growth; (6) GSH depletion in proliferating tumour cells promotes an inhibition of the PKC activity and a decrease in pH_i .

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