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Tumor Cell Glutathione Metabolism Therapeutic Implications

Bradley Alan Arrick

TUMOR CELL GLUTATHIONE METABOLISM:

THERAPEUTIC IMPLICATIONS

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Bradley A. Arrick, B.A.

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ABSTRACT

In response to oxidative injury, murine tumors depend upon the GSH oxidation-reduction cycle. The susceptibility of tumor cells to lysis by a flux of H_2O_2, such as generated by granulocytes or activated macrophages, or by the enzyme glucose oxidase, was augmented by interference with the GSH redox cycle. Depletion of tumor cell GSH was by 2 methods: incubation of cells with buthionine sulfoximine (BSO), a nontoxic inhibitor of GSH synthesis, or with 1-chloro-2,4-dinitrobenzene, a substrate for GSH S-transferase. Sensitization to oxidative cytolysis correlated with GSH depletion by either method by the critera of both dose-response and the time course of onset and recovery. A third approach involved inhibition of glutathione reductase (GR) with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Here too, interference in tumor cell GSH metabolism enhanced cytolysis by a flux of H_2O_2. In contrast, inhibition of catalase with aminotriazole had little effect.

This theme was extended into another arena of GSH metabolism, the detoxification of reactive electrophiles by the formation of thioether conjugates with GSH. The interaction of tumor cell GSH with four sesquiterpene lactones (SLs), a class of sulfhydryl-reactive antineoplastic agents, was evaluated. SLs are potent depletors of cellular GSH. Prompt resynthesis of GSH is a critical component of tumor cell recovery following exposure to SLs. Cytolysis by SLs was augmented by inhibition of GSH synthesis with BSO. Potent synergy between BSO and the SL vernolepin was evident even if BSO was not present during but

added just after exposure to vernolepin. For six additional, unrelated antineoplastics, sulfhydryl reactivity, in terms of their ability to deplete GSH, correlated with synergistic lysis in the presence of BSO. Lysis by BCNU, however, was not enhanced by BSO, in spite of GSH depletion.

Vernolepin-mediated cytolysis of P815 cells was dependent upon exogenous cystine, as was GSH synthesis. This dependence upon cystine for cytolysis was not due to a requirement for protein synthesis or GSH synthesis. The formation of H_2O_2 as a result of cysteine autoxidation may contribute to the toxicity of vernolepin. In accord with this hypothesis, inhibition of GR by BCNU significantly enhanced the toxicity of vernolepin. Inhibition of catalase by aminotriazole resulted in less dramatic augmentation of vernolepin-mediated lysis.

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ABBREVIATIONS

AMSA	4'-(9-acridinylamino)methanesulfon-m-anisidide
Ara-C	Cytosine-1-β-D-arabinofuranoside hydrochloride
BCG	Bacille Calmette-Guérin
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BSO	Buthionine sulfoximine
CDNB	1-Chloro-2,4-dinitrobenzene
GPO	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
i.p.	Intraperitoneal
LD_{50}	Concentration of lytic agent or flux of H_2O_2 causing 50% specific release of ^{51}Cr label from the cells
MEM	Eagle's minimum essential medium
α-MEM	Eagle's minimum essential medium, alpha variant
NPSH	Non-protein sulfhydryl
PMA	Phorbol myristate acetate
ROI	Reactive oxygen intermediates
SLs	Sesquiterpene lactones

TABLE OF CONTENTS

INTRODUCTION

Recent developments in our understanding of the process of oncogenesis at the molecular level, as well as the arising technology by which human monoclonal antibodies of desired specificity may be produced in quantity, evoke high expectations for new therapeutic approaches to neoplastic diseases. Still, we need not rely solely on the discovery of new drugs or novel treatments. Elucidation of the biochemistry of antineoplastic action, as well as the defense mechanisms employed by tumor cells in response to anti-cancer therapy, can help us to use more effectively the tools we already have. Analysis of tumor cell defenses could be of use in the design of synergistic therapeutic combinations and in the analysis of treatment failure.

Much of our pharmacologic armamentarium for the treatment of infectious and neoplastic diseases has developed from the isolation and characterization of cell-derived products, presumably of importance in cell-cell interactions, such as the establishment of territorial niches. Within the mammalian organism, cytotoxicity by cells of the host immune system is a well studied phenomenon, though identification of the substances mediating cytotoxicity is still the subject of active research. Cytolysis of tumor cells by granulocytes and activated macrophages in certain settings has been shown to proceed by the secretion of H_2O_2, and is thus the result of oxidative injury. It

can be expected that tumor cell antioxidant defenses are an important factor in such injury, analysis of which might suggest ways to enhance both cell-mediated cytotoxicity and alternative oxidant-injury delivery systems.

In mammalian cells, the glutathione oxidation-reduction cycle fulfills an important function in antioxidant defense. Our research group first became interested in tumor cell glutathione metabolism as a potential determinant of susceptibility to cytolysis by peroxide-producing leukocytes. This work will be discussed in Section I of Results. In view of the importance of glutathione for the detoxification of reactive electrophiles via thioether conjugate formation, we next focused on the role of glutathione in lysis of tumor cells by sulfhydryl-reactive antineoplastic agents. Sections II and III of Results will describe this topic in detail.

First, I will briefly review some relevant aspects of glutathione metabolism, followed by a discussion of the various ways in which glutathione metabolism may be of importance in antineoplastic therapy.

Glutathione (GSH, Fig. 1) has been identified as the predominant intracellular non-protein sulfhydryl (NPSH) in a wide range of cells, both prokaryotic and eukaryotic, plant and animal. The now vast literature concerning the occurence and properties of GSH probably began with the report by Rey-Pailhade (1888) that a component of aqueous yeast extracts could reduce sulfur to hydrogen sulfide. He named this new cell-derived reducing principal "philothion". Twenty-three years later, across the English Channel, Hopkins (1921) isolated

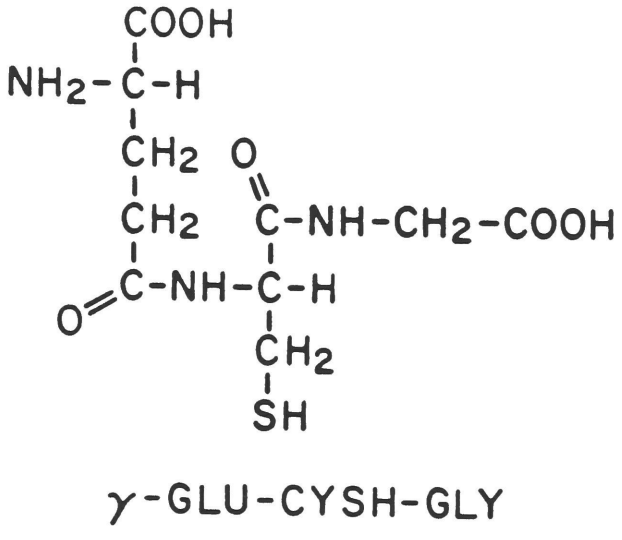

Figure 1. Structure of GSH.

a thiol-containing peptide from a variety of cell types, including yeast and mammalian liver, which he proposed to be equivalent to Rey-Pailhade's philothion. Hopkins further demonstrated the existence of factors within cells capable of catalyzing both the oxidation and reduction of this peptide, suggesting that such a sulfhydryl-disulfide redox cycle might be functionally important. At that time, Hopkins thought philothion was a dipeptide of glutamate and cysteine, and so renamed it glutathione ("gluta--" for glutamate, "--thi-" for thiol, and "---one" as in peptone or simple peptide).

A number of monographs and review articles devoted to various aspects of GSH metabolism and function have been published in the past decade (Flohe et al. 1974; Meister and Tate 1976; Arias and Jakoby 1976: Kosower and Kosower 1978; Sies and Wendel 1978; Meister et al. 1980). As background for a consideration of the importance of GSH metabolism in antineoplastic therapy, I will briefly outline some of this information.

Biosynthesis of GSH occurs in two successive ATP-requiring steps. First, γ-glutamylcysteine synthetase catalyzes the formation of an amide linkage between cysteine and the γ-carboxyl of glutamate. GSH synthetase then mediates the reaction of glycine with the cysteine carboxyl of γ-glutamylcysteine to form the tripeptide γ-glutamyl-cysteinylglycine.

In mammalian cells, GSH exists in at least three, possibly four, dynamically interchanging forms. Under normal steady-state conditions, the majority of GSH exists in the reduced form (0.5-10 mM). Oxidation of reduced GSH, either non-enzymatically or by the action of

glutathione peroxidase (GPO), yields glutathione disulfide (GSSG). NADPH-dependent reduction of GSSG by glutathione reductase (GR), as well as efflux, effectively maintains the intracellular concentration of GSSG at very low levels (usually 5-50 μM). The extent to which GSH oxidation is occuring under normal conditions, that is the flux rate of GSH through the redox cycle in the absence of an imposed oxidant stress, has not been determined either in tissue culture or in vivo.

A third form of cellular GSH which has been evaluated in a number of cell types involves participation of GSH in mixed disulfides with both protein and non-protein sulfhydryls. In one such study with an Ehrlich ascites tumor cell line, Modig (1968) found that approximately 35% of total cellular GSH was in disulfide linkage to protein.

Thiol esters of GSH constitute yet another potentially significant form of cellular GSH. Although the occurence and metabolism of such thiol esters have not been adequately studied, the identification by Uotila (1973) of at least three distinct GSH thiol esterases in human liver is suggestive of functional significance.

The numerous reactions of GSH in mammalian cells can be divided into those involving principally the γ-glutamyl portion of the tri-peptide, such as the γ-glutamyl cycle, versus those of the sulfhydryl moiety. Sulfhydryl-dependent metabolism of GSH can be further sub-divided into oxidation-reduction reactions versus nucleophilic reactions in which the reduced sulfhydryl reacts with an electrophile to form a thioether (Fig. 2). By forming such thioethers, the cell may detoxify potentially harmful compounds.

An alternative scheme by which GSH metabolism can be categorized

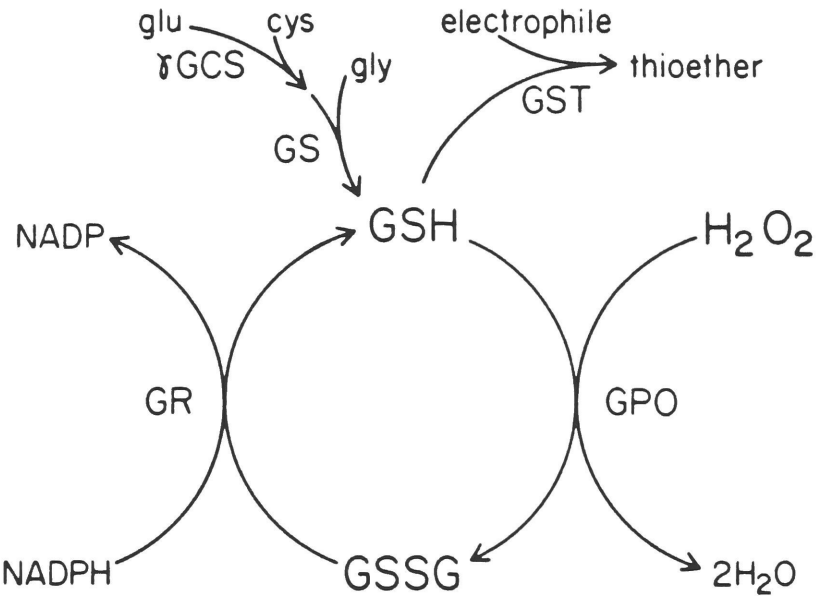

Figure 2. Aspects of sulfhydryl-dependent GSH metabolism: oxidation-reduction cycle and thioether formation. The enzymes shown are: GPO; GR; γGCS, γ-glutamylcysteine synthetase; GS, glutathione synthetase; GST, glutathione S-transferase.

distinguishes between reversible and irreversible loss of reduced GSH. Reactions involving the formation of GSSG, mixed disulfides, or thiol esters result in reversible loss since resynthesis of GSH from its amino acid constituents is not required for maintenance of cellular content of GSH in the reduced form. In contrast, irreversible loss of GSH is the consequence of reactions resulting in thiol conjugate formation, such as the mercapturic acid pathway, or efflux of GSH from the cell, where it may be degraded by the externally disposed enzyme γ-glutamyl transpeptidase as part of the γ-glutamyl cycle. Synthesis is required for restoration of cellular GSH levels in response to irreversible loss.

GSH has been shown to play a critical role in cellular defense against a variety of injurious agents. In this regard, the sulfhydryl group assumes primary importance. Thus, maintenance of structural and functional viability in spite of the ongoing production by cells of reactive oxygen intermediates (ROI), an apparently unavoidable consequence of aerobic metabolism, as well as defense against acute oxidant injury, relies heavily upon the GSH redox cycle. The capacity of the redox cycle may be related to the cellular content of GSH, GR, and/or GPO, as well as to the ability of the cell to regenerate NADPH via the pentose phosphate shunt. Similarly, as the non-protein sulfhydryl of greatest abundance, GSH protects against toxic electrophiles by thioether formation. The effectiveness of this pathway may depend upon the intracellular concentration of GSH, the presence of GSH S-transferases of appropriate specificity, and/or the capacity of the cell for rapid resynthesis of GSH.

The importance of GSH-mediated detoxification in the pharmacology and toxicology of various agents has been discussed recently in a number of reviews (Mitchell et al. 1976; Jakoby 1978; Orrenius and Jones 1978; Reed and Beatty 1980). However, a comprehensive evaluation of the implications of GSH metabolism within the context of therapeutic intervention in disease states has not been provided.

The rest of this introduction will focus on a consideration of the various ways in which GSH metabolism may be of importance in anti-neoplastic therapy. I will begin by outlining four major determinants of therapeutic efficacy in which GSH metabolism may be involved.

A. Detoxification. GSH may mediate protection via detoxification of the active species and/or repair of the injury once incurred.

B. Toxification. GSH may be involved in formation of the active species, either within the tumor target or normal cells of the host.

C. Delivery. Drug targetting, drug uptake, and other aspects concerning delivery of the toxic agent to its site of action may be influenced by GSH metabolism.

D. Therapeutic Interactions. An alteration in the GSH status of tumor or host as a result of a particular therapeutic intervention may influence the effectiveness or toxicity of

a concurrent therapy.

For each of the above categories, specific examples of antineoplastic inverventions will be discussed. In this regard, it will be important to distinguish between GSH metabolism of tumor and host.

A. Detoxification.

1. Radiation. The attenuated radiosensitivity of cells in an oxygen-deficient atmosphere compared to irradiation in air has been termed the "oxygen effect". Neither nucleic acids nor enzymes in aqueous suspension exhibit such an effect. Working with T2 bacteriophage, Howard-Flanders (1960) showed its sensitivity to inactivation by irradiation to be independent of the concentration of oxygen. The addition of cysteamine, however, decreased the extent of inactivation when irradiation was conducted in a nitrogen atmosphere, while having no effect in the presence of oxygen. Similar studies concerning inactivation of DNA and trypsin by irradiation in the presence and absence of GSH helped to focus attention on cellular thiols as mediators of the oxygen effect (Hutchinson 1961).

The notion that cellular NPSH might be responsible for anoxic radioresistance has been evaluated in a variety of settings. Revesz et al. (1963) studied a sub-line of an Ehrlich ascites tumor obtained by sub-lethal in vitro irradiation between passages, for twenty-three passages, as well as a series of single-cell clones form this sub-line. In comparison to the parental cell line, all of the thus derived cell

populations, with one exception, exhibited both enhanced anoxic radioresistance and increased NPSH content. In the presence of oxygen, all sub-lines tested were as susceptible to the effects of radiation as the parental line.

The role of GSH in cellular response to irradiation has been further evaluated by experiments with fibroblast cell cultures from a child suffering from 5-oxoprolinuria due to homozygous glutathione synthetase deficiency, and from his healthy brother (Deschavanne et al. 1981a, 1981b; Midander et al. 1982). The deficient cells (containing approximately 6% of control GSH) and normal cells were of equivalent sensitivity when irradiated in an atmosphere of 100% oxygen. However, with radiation exposure under anoxic conditions, damage to the normal cells was diminished, while the susceptibility of GSH-deficient cells was unchanged. Radiosensitization of hypoxic cells by removal of GSH has been demonstrated with diamide (Harris and Power 1973; Harris 1979), although doses in excess of that necessary to oxidize GSH to GSSG are required, and with diethyl maleate (Bump et al. 1982), which resulted in a greater than 95% depletion of GSH. Dethmers and Meister (1981) have examined the euoxic radiosensitivity of a human cell line after a 24 hour incubation with buthionine sulfoximine, an inhibitor of GSH biosynthesis, which results is approximately a 95% depletion of GSH content. Compared to control cells, these GSH-deficient cells were somewhat more radiosensitive, though radiation survival curves were not generated (the highest dose resulted in a survival fraction of 0.5).

Theoretically, the importance of GSH as a determinant of hypoxic cell radiosensitivity could result from a role in the detoxification of

radiation-induced toxins, such as free radicals, and/or the repair of critically damaged cell structures. GSH can engage in one-electron reactions with potentially harmful free radicals by hydrogen atom donation, thus forming the relatively stable thiyl radical, and ultimately GSSG. (reviewed by Kosower and Kosower 1976). Analysis of the rate at which irradiation-induced single-strand DNA breaks were repaired by the GSH-deficient human fibroblasts described earlier failed to demonstrate a role for GSH under conditions of anoxia. In contrast, GSH content was a determinant of the repair capacity of cells following aerobic irradiation (Edgren et al. 1981). Thus, the precise mechanism(s) by which GSH is involved in hypoxic cell defense against radiation damage have not been determined. It is clear, however, that the presence of oxygen serves to change the kinetics, magnitude, and/or nature of the injury such that GSH is no longer protective.

Hypoxic, poorly vascularized cells within solid tumors, including microscopic metastatic foci, limit the efficacy of both radiotherapy and blood-borne chemotherapy (Kennedy et al. 1980a). One approach to the problem of hypoxic cell radioresistance has been the development of chemical radiosensitizers, which by virtue of their affinity for electrons serve to mimic the effects of oxygen. A number of the more effective radiosensitizers are metabolized by an oxygen-inhibited pathway, resulting in the generation of cytotoxic species (Taylor and Rauth 1980). Furthermore, incubation of cells under hypoxic conditions with a variety of nitroimidazole sensitizers, such as misonidazole, resulted in significant loss of GSH (Varnes et al. 1980; Astor et al. 1982; Biaglow et al. 1982; Hall et al. 1982). The potential signifi-

cance of this depletion of GSH to either the mechanism(s) of radiosensitivity or the differential toxicity of these compounds towards hypoxic cells has yet to be carefully analyzed.

2. Mechlorethamine and related nitrogen mustards. The ability of alkylating agents such as the nitrogen mustards to react with a variety of cellular nucleophiles, including sulfhydryl groups, has long been recognized. The toxicity of mechlorethamine in experimental animals was shown to be diminished by appropriately timed administration of cysteine (Brandt and Griffin 1951) or cysteinamine (Peczenik 1953). In this regard, it should be noted that in vitro exposure of cells to cysteamine can result in a significant increase in intracellular GSH, presumably by release from mixed disulfides with proteins (Revesz and Modig 1965).

Analyses of the sulfhydryl content of murine tumors of varying sensitivity to mechlorethamine and its N-oxide derivative have revealed potentially significant correlations. Hirono (1961) generated resistant variants of three ascites tumors by repeated passage through treated animals and found in all three cases that their NPSH content was increased. Among the original cell lines, relative drug susceptibility did not correlate with the content of NPSH, suggesting a mechanistic distinction between innate versus acquired resistance to this agent. Similarly, Goldenberg (1969) demonstrated increased NPSH content in two variants of a lymphoblast cell line made resistant to mechlorethamine by in vitro exposure.

Comparing six murine tumors of varying sensitivity to merophan,

an isomer of melphalan, Calcutt and Connors (1963) demonstrated a relationship between inhibition of tumor growth by this agent and the ratio of protein sulfhydryl to NPSH. Ball et al. (1966) evaluated the content of protein, DNA, RNA, nucleotides, and NPSH in a melphalan-resistant Yoshida sarcoma relative to the sensitive parental cell line growing in the contralateral limb, and found the only difference to be an increased level of NPSH in the resistant tumors. More recently, Suzukake et al. (1982) have shown that an L1210 leukemia cell line made resistant to melphalan and containing a 2-fold greater content of GSH could be re-sensitized by lowering its GSH content. This was achieved by incubation of the cells in medium deficient in cystine for 24 hours prior to exposure to melphalan. A 2-fold increase in NPSH content has also been documented for a melphalan-resistant subline of Chinese hamster ovary cells (Begleiter et al. 1983).

3. Cyclophosphamide. Cyclophosphamide is a widely used nitrogen mustard, which undergoes metabolic activation by hepatic mixed-function oxidases. The generally accepted mechanism for the generation of active metabolites involves oxidation to 4-hydroxycyclophosphamide, which would be in tautomeric equilibrium with aldophosphamide. Nonenzymatic cleavage of aldophosphamide results in the formation of two toxic species, phosphoramide mustard and acrolein. Phosphoramide mustard is considered to be the therapeutically active species while acrolein is believed to be responsible for some aspects of host toxicity, such as hemorrhagic cystitis. An alternative metabolic pathway for 4-hydroxy-cyclophosphamide, involving loss of water to form a sulfhydryl-

reactive species, has been suggested by the work of Draeger et al. (1976) and Cates and Li (1982). Such sulfhydryl derivatives of cyclophosphamide, themselves inactive, may serve as chemically stable prodrugs, able to release by hydrolysis an active cyclophosphamide metabolite (Peter and Hohorst 1979).

Working with mice, Gurtoo et al. (1981) have demonstrated significant dose-dependent depletion of hepatic NPSH by both cyclophosphamide and acrolein, but not phosphoramide mustard. They further showed that cyclophosphamide-induced inhibition in weight gain in rats was enhanced by coadministration of a GSH depletor, diethyl maleate, and diminished by prior injections of cysteine. Cysteine also significantly delayed the onset of hematuric cystitis in cyclophosphamide-treated rats. Ashby et al. (1976) have reported that injection of cysteine or GSH prior to cyclophosphamide protected against teratogenesis in pregnant rats. The antitumor activity of cyclophosphamide appears not to be inhibited by cysteine administration (Gurtoo et al. 1981). In a similar manner, N-acetylcystine protected mice from the toxic effect of isophosphamide, an analog of cyclophosphamide, without interfering with its antitumor activity against L1210, allowing for the safe administration of higher doses of isophosphamide, and thus enhanced therapeutic efficacy (Venditti and Goldin 1974).

4. Nitrosoureas. Inhibition of GR in the erythrocytes of patients receiving therapeutic doses of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) was reported by Frischer and Ahmad (1977). Babson and Reed (1978) have demonstrated that the isocyanates derived from the break-

down of BCNU and other nitrosoureas are responsible for the irreversible inactivation of GR. They further suggest a correlation between the inhibition of GR and nitrosourea-induced myelosuppression. There is, however, disagreement about the ability of nitrosoureas other than BCNU to inactivate GR (Frischer and Ahmad 1977; Shinohara and Tanaka 1979). The relevance of this finding to the therapeutic action or toxicity of BCNU is not at all clear. Administration of BCNU to tumor-bearing mice resulted in a significant decrease in hepatic GSH content, accounting for greater than 50% of the dose of BCNU, assuming a one-to-one reaction stoichiometry (McConnell et al. 1979). It has been suggested by Nathan et al. (1981) that inhibition of GR activity of alveolar macrophages and other pulmonary cells may play a role in the development of pulmonary toxicity observed following repeated therapy with BCNU.

5. 6-Thiopurine. The cytochrome P-450 system activates 6-thiopurine, in vivo or in vitro, to a reactive intermediate which binds irreversibly through disulfide linkage to protein (Hyslop and Jardine 1981a, 1981b). In the presence of GSH, protein binding was diminished and GSSG was formed. These authors therefore proposed that GSH competes with protein sulfhydryls for reaction with the drug metabolite, purine-6-sulfenic acid, forming thiopurine-GSH mixed disulfide. This then reacts with another GSH molecule to regenerate 6-thiopurine and GSSG. Depletion of hepatic GSH concentration by feeding rats a protein-free diet prior to injection of radiolabeled 6-thiopurine resulted in increased protein binding (Hyslop and Jardine (1981b). It is not yet

clear whether these findings are relevant to the toxicity of 6-thiopurine.

6. 4'-(9-acridinylamino)methanesulfon-m-anisidide (AMSA). AMSA (NSC 141549) is one of the new antineoplastic agents arising from investigations into the synthesis and antitumor activity of acridine derivatives. Studying the pharmacokinetics of AMSA in mice, Cysyk et al (1977) reported a 40% drop in hepatic GSH content within 2-3 hours after intraperitoneal injection (100 mg/kg). They further proposed that excretion in the bile of a GSH-acridine thioether conjugate was the predominant pathway of host metabolism and detoxification. A few years later, Shoemaker et al. (1980) demonstrated that biliary excretion of AMSA, administered intravenously to rats (10 mg/kg), was markedly diminished by pretreatment with diethyl maleate, which itself resulted in a 90% depletion of hepatic GSH. A role for the mixed-function oxidase system was also suggested by the observation that excretion was enhanced by pretreatment with phenobarbital and diminished by pretreatment with metyrapone, agents with induce and inhibit, respectively, the oxidase system (Shoemaker et al. 1980). Careful analysis of the fate of radiolabeled AMSA in rats has confirmed the principal biliary metabolite to be a GSH thioether, with GSH linked to the 5' position of the anisidine ring. Conjugation with GSH presumably occurs after oxidation of AMSA by the mixed-function oxidase system to a quinone-like diimine (Shoemaker et al. 1982).

Although the importance of similar metabolic transformations of AMSA within tumors is not known, it is clear that host tissue GSH- and

mixed function oxidase-dependent pathways are important determinants of drug plasma levels (Shoemaker et al. 1980).

7. Generators of oxidant injury.

a. Cells of the host immune system. Various cells of the host immune system have the capacity to lyse tumor cells. An understanding of the biochemical mechanisms underlying cell-mediated cytotoxicity could be of considerable value for the development of effective immunotherapy. Over the past eight years, numerous reports have described oxygen-dependent mechanisms of cytolysis by granulocytes and activated macrophages (Clark and Klebanoff 1975, 1977, 1979; Hafeman and Lucas 1979; Simchowitz and Spilberg 1979; Borregaard and Kragballe 1980; Weiss 1980; Slivka et al. 1980; Weiss and LoBuglio 1980; Nathan et al. 1979b; Nathan and Cohn 1980; Nathan and Klebanoff 1982; Clark and Szot 1981). These effector cells can be stimulated to release H_2O_2 and other ROI at substantial rates, resulting in concentrations sufficient to lyse target cells (Repine et al. 1974; Nathan and Root 1977; Nathan et al. 1979a). Indeed, exposure to H_2O_2 appears to be the principal injurious event resulting in tumor cell lysis by phorbol myristate acetate (PMA)-triggered granulocytes and macrophages, by macrophages in the presence of antitumor antibody, and by activated macrophages without the addition of triggering agents if the tumor cells are first exposed to the cytophilic peroxidase of eosinophils (EPO) (Nathan 1982). Nathan and co-workers have recently demonstrated that the susceptibility of various tumor cells to effector cell-

mediated lysis in these settings is largely a measure of the anti-oxidant capabilities of the target cell, chiefly the GSH redox cycle (Nathan et al. 1981; Arrick et al. 1982). This work will be discussed in detail in Section I of Results. In short, we have found that the relative susceptibility of six murine tumors to lysis by H_2O_2 correlated with cellular GSH content. Further, depletion of GSH, or interference with its utilization in the GSH redox cycle by inhibition of GPO or GR, sensitized tumor cells to various forms of peroxide-dependent lysis.

It should be noted that self protection of granulocytes and macrophages from the H_2O_2 and ROI they themselves generate is probably a function of GSH-dependent detoxification as well. Basford (1980) has recently reviewed the numerous reports of auto-oxidative damage in leukocytes with enzyme defects affecting the GSH redox cycle, such as inherited deficiencies in GR or GSH synthetase.

b. Quinone-containing drugs. Antineoplastic agents which contain a quinone moiety are believed to undergo metabolic activation by microsomal enzymes to free radicals, themselves toxic, which can interact with oxygen to generate ROI via superoxide anion (Bachur et al. 1978). Examples of such drugs include doxorubicin and daunorubicin (benzanthraquinones), mitomycin C and streptonigrin (N-heterocyclic quinones), and lapachol (a naphthoquinone). While it is believed that such oxygen-dependent metabolism of streptonigrin forms the basis of its antitumor activity, the observation by Teicher et al. (1981) that mitomycin C and doxorubicin are preferentially toxic to hypoxic tumor

cells suggests the existence of oxygen-independent or -inhibitable pathways of metabolic activation. Still, the generation of free radicals and ROI by these agents may be an important determinant of drug toxicity.

In particular, substantial evidence now exists implicating a major role for host GSH metabolism as a determinant of host tissue damage by doxorubicin, principally cardiac toxicity. Analysis of the antioxidant defenses of mouse heart has identified selenium-dependent GPO, and thus the GSH redox cycle, as a major pathway for the detoxification of ROI, such as that generated by doxorubicin (Doroshow et al. 1980). It has further been demonstrated that administration of doxorubicin to mice resulted in a dose-dependent decrease in the activity of cardiac GPO with no effect on the hepatic enzyme (Doroshow et al. 1980). Obtaining similar results with doxorubicin-treated rabbits, Revis and Marusic (1978) also noted a significant decrease in the cardiac content of selenium after drug treatment. GSH content was diminished slightly in both liver and heart after injection of mice with doxorubicin (Doroshow et al. 1979; Olson et al. 1980). The lethal toxicity of doxorubicin to mice could be markedly increased by prior depletion of GSH by pre-treatment with diethyl maleate, itself without lethality (Olson et al. 1980). Working with isolated hepatocytes, Babson et al. (1981) have shown that pretreatment with BCNU, resulting in a >90% inhibition of GR and a 70% depletion of GSH, augmented the toxicity of doxorubicin and increased the degree of lipid peroxidation. Equivalent GSH depletion by diethyl maleate had no effect, indicating a predominant role for GR as the rate-limiting component of the GSH redox cycle in this system.

Administration of various antioxidants to mice, such as tocopherol, cysteamine, and N-acetylcysteine, has been shown to diminish the host toxicity of doxorubicin, without interference with its antitumor activity, resulting in an increase in the therapeutic index (Myers et al. 1977; Freeman et al. 1980; Doroshow et al. 1981).

In summary, as regards generators of oxidant injury, be they cells of the host immune system or quinone-containing drugs, GSH metabolism of both tumor and host may be an important detoxification pathway, influencing both toxicity and antineoplastic efficacy.

8. Sulfhydryl-reactive antineoplastic agents. A high degree of sulfhydryl reactivity is a shared feature of a variety of experimental antineoplastic agents, many of which have been isolated from natural sources. The most recent overall review of the in vitro sulfhydryl reactivity of such antitumor alkylating agents was provided by Fujita and Nagao (1977). However, very little information concerning the interaction of such compounds with intracellular sulfhydryl groups, such as GSH, is available. As will be detailed in Section II of Results, we have studied the effect of four sesquiterpene lactones, and the related diterpene jatrophone, on tumor cell GSH content. We have found these agents to be potent depletors of cellular GSH. Furthermore, resynthesis of GSH after drug exposure was an important component of tumor cell response and repair; inhibition of GSH resynthesis markedly augmented cytolysis (Arrick et al. 1983). Jackson et al. (1976) have reported that incubation of Yoshida sarcoma or L1210 cells with cytembena (cis-β-4-methoxybenzoyl-β-bromoacrylate), an anticancer drug

susceptible to nucleophilic addition by sulfhydryls, resulted in substantial depletion of GSH. It is clear that as sulfhydryl-reactive agents gain wider use in the treatment of cancer, an understanding of the role of cellular GSH in detoxification and protection of vital cell structures becomes all the more important.

B. Toxification.

1. Azathioprine. Azathioprine is a prodrug which is converted into 6-thiopurine and various methylnitroimidazole metabolites. Thiolysis by GSH, with the formation of the GSH-imidazole adduct, is believed to be the major route of azathioprine toxification (de Miranda et al. 1973). Kaplowitz and Kuhlenkamp (1978) have implicated a role for GSH S-transferases in the thiolysis of azathioprine; both the depletion of hepatic GSH and formation of 6-thiopurine observed after azathioprine administration were inhibited by prior inhibition of GSH S-transferases with probenicid.

Chalmers (1974) has shown that the GSH-imidazole adduct discussed above is further metabolized to 5-thio-1-methyl-4-nitroimidazole. This compound, itself without activity as an immunosuppressant or stimulant, significantly diminished the degree of immunosuppression obtained with a concurrent injection of 6-thiopurine (Garattini et al. 1974).

2. Bleomycin. Bleomycin, a mixture of two structurally similar glycopeptides, is believed to act as an antitumor agent via an oxygen-dependent mechanism of DNA cleavage. Specifically, a ferrous iron-

bleomycin complex reacts with oxygen to generate superoxide anion, and the iron-bleomycin complex itself is oxidized to the ferric state. Caspary et al. (1981) have recently reported that GSH is able to bind to this ferric iron-bleomycin complex and reduce it to the active ferrous-iron complex. They therefore proposed a catalytic redox cycle in which reducing equivalents from GSH are ultimately transferred to oxygen resulting in the formation of superoxide anion and other ROI. Other investigators have not detected significant interaction of GSH with the ferric iron-bleomycin complex, while still reporting enhanced DNA strand scission by ferrous iron-bleomycin in the presence of GSH (Antholine et al. 1981). A role for GSH in the toxification of copper(II)-bleomycin, a form of the drug with in vivo activity yet without activity in cell-free systems, has been suggested by the work of Freedman et al. (1982). They suggest that metal-free bleomycin first chelates copper(II), forming an inactive complex which upon entry into cells is converted back into metal-free bleomycin by the action of GSH, via a copper(I) intermediate. This then binds any available iron(II), thus forming the active species.

Involvement of GSH in intracellular reductive toxification, such as in the iron-bleomycin catalytic cycle, may be characteristic of other drug interactions. Neocarzinostatin is another antitumor agent which results in oxygen-dependent cleavage of DNA in a manner which is dependent upon the presence of thiols, though evidence has been presented both for (Sim and Lown 1978) and against (Kappen and Goldberg 1978; Kappen et al. 1980) a role for copper(II) in this process. Recently, Myers et al. (1982) have reported that the oxidative

destruction of erythrocyte ghost membranes by an iron-doxorubicin complex is dependent upon the presence of GSH.

C. Delivery.

1. Methotrexate. The uptake of methotrexate by cultured rat hepatocytes has been shown to be significantly accelerated by the addition of GSH to the medium (Leszczynska and Pfaff 1982). A role for externally disposed membrane sulfhydryl groups was indicated by the demonstration that methotrexate uptake was inhibited by exposure of the cells to an analog of diamide which is unable to enter cells, and thus oxidizes only extracellular sulfhydryl groups (the bis-N'-methyl quaternary salt of diazenedicarboxylic acid bis(N'-methylpiperazide)). GSH reversed this inhibition, presumably by regeneration of critical sulfhydryl groups. Leszczynska (1981) has reported that both the pharmacokinetics of clearance from blood as well as ultimate tissue distribution of rifamycin SV in rats is significantly affected by coadministration of GSH, but not GSSG. Interestingly, GSH served to enhance drug uptake by the lung, while reducing drug levels in the liver.

It should be kept in mind that host tissue GSH-dependent metabolism and excretion of systemically administered anticancer agents, as discussed for AMSA and azathioprine, are obvious determinants of the delivery of active drug to its target. A role for GSH metabolism as a determinant of drug uptake by cells, such as suggested above for methotrexate, represents a different type of GSH-drug interaction.

2. γ-Glutamyl derivatives as possible prodrugs. It has been suggested by Meister and Griffith (1979) that γ-glutamyl derivatives of some chemotherapeutic agents might be transported into certain tumors more readily than the agents themselves since many tumors exhibit increased γ-glutamyl transpeptidase activity. The feasibility of this approach to prodrug targetting has been demonstrated for agents directed to the kidney (Wilk et al. 1978; Orlowski et al. 1980).

D. Therapeutic interactions.

From the discussion above, it is evident that many antineoplastic agents alter the GSH status of host and/or tumor by depletion of cellular GSH content and/or inhibition of associated enzymes. Conceivably, this could influence both the toxicity and therapeutic efficacy of concurrent therapeutic interventions. In other words, changes in GSH metabolism may constitute the biochemical basis of some therapeutic interactions. Examples of a few such interactions, both actual and theoretical, will illustrate this point.

Inhibition of GR by BCNU, as noted above, augmented macrophage-mediated tumor cell lysis (Nathan et al. 1981). Similarly, the in vivo antitumor activity of glucose oxidase-generated H_2O_2 was enhanced by BCNU at sub-therapeutic yet GR-inhibiting doses (Nathan and Cohn 1981). In view of the role of the GSH redox cycle in the antioxidant defenses of the heart, BCNU might serve to increase the cardiotoxicity of doxorubicin. Another possibility is that the effectiveness of bleomycin may be dependent upon a properly functioning GSH redox cycle within the

tumor target, and thus BCNU would interfere with the action of bleomycin. Inhibition of GR could result in increased protein binding of 6-thiopurine metabolites, leading to enhanced toxicity. Finally, inactivation of the GSH redox cycle by BCNU could impair cellular response to radiation damage.

It has previously been pointed out that alterations in GSH content can affect the detoxification and effectiveness of a variety of antineoplastics, including nitrogen mustards, cyclophosphamide, and AMSA. Such alterations in GSH content can arise as a result of the concurrent use of agents which deplete cellular GSH, such as misonidazole, BCNU, and sesquiterpene lactones. GSH is translocated out of cells, and thus intracellular GSH may be the major source of reducing equivalents for the external surface of cell membranes (Griffith and Meister 1979a). This raises the possibility that GSH depletion may result in diminished permeability of cells to methotrexate. Griffith and Meister (1979b) have reported that systemic administration to mice of an inhibitor of γ-glutamyl transpeptidase resulted in a three-fold increase in plasma GSH. Thus, the pharmacokinetics and distribution of methotrexate may also be altered by agents which inhibit human γ-glutamyl transpeptidase, such as the antineoplastic glutamine antagonists 6-diazo-5-oxo-L-norleucine (DON) (Tate and Ross 1977) and α-amino-3-chloro-2-isoxazoline-5-acetic acid (AT-125) (Allen et al. 1980).

In summary, a role for GSH metabolism as a determinant of therapeutic efficacy has been suggested for a wide variety of antineoplastic interventions (Table I). In this thesis, I will discuss the

importance of tumor cell GSH as a determinant of susceptibility to lysis by two forms of injury: exposure to oxidant injury (Section I of Results), and incubation with sulfhydryl-reactive antineoplastics (Sections II and III of Results).

TABLE I
Examples of Antineoplastic Interventions for which GSH Metabolism may Influence Therapeutic Efficacy

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A. Detoxification

1. Radiation
2. Mechlorethamine and related nitrogen mustards
3. Cyclophosphamide
4. Nitrosoureas
5. 6-Thiopurine
6. AMSA
7. Generators of oxidant injury
   a. Cells of the host immune system
   b. Quinone-containing drugs
8. Sulfhydryl-reactive agents

B. Toxification

1. Azathioprine
2. Bleomycin

C. Delivery

1. Methotrexate
2. γ-Glutamyl derivatives as possible prodrugs

MATERIALS AND METHODS

Eagle's minimum essential medium, alpha variant (α-MEM), streptomycin, penicillin, and horse serum were obtained from Flow Laboratories. The following were from Sigma: glucose oxidase (type V), Triton X-100, dimethyl sulfoxide, 5-sulfosalicylic, CDNB, NADPH, GSH, GSSG, yeast GR, catalase (thymol free), 3-amino-1,2,4-triazole, 5,5'-dithiobis-(2-nitrobenzoic acid), mitomycin C, Ara-C, and vinblastine sulfate. Superoxol (30% hydrogen peroxide) was from Mallinckrodt Chemical Works. PMA was obtained from Consolidated Midland. Dulbecco's phosphate-buffered saline and Eagle's minimum essential medium (MEM), provided as a kit in which the separate amino acids can be added as desired, was obtained from Grand Island Biological Co. $Na_2{}^{51}CrO_4$ was from New England Nuclear. DL-Buthionine-SR-sulfoximine (BSO) was either synthesized and provided by Dr. Owen Griffith of Cornell Medical College, Department of Biochemistry, or it was purchased from Chemical Dynamics Corp. The following compounds were obtained through the courtesy of Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, and Dr. J. D. Douros, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute: helenalin (NSC 85236); vernolepin (NSC 106398); elephantopin (NSC 100046); eriofertopin (NSC 283439); jatrophone (NSC 135037); maytansine (NSC 153858); BCNU (NSC 409962); and doxorubicin hydrochloride (NSC 123127).

Tumors. Tumor cell lines were maintained by intraperitoneal passage in histocompatible mice as described (Nathan et al. 1979a, 1981). For experiments, these tumors were grown in stationary suspension cultures in α-MEM, supplemented with 100 μg/ml of streptomycin, 100 U/ml of penicillin, and 10% heat-inactivated horse serum. J774 cells were maintained in spinner culture with 5% fetal bovine serum plus antibiotics.

Effector Cells. Activated macrophages and granulocytes were collected as described (Nathan et al. 1981). In brief, effector cells were obtained by peritoneal lavage of $CD2F_1$ female mice 10–42 days after i.p. injection of viable BCG (BCG-activated macrophages) or 12–18 h after i.p. injection of thioglycollate medium (granulocytes).

Use of Pharmacologic Agents. GSH depletion by BSO (0.2 mM) was achieved by incubation of cells in α-MEM, 10% horse serum at 37°C in 5% CO_2, 95% air. BSO stock solution (20–45 mM) was stored at 0°C for up to 3 weeks before use. CDNB was dissolved in absolute ethanol just before use and added to tumor cells in α-MEM, 5% horse serum (final ethanol concentration, 0.2–1%). In some experiments, CDNB was included in the 4.5-h cytolysis assay. In other experiments, tumor cells were pretreated with CDNB for 15 min at 37°C in the presence of BSO (0.2 mM), washed twice, and then assayed for GSH content and susceptibility to lysis in the presence of BSO. The inclusion of BSO was necessary to prevent resynthesis of GSH by the CDNB-treated cells. In experiments with SLs and other potential sulfhydryl-reactive agents, tumor cells in α-MEM, 5% horse serum (1.0×10^6 – 1.7×10^6/ml) were incubated at 37°C with the test compounds dissolved in dimethyl

sulfoxide, or vehicle alone, for the indicated times. Dimethyl sulfoxide content ranged from 0.1 to 1% and did not affect GSH levels or specific release of ^{51}Cr from the cells. Inhibition of GR by BCNU was as follows: In Section I of Results, tumor cells (5×10^6 - 1×10^7/ml in α-MEM, 1% horse serum) were incubated with BCNU or vehicle (final ethanol concentration, 0.09%) at 37°C for 10 min, and then washed. In Section III of Results, tumor cells (1.5×10^6 - 3×10^6/ml in cystine-free MEM, 5% horse serum) were incubated with BCNU or vehicle alone (final dimethyl sulfoxide concentration, 0.5%) at 37°C for 15 min, and then washed. BCNU was stored at -20°C and dissolved immediately before use.

Biochemical Assays. To measure total cellular glutathione (GSH plus GSSG), 1×10^6 - 2×10^6 tumor cells were washed 3 times in ice-cold phosphate buffered saline, lysed with 0.2% Triton X-100, and made to contain 2.5% sulfosalicylic acid in a total final volume of 0.12 ml. After centrifugation, acid-soluble extracts were stored at -70°C and assayed within 2 weeks by a minor modification of the method of Tietze (1969), in which the rate of 5,5'dithiobis-(2-nitro-benzoic acid) reduction obtained with 0.05 ml of extract was analyzed. With each assay a standard curve was generated with known amounts of GSH (0-2 nmol/0.05 ml) in the same concentrations of Triton X-100 and sulfosalicylic acid as the samples. Total glutathione is expressed as nanomoles of the tripeptide per milligram of cell protein. Extracts of cells treated with compounds that were found to cause GSH depletion were mixed with known amounts of GSH and then assayed to rule out the presence of an inhibitor of the Teitze assay, which might have

accounted for the observed loss of GSH. The activity of glucose oxidase used in cytolysis experiments was measured with the scopoletin assay for H_2O_2 as described (Nathan and Root 1977). GR was assayed by the method of Roos et al. (1979) as described by Nathan et al. (1981). GPO activity was measured by the method of Paglia and Valentine (1967). Catalase was measured according to the method of Baudhuin et al. (1964). Superoxide dismutase was assayed according to the method of McCord and Fridovich (1969). GSH S-transferase activity was measured with CDNB (1 mM) as substrate by the method of Habig et al. (1974). Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Cytolysis Assays. Tumor cells were labeled with $Na_2{}^{51}CrO_4$ as described (Nathan et al. 1979a, 1981). Calculation of percent specific release was as suggested by Nathan et al. (1979a). In some experiments, the ^{51}Cr release assay was validated by comparison to the trypan blue dye exclusion test as described (Nathan et al. 1981).

In Section I of Results, cytolysis assays were performed as follows: susceptibility of tumor cells to cytolysis by H_2O_2 was determined by incubation of 4×10^4 labeled cells with dilutions of glucose oxidase or preformed H_2O_2 (Superoxol, 8.82 M), plus BSO (0.2 mM) in 0.22 ml of α-MEM, 5% horse serum at 37°C for 4.5 h. The amount of glucose oxidase or preformed H_2O_2 causing 50% specific release of the ^{51}Cr label (LD_{50}) was determined by interpolation. For evaluation of tumor cell susceptibility to cell-mediated cytotoxicity, 2×10^4 labeled tumor cells were incubated with various numbers of effector cells in 0.2 ml of α-MEM, 5% horse serum in the presence of 100 ng/ml

PMA or vehicle alone (0.033% dimethyl sulfoxide) plus BSO (0.2 mM) for 5 h at 37°C. Each reported data point represents the mean of triplicate determinations. Effector:target ratios are based on the proportion of effector cells in the peritoneal cell population used in each experiment, as determined by differential counting. The presence of BSO during the cytolysis assay did not effect the lysis of cells which had not previously been treated with CDNB.

In Section II of Results, cytolysis was studied in 2 experimental settings: (a) 4×10^4 labeled cells were incubated with various concentrations of lytic agent or vehicle alone (0.1-1% dimethyl sulfoxide) in the presence or absence of BSO (0.2 mM) in 0.22 ml of α-MEM, 5% horse serum at 37°C for 18 h. (b) Cells (1.5×10^6/ml in α-MEM, 5% horse serum) were incubated with various concentrations of vernolepin or vehicle alone (0.1% dimethyl sulfoxide) plus $Na_2{}^{51}CrO_4$ for 1 h, washed extensively (4 centrifugations), and then incubated in α-MEM, 5% horse serum (1.5×10^5 in 1 ml) for 18 h. BSO (0.2 mM) was added to the medium at various times as indicated in each experiment. After the 18 h incubation, supernatant (0.1 ml for a, 0.5 ml for b) was removed after centrifugation for gamma counting and the percent specific release was calculated. The LD_{50} was determined from a dose-response curve by interpolation. In protocol b, if ^{51}Cr was measured after 26 h rather that 18 h, the LD_{50} was reduced by a factor of 1.2 while the spontaneous release increased by a factor of 1.4. Each reported data point represents the mean of triplicate determinations.

In Section III of Results, cytolysis was studied in 2 experimental

settings: (a) P815 cells were washed into cystine-free MEM, 5% horse serum and incubated for 1 h with vernolepin, or vehicle alone (0.1-0.5% dimethyl sulfoxide) with $Na_2{}^{51}CrO_4$ (1.5×10^6/ml), washed, and then incubated for 18-20 h in MEM, 5% horse serum (1.5×10^5/ml in 0.5 ml) with or without cystine (0.09 mM), with or without BSO (0.2 mM). At this time, cells were centrifuged and 0.2 ml of supernatant sampled for gamma counting. Reported data points represent the mean of duplicate or triplicate determinations, as indicated. (b) 4×10^4 labeled cells were incubated with dilutions of glucose oxidase plus vernolepin or vehicle alone (0.1% dimethyl sulfoxide) in the presence or absence of BSO (0.2 mM) in 0.22 ml of α-MEM, 5% horse serum at 37°C for 5 h. At this time, 0.1 ml of supernatant was removed for gamma counting. Each reported data point represents the mean of triplicate determinations.

RESULTS

I. Oxidative Cytolysis

A. Introduction

As discussed in the Introduction, the secretion of H_2O_2 by granulocytes and activated macrophages following appropriate stimulation constitutes the main cytolytic component of effector cell response in some in vitro systems. Comparison of the susceptibility of various tumor cell lines to lysis by H_2O_2 or peroxide-producing leukocytes reveals considerable variation (Nathan 1979; Nathan et al. 1979a, 1981). Thus, a potentially cytolytic event is perhaps best viewed as an interaction in which peroxide generation by leukocytes and the antioxidant capacity of the target cell each play a significant role. The principal pathways of H_2O_2 detoxification in cells are the GSH oxidation-reduction cycle and catalase.

In this section, I will present data indicating a correlation between the susceptibility of six murine tumor cell lines to H_2O_2-mediated lysis and their content of GSH. I will further document sensitization of tumor cells to lysis by a flux of H_2O_2, such as that generated by the enzyme glucose oxidase or peroxide-producing leukocytes, as a result of GSH depletion or inhibition of GR, but not inhibition of catalase.

B. Results

Correlation of GSH Content with Susceptibility to Oxidative Cytolysis. Four lymphomas, a mastocytoma, and a histiocytoma, spanning a 54-fold range in susceptibility to lysis by H_2O_2, were studied. We evaluated the susceptibility of ^{51}Cr-labeled tumor cells to lysis by a flux of H_2O_2 by measuring the ^{51}Cr released into the supernatant after a 4.5-h incubation with varying amounts of the H_2O_2-generating enzyme, glucose oxidase. The levels of catalase, GR, or GPO in these cells did not correlate with the resistance of the tumors to H_2O_2 (Nathan et al. 1981). In contrast, the cellular content of glutathione (per mg of cell protein) correlated with the logarithm of the flux of peroxide causing 50% lysis (Fig. 3).

Depletion of Tumor Cell GSH by Buthionine Sulfoximine (BSO). BSO is a selective inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister 1979c). GSH depletion in the presence of BSO reflects ongoing GSH catabolism, efflux, and cell division in the absence of new synthesis. Incubation of intact P815 mastocytoma or P388 lymphoma cells with 0.2 mM BSO resulted in a gradual decline in cellular GSH (Fig. 4). Tumor cell GSH levels were reduced to 50% of normal values after 4.5-5 h of exposure to BSO. Incubation with BSO for 15 h or longer resulted in >90% depletion of GSH. Similar kinetics of GSH depletion were observed with the TLX9 lymphoma (not shown). Incubation of P815 cells with 0.02 mM BSO resulted in a slower rate of GSH depletion, while 1 mM was no more effective that 0.2 mM BSO (not shown). Thus, 0.2 mM BSO, which produced complete inhibition of the isolated enzyme (Griffith and Meister 1979c), is assumed to achieve virtually complete inhibition of

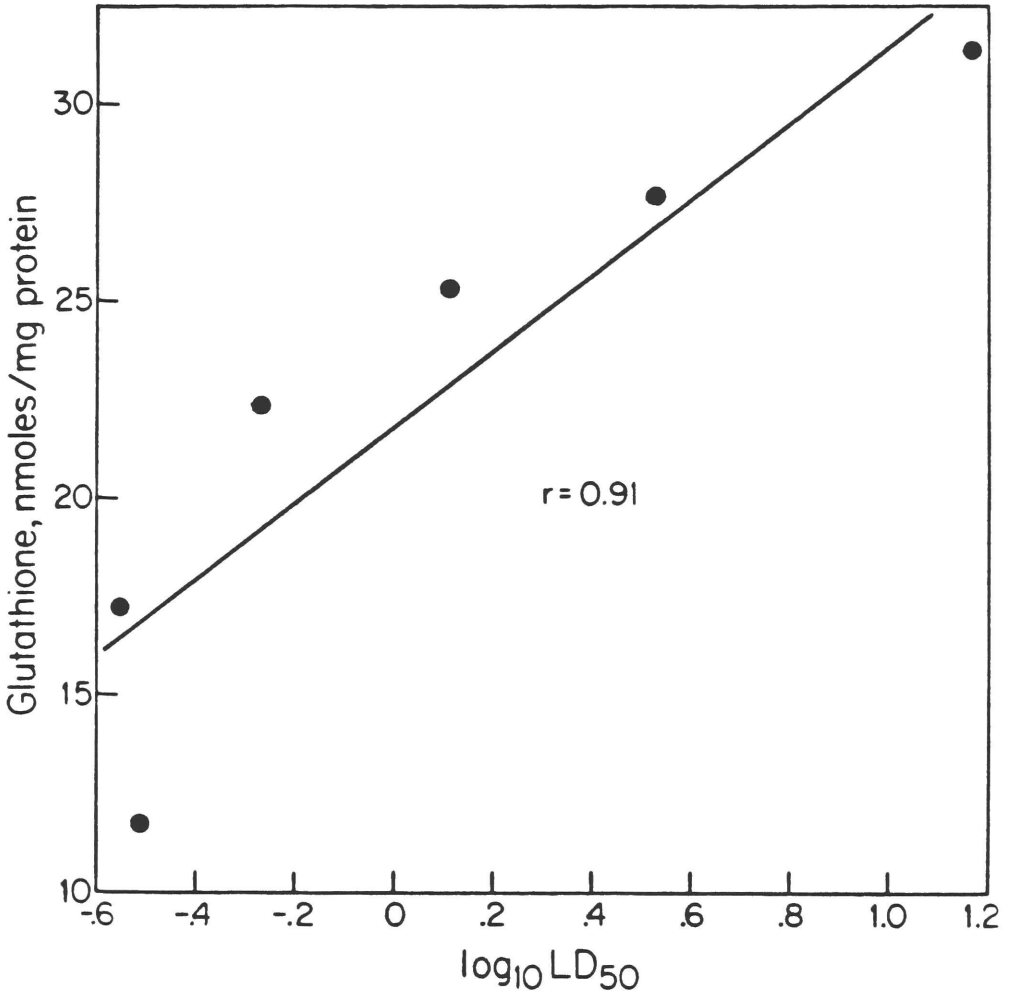

Figure 3. Correlation of the GSH content of 6 tumor cell lines with their sensitivity to enzymatically generated H_2O_2. The latter is expressed as the logarithm of the activity (nanomoles of H_2O_2/min) of glucose oxidase necessary to cause 50% lysis of the tumors in a 4.5-h ^{51}Cr-release assay. Each point represents 1 tumor and is the mean of 3-6 GSH determinations and 2-19 LD_{50} determinations.

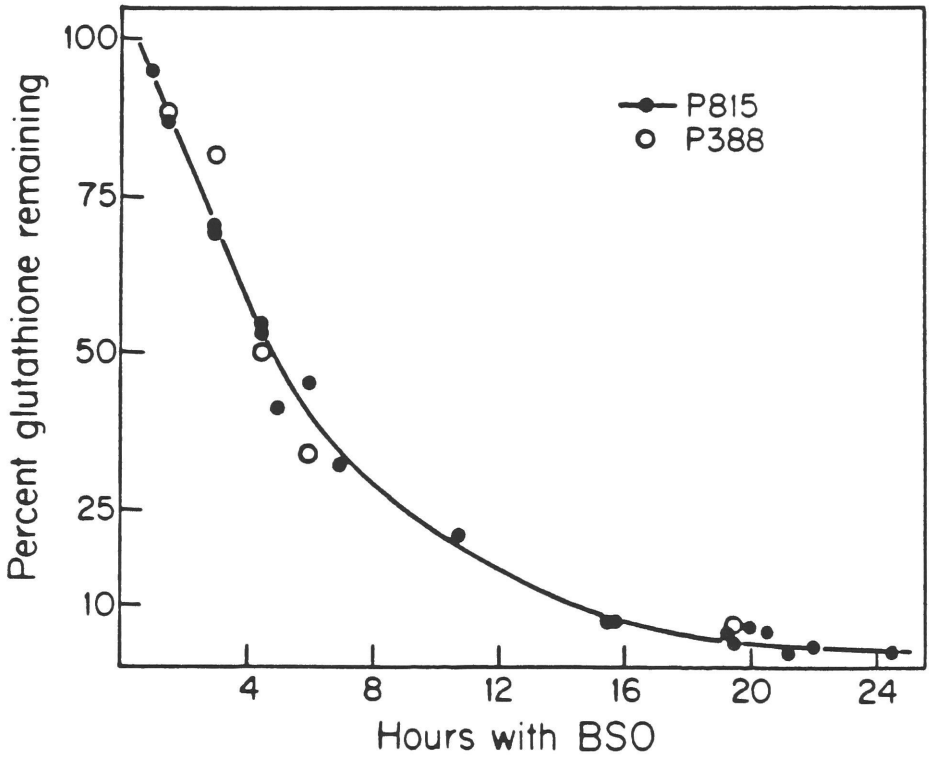

Figure 4. Time course of GSH depletion by BSO. The intact cells were incubated with 0.2 mM BSO in α-MEM, 10% horse serum at 37°C for the indicated times and then assayed for glutathione content. Data for P815 are from 7 experiments with initial GSH levels of 26.1 ± 2.1 nmol/mg of protein. Data for P388 are from 1 experiment in which the initial GSH level was 11.6 nmol/mg of protein.

GSH biosynthesis in intact cells grown in tissue culture medium. In all subsequent experiments, BSO was used at a concentration of 0.2 mM.

Incubation of P815 cells with 2 mM BSO for 18 h, or 0.2 mM for up to 3 weeks, was nontoxic, as judged by exclusion of trypan blue. Furthermore, the rate of cell division of P815 cells was unaffected by BSO (generation time of ~11 h). Incubation of P388 cells with BSO for 16 h had no significant effect on the specific activities of GR, GPO, catalase, or superoxide dismutase (not shown). Inclusion of BSO in the assay reaction mixture was similarly without effect for any of the above enzymes (not shown).

Depletion of Tumor Cell GSH by CDNB. GSH S-transferases catalyze the formation of thioether conjugates between GSH and a wide variety of electrophilic compounds (Habig et al. 1974). CDNB serves as an effective substrate and has previously been used to reduce the GSH content of intact cells (Novogrodsky et al. 1979). We first verified the presence of GSH S-transferase in P815 lysates (0.05 unit/mg of protein). We then measured the GSH content of P815 and P388 cells after exposure to CDNB for 15 min (Fig. 5). Treatment of P815 cells with CDNB resulted in a dose-dependent loss of GSH, with a maximal depletion of approximately 70% of initial values obtained with 10 μM CDNB. At concentrations above 30 μM, CDNB was toxic to both P815 and P388, as judged by significant elevation in the spontaneous release of ^{51}Cr over 4.5 h.

The activities of GR, GPO, catalase, and superoxide dismutase in lysates prepared from P388 cells treated with 20 μM CDNB for 15 min and then washed twice were not significantly different from untreated

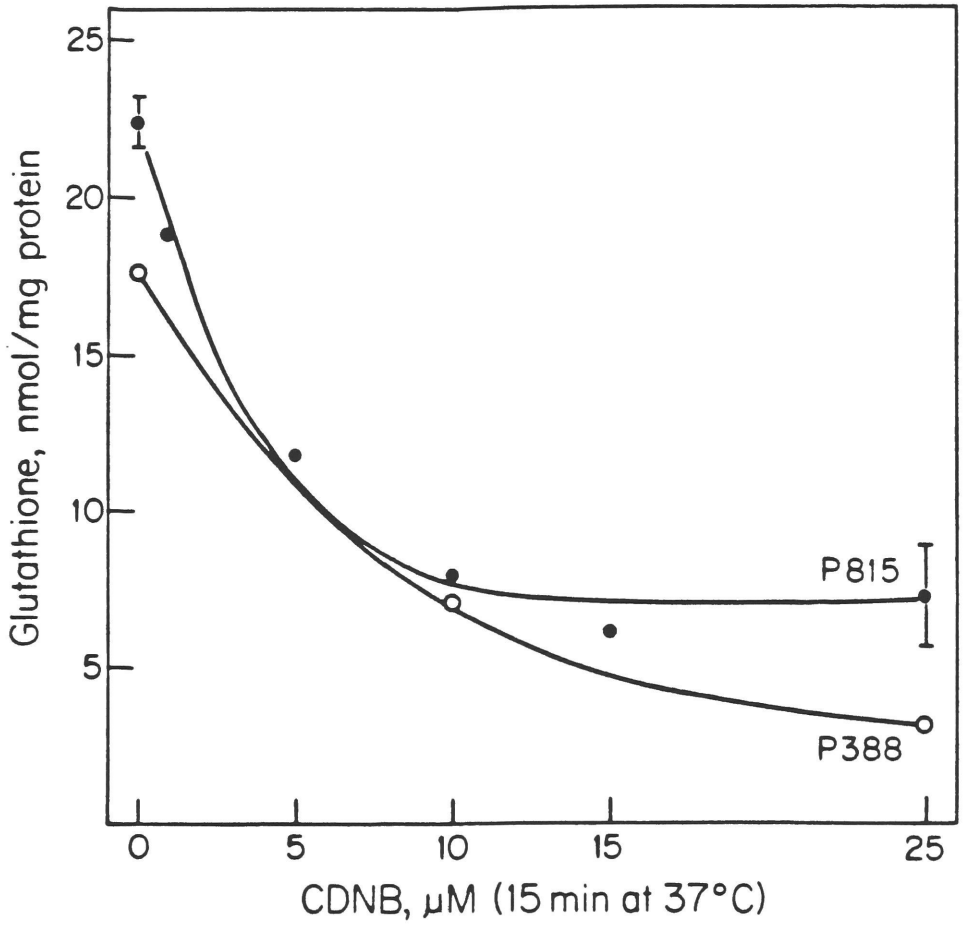

Figure 5. Effect of CDNB on cellular GSH content. The intact cells were treated with CDNB in α-MEM, 5% horse serum as indicated and then assayed for glutathione content. Data for P815 treated with 0 and 25 μM CDNB represent the average of 4 experiments ± S.E. The remaining points for P815 and P388 represent single determinations.

control lysates (not shown). However, experiments with yeast GR demonstrated concentration-dependent inhibition by CDNB, with maximal inhibition of 42% achieved with 35 μM CDNB. Inhibition of GR by CDNB was not dependent upon pre-exposure of the enzyme to CDNB, in either the presence or absence of NADPH, and enzyme activity could be restored by dilution, suggesting a reversible, noncovalent mode of inhibition.

Effect of GSH Depletion on Susceptibility of Tumor Cells to Lysis by H_2O_2. Tumor cells were exposed to a continuous flux of nascent H_2O_2 by the addition of glucose oxidase to the glucose-containing medium, or where indicated, were exposed to a bolus of preformed H_2O_2. As before, the susceptibility of ^{51}Cr-labeled tumor cells to lysis by a flux of H_2O_2 was determined after a 4.5-h incubation with varying amounts of glucose oxidase. In one such experiment, incubation of P815 cells with BSO for 19 h reduced the amount of glucose oxidase required to cause 50% specific ^{51}Cr release (LD_{50}) by a factor of 3.5 (Fig. 6). The correlation between the time of incubation with BSO, and therefore the extent of GSH depletion, and increased sensitivity to lysis by glucose oxidase-generated H_2O_2 is illustrated in Figure 7 for P815. With P388, a cell line with a native sensitivity to H_2O_2 about 4-fold greater than that of P815, incubation with BSO for 13-16.5 h reduced the LD_{50} for glucose oxidase by a factor of 1.68 ± 0.1 (5 experiments). In contrast, incubation of TLX9 cells with BSO for 24 h reduced the GSH content to <5% of its initial level (18.1 nmol/mg of protein), yet did not affect sensitivity to lysis by glucose oxidase. In all cases, lysis by glucose oxidase was abolished by exogenous catalase (not shown).

The inclusion of CDNB with the labeled tumor cells and glucose

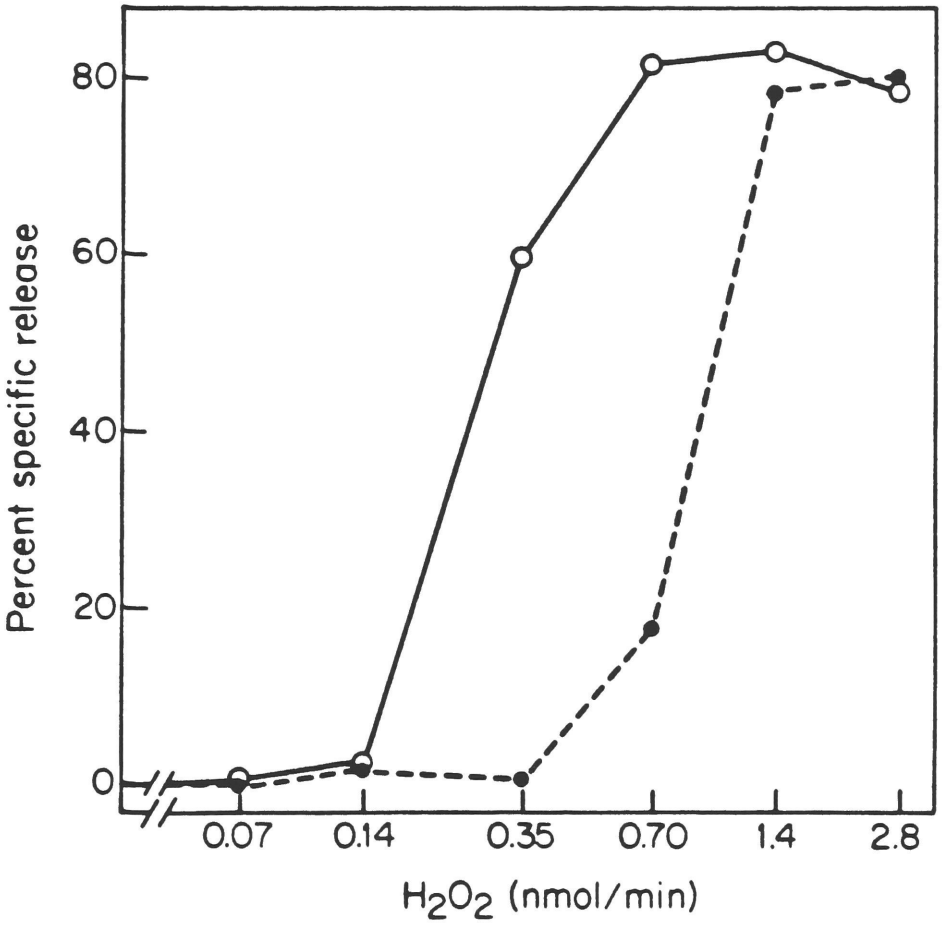

Figure 6. Effect of GSH depletion on the sensitivity of P815 cells to lysis by a flux of H_2O_2. ^{51}Cr release was measured after a 4.5-h incubation with dilutions of glucose oxidase. Prior to assay, the tumor cells were incubated with (o) or without (•) 0.2 mM BSO for 19 h. Points are means of triplicate determinations. S.E. averaged 1.1%. Spontaneous release of ^{51}Cr label was <8%.

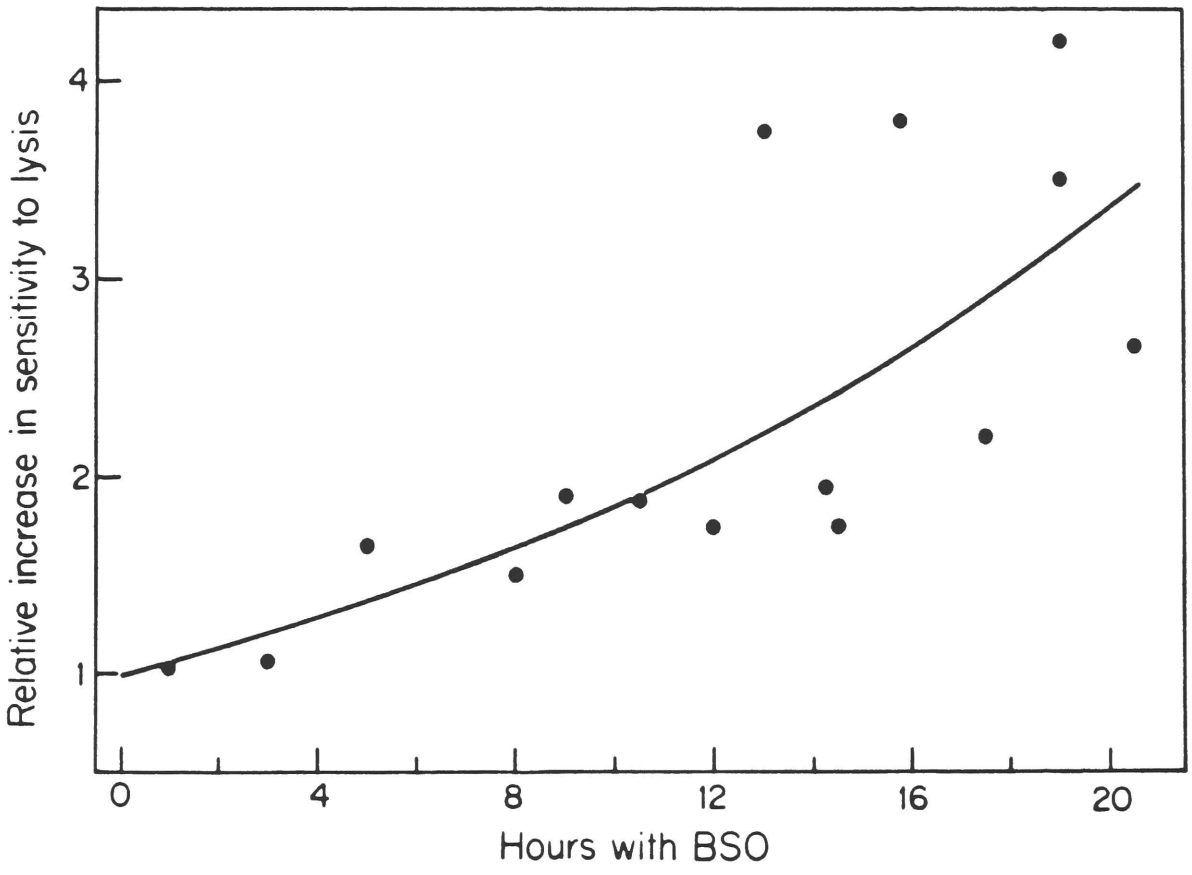

Figure 7. Time course of the effect of 0.2 mM BSO on sensitivity of P815 cells to lysis by a flux of H_2O_2. Relative increase in sensitivity to lysis = LD_{50}(control)/LD_{50}(treated) for the activity of glucose oxidase causing 50% lysis in 4.5 h. LD_{50} values were calculated by interpolation from curves like those in Figure 6. LD_{50} (control) averaged 1.04 ± 0.04 nmol of H_2O_2/min (N=8). Spontaneous release averaged 8.1 ± 1.6% (N=15). The fit of the data to an exponential curve is shown (r^2=0.72).

oxidase during the 4.5-h cytolysis assay resulted in a dose-dependent increase in lysis of P815, reaching a 4- to 6-fold relative increase in sensitivity to lysis with 15 μM CDNB (Fig. 8). When the same experiment was performed with P815 cells which had already been depleted of GSH by prior incubation with BSO for 13 h, CDNB did not result in increased lysis relative to BSO-treated cells in the absence of CDNB (Fig. 8).

Exposure of labeled P815 cells to 10-30 μM CDNB for 15 min prior to but not during the 4.5-h incubation with glucose oxidase made the cells more susceptible to lysis by a factor of 1.70 ± 0.11 compared to vehicle-treated controls (10 experiments). BSO was present during the 4.5-h cytolysis assay to prevent resynthesis of GSH by the treated cells (see below).

It has previously been shown that inhibition of catalase by incubation of P815 cells with 25 mM aminotriazole is not associated with increased lysis by either enzymatically generated H_2O_2 or preformed H_2O_2 added as a bolus (Nathan et al. 1981; see Fig. 14 below). The data presented in Table II confirm these observations and further demonstrate that whereas depletion of GSH is similarly without effect on tumor cell lysis by preformed H_2O_2, the combination of GSH depletion and catalase inhibition markedly sensitizes P815 cells to a bolus of H_2O_2. Such synergy is not evident when glucose oxidase is used to deliver a steady flux of H_2O_2.

The marked sensitization to H_2O_2-mediated lysis observed with GSH depletion by BSO and CDNB was also evident when cytotoxicity was evaluated by trypan blue exclusion (not shown), confirming the validity of the ^{51}Cr release assay under the experimental conditions. Under

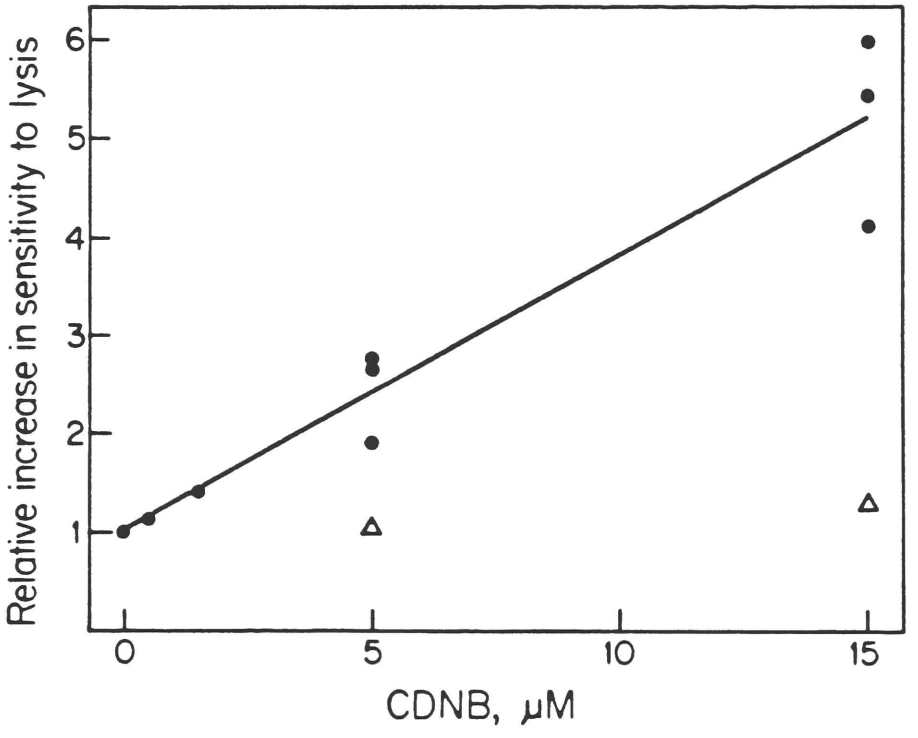

Figure 8. Effect of CDNB on sensitivity of P815 to lysis by a flux of H_2O_2. CDNB at the indicated concentrations was included in the 4.5-h incubation with glucose oxidase and the increase in sensitivity to lysis relative to no CDNB (LD_{50} (-CDNB)/LD_{50}(+CDNB)) was determined as in Figure 7 for normal cells (•) and cells which had been incubated with 0.2 mM BSO for 13 h to deplete their GSH (Δ). For the normal cells, LD_{50}(-CDNB) = 1.25 ± 0.04 nmol of H_2O_2/min (N=3). For the BSO-treated cells, LD_{50}(-CDNB) = 0.34 nmol of H_2O_2/min. Spontaneous release averaged 12.6 ± 1.5% (N=14).

Table II

Effect of GSH Depletion and/or Inhibition of Catalase on the Sensitivity of P815 Cells to Lysis by Glucose Oxidase or Preformed H_2O_2

Treatment‖	Relative increase in sensitivity to lysis by*	
	Glucose oxidase + glucose	Preformed H_2O_2
Experiment 1		
BSO	3.5	1.2
Aminotriazole	1.2	1.0
BSO + aminotriazole	3.3	2.6
Experiment 2		
BSO	1.9	1.0
Aminotriazole	0.9	1.2
BSO + aminotriazole	2.3	3.9

*LD_{50}(control)/LD_{50}(treated) for the activity of glucose oxidase or concentration of H_2O_2 added as a bolus causing 50% specific ^{51}Cr release in 4.5 h in α-MEM, 5% horse serum. LD_{50}(control) for glucose oxidase = 1.01 nmol of H_2O_2/min in both experiments. LD_{50}(control) for preformed H_2O_2 = 5.60×10^{-4} M in experiment 1 and 7.28×10^{-4} M in experiment 2. Average spontaneous ^{51}Cr release = 5.9 ± 0.3% (N=16).

‖Tumor cells were incubated in α-MEM, 10% horse serum with 0.2 mM BSO for 19 h (experiment 1) or 18 h (experiment 2). Treatment with aminotriazole (25 mM) was for 45 min prior to and during the cytolysis assay.

these same conditions, neither BSO, CDNB, aminotriazole, nor any combination of these agents elevated the spontaneous release of ^{51}Cr from the tumor cells.

Recovery from the Effects of BSO and CDNB. P815 cells were incubated with BSO for 20 h and then incubated without BSO for 0, 5, 10, or 20 h, at which time the cells were assayed for both GSH content and sensitivity to lysis by glucose oxidase (Table III). By 5 h, restoration of cellular GSH was modest, perhaps due to slow dissociation of the enzyme-inhibitor complex, and the cells continued to exhibit increased sensitivity to lysis, although some recovery was evident. Resynthesis of GSH to 89% of initial levels, seen at 10 h, was associated with near-complete recovery of the innate resistance to H_2O_2 of untreated cells.

In a similar fashion, P815 cells were treated with 25 μM CDNB for 15 min, and then incubated with or without BSO. At various times the cells were assayed for cellular GSH (Fig. 9A), or for sensitivity to lysis by glucose oxidase relative to untreated controls (Fig. 9B). Recovery of both parameters after exposure to CDNB was rapid and was inhibited completely by BSO. The slightly increased resistance to H_2O_2-mediated lysis displayed by cells which had been treated with CDNB and then incubated without BSO for 4.5 h may have reflected supernormal levels of GSH, although such a correlation is not firm (Fig. 9 and Table III).

It was expected that incubation of cells with γ-glutamylcysteine might obviate the need for a functional γ-glutamylcysteine synthetase and thereby specifically bypass the effect of BSO. However, attempts to

Table III

Time Course of Recovery of Cellular GSH and Resistance to H_2O_2 After Incubation of P815 Cells with BSO for 20 h

Treatment	% of initial GSH[≡]	Relative increase in sensitivity to lysis[×]
None	100	1.0
20 h with BSO[@]	7	3.1
20 h with BSO, then 5 h without	11	2.1
20 h with BSO, then 10 h without	89	1.1
20 h with BSO, then 20 h without	140	1.0

[≡]Initial GSH = 33.8 nmol/mg of protein.

[×]LD_{50}(control)/LD_{50}(treated) for the activity of glucose oxidase causing 50% lysis in 4.5 h in α-MEM, 5% horse serum. LD_{50}(control) = 1.3 nmol of H_2O_2/min. Spontaneous release of ^{51}Cr averaged 5.7 ± 0.3% (N=5).

[@]Incubations in α-MEM, 10% horse serum at 37°C. BSO, 0.2 mM.

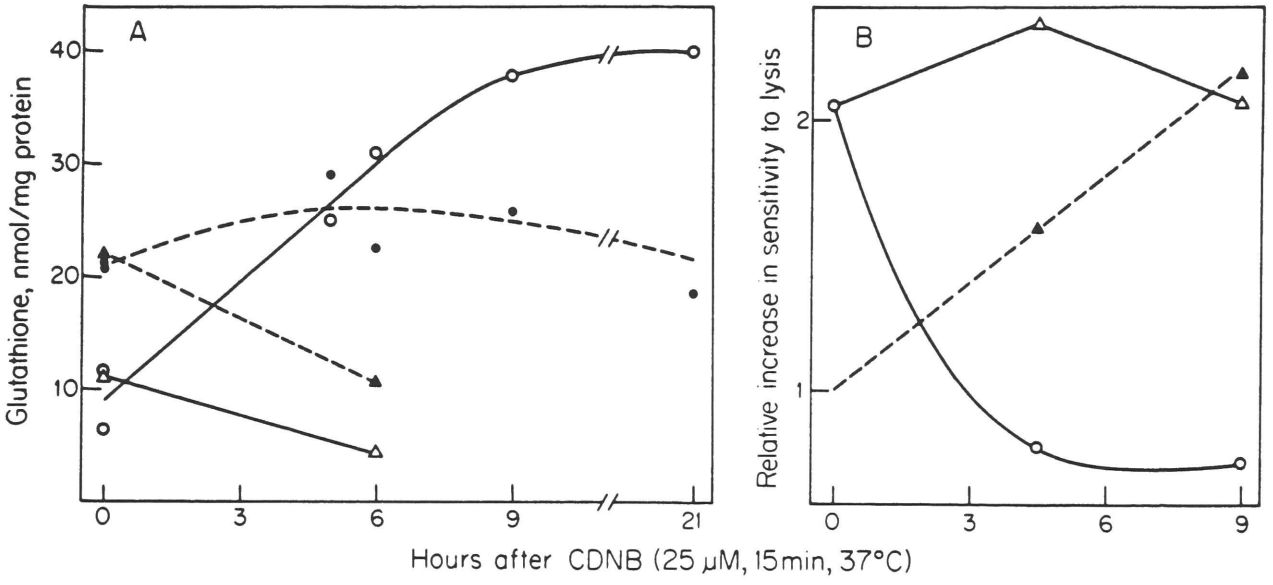

Figure 9. Time course of recovery of cellular GSH and resistance to H_2O_2 after treatment of P815 cells with CDNB. Cells were treated with CDNB (o,Δ) or vehicle alone (•,▲), incubated with (Δ,▲) or without (o,•) 0.2 mM BSO for various times, and assayed for cellular GSH (A) or for sensitivity to lysis by glucose oxidase relative to untreated controls as in Figure 7 (B). LD_{50} (control) = 1.79 ± 0.12 nmol of H_2O_2/min (N=3). Spontaneous release averaged 6.2 ± 0.7% (N=10). Recovery of both GSH content and resistance to H_2O_2 was rapid in the absence of BSO (o) and totally inhibited by 0.2 mM BSO (Δ).

induce resynthesis of GSH in the presence of BSO by incubation of GSH-depleted cells with 1 mM γ-glutamylcysteine or bis-γ-glutamyl-cystine for 5 h were without success. Presumably, either these peptides did not enter the cells or they were preferentially degraded by γ-glutamylcyclotransferase and thus not available to GSH synthetase.

Effect of GSH Depletion on Tumor Cell Lysis by Granulocytes and BCG-Activated Macrophages in the Presence of Phorbol Myristate Acetate (PMA). We also evaluated the sensitivity of GSH-depleted tumor cells to lysis by PMA-stimulated granulocytes and activated macrophages. Figure 10 shows that 16 h incubation of both P388 (A) and P815 cells (B) with 0.2 mM BSO resulted in a pronounced enhancement of granulocyte-mediated cytolysis in the presence of PMA. Lysis of BSO-treated and untreated tumor cells was inhibited by exogenous catalase.

Similarly, incubation of P388 cells with BSO for 16 h markedly augmented lysis by BCG-activated macrophages (Fig. 11). Cytolysis was inhibited by catalase an average of 58 ± 7% (N=4).

Inhibition of Tumor Cell GR by BCNU. To further investigate the role of the glutathione in tumor cell antioxidant defense, we sought a means of inhibiting GR, thereby interupting the cyclic oxidation-reduction of GSH. BCNU was recently reported to inhibit GR in human erythrocytes without influencing the activity of any other enzyme tested (Frischer and Ahmad 1977). We assayed GR in lysates of P815 cells after exposing the intact cells to BCNU for 10 min (Fig. 12). GR was 50% inhibited by 2.3 μg/ml (11 μM) BCNU and 95% inhibited by 100 μg/ml. Doses up to 300 μg/ml did not affect the activity of GPO or

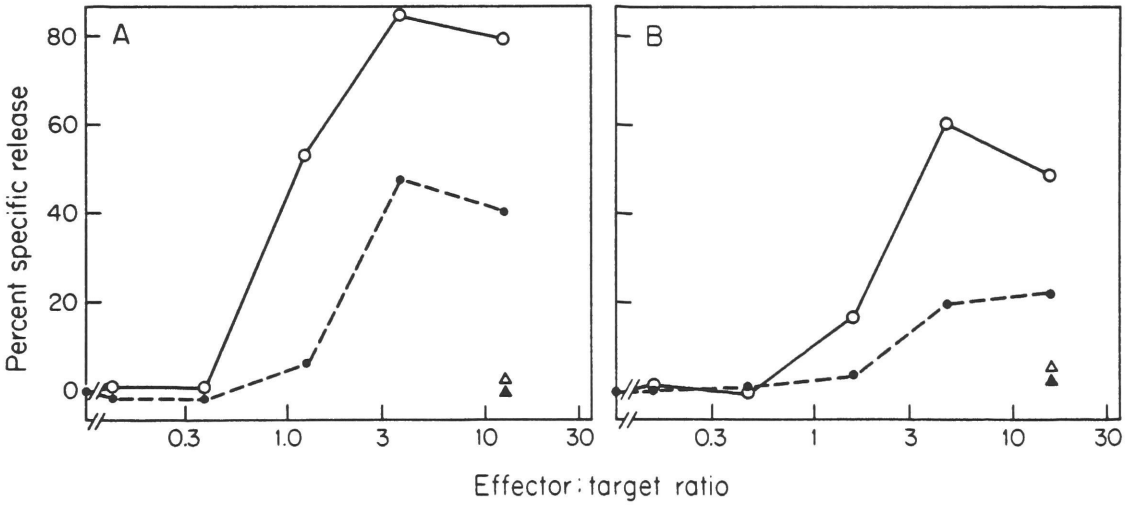

Figure 10. Effect of GSH depletion on the susceptibility of P388 cells (A) and P815 cells (B) to lysis by PMA-stimulated granulocytes. Prior to assay, the tumor cells were incubated with (o) or without (•) 0.2 mM BSO for 16 h. Catalase (3000 units/ml) inhibited lysis of both BSO-treated (Δ) and untreated (▲) cells. Points are means of triplicates. S.E. averaged 1.6%. Spontaneous release of ^{51}Cr was <11%.

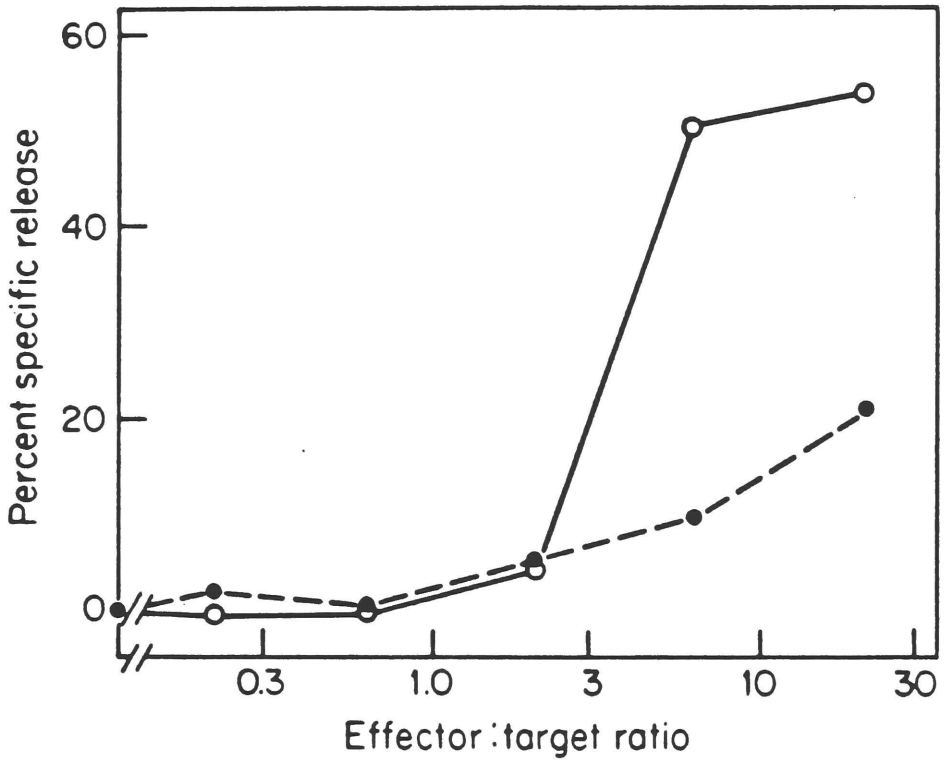

Figure 11. Effect of GSH depletion on the susceptibility of P388 cells to lysis by BCG-activated macrophages in the presence of 100 ng/ml of PMA. Prior to assay, the tumor cells were incubated with (o) or without (•) 0.2 mM BSO for 16 h. Points are means of triplicates. S.E. averaged 1.4%. Spontaneous release of ^{51}Cr was <12%.

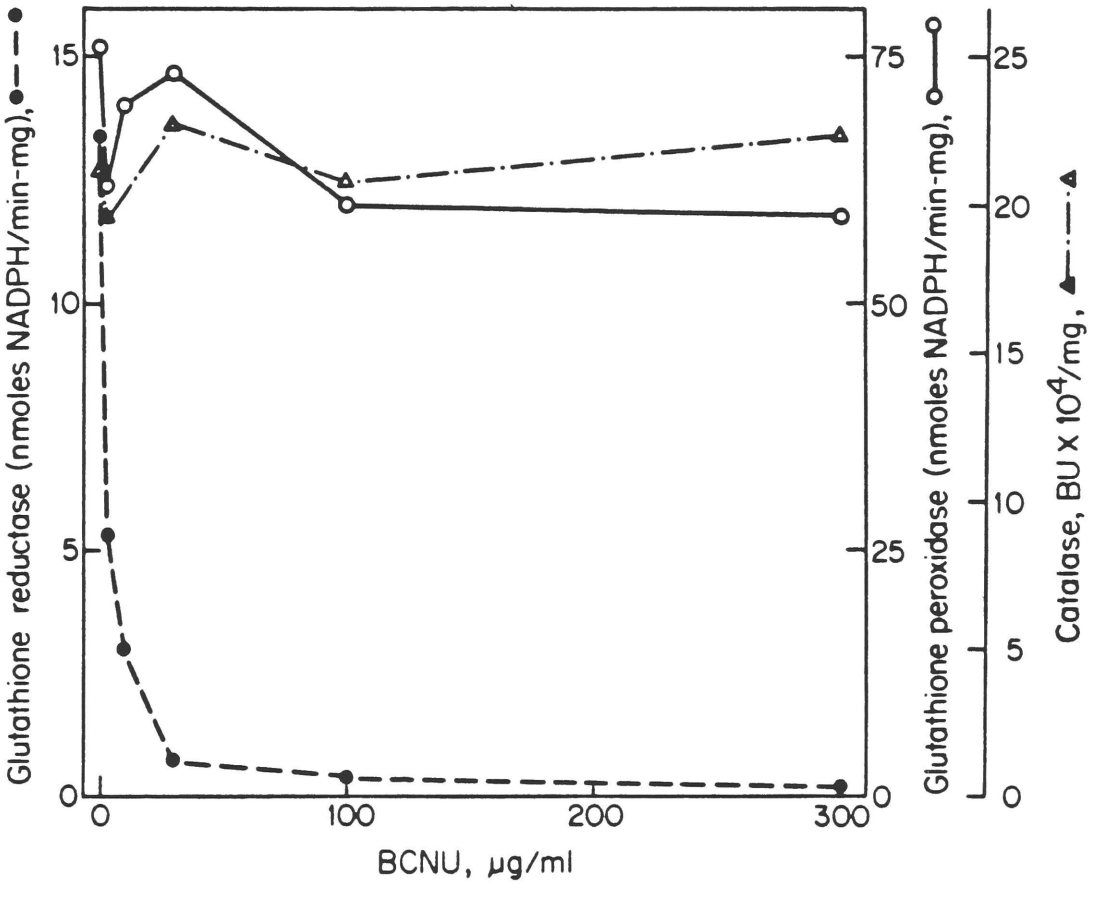

Figure 12. Effect of BCNU on the activity of GR, GPO, and Catalase in P815 cells. The intact tumor cells were incubated with the indicated doses of BCNU for 10 min at 37°C, washed, lysed, and assayed. BU, Baudhuin units.

catalase in the same cells (Fig. 12). Similar results were obtained with P388 and TLX9 lymphomas (not shown).

Effect of BCNU on the Susceptibility of Tumor Cells to Oxidative Cytolysis. Exposure of P815 mastocytoma cells to 100 μg/ml of BCNU for 10 min was nontoxic, as judged by exclusion of trypan blue and release of ^{51}Cr measured over a period of 18 h. However, BCNU treatment made the tumor cells much more susceptible to lysis by small fluxes of H_2O_2. For example, in the experiment illustrated in Figure 13, 18.7 times less glucose oxidase was required to cause 50% specific release of ^{51}Cr from P815 cells after pulsing them with BCNU. Similar results were obtained with all six of the tumors studied; representative experiments with P388 and J774 cells are also illustrated in Figure 13. Lysis of the BCNU-treated tumors by glucose oxidase was abolished by exogenous catalase, although inhibition of intracellular catalase with amino-triazole had little effect (Fig. 13).

C. Discussion

In this section, the importance of tumor cell antioxidant defense as a determinant of susceptibility to oxidative cytolysis is addressed.

The innate resistance of six murine tumor cell lines to lysis by the H_2O_2-producing enzyme glucose oxidase correlated with the cellular content of GSH, and not with the specific activities of catalase, GR, or GPO. However, such a comparison of lysed cell preparations may not accurately reflect conditions in the intact cell, especially as regards compartmentalization.

Depletion of intracellular GSH was accomplished by two independent

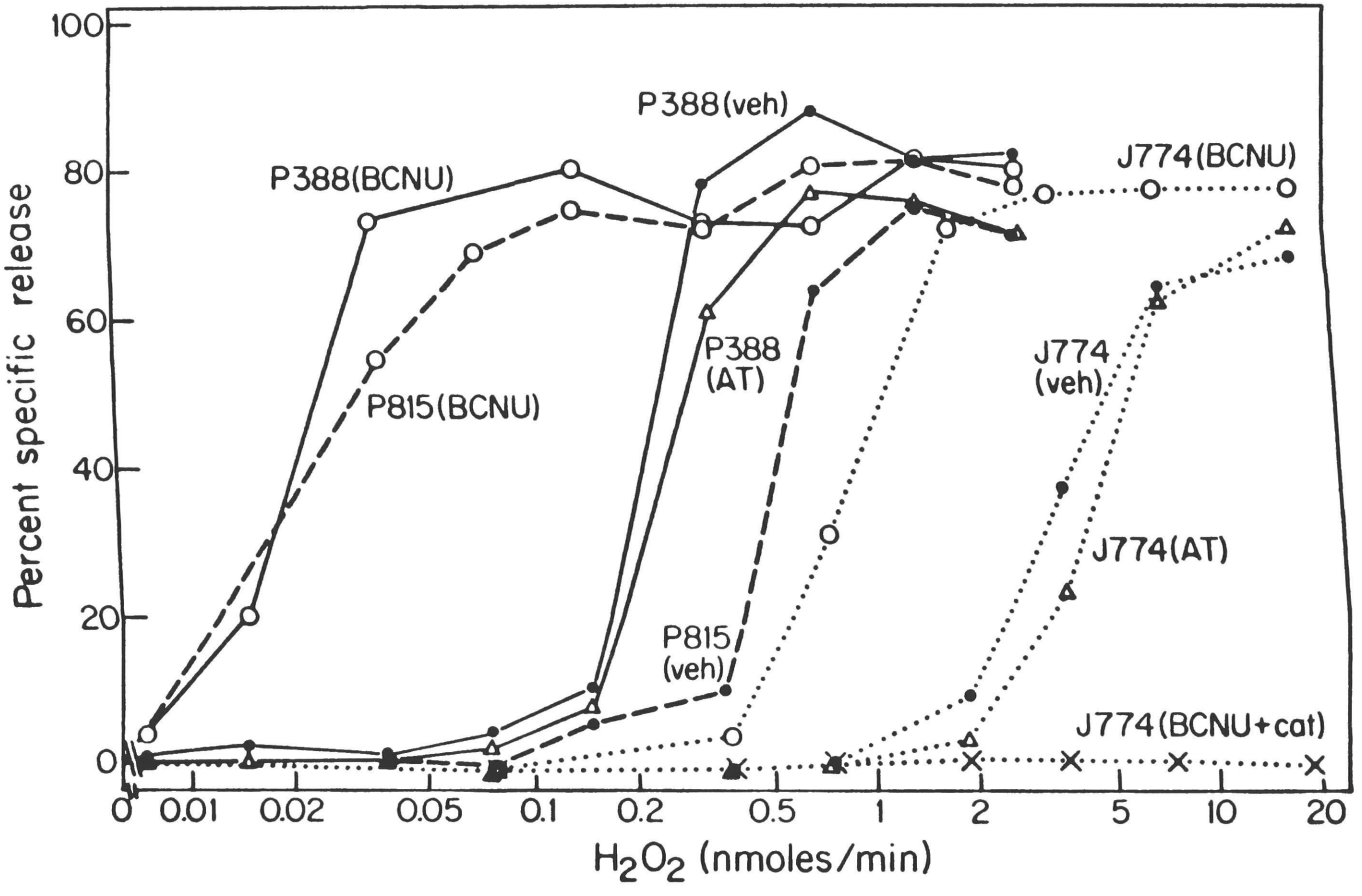

Figure 13. Effect of BCNU and aminotriazole on sensitivity of P388 (—), P815 (– –), and J774 (·····) to lysis by fluxes of H_2O_2. ^{51}Cr-release was measured from the tumor cells after a 4.5-h incubation with dilutions of glucose oxidase. Before assay, the cells were treated with vehicle alone (veh,•) or 100 µg/ml BCNU for 10 min (o), or incubated with 25 or 50 mM aminotriazole (AT,Δ) for 45-60 min before and then throughout the assay. Catalase (cat) (3,000 units/ml) abolished lysis in all cases, as shown for J774 (×). Points are means of triplicates. SEM averaged 2.0%.

methods. First, de novo GSH biosynthesis was inhibited by incubation of cells with BSO, a selective inhibitor of γ-glutamylcysteine synthetase. The rate of GSH depletion seen with inhibition of synthesis represents a combination of GSH catabolism, efflux, and dilution by continuing cell division. Incubation of P388 lymphoma or P815 mastocytoma cells with 0.2 mM BSO resulted in a steady decline in GSH content, such that 50% reduction was reached by 4.5-5 h and 90% depletion by 15 h. As an alternative approach to GSH depletion, we made use of the endogenous enzyme GSH S-transferase by treatment of intact cells with exogenous substrate (CDNB). Addition of 10 μM CDNB to P815 cells resulted in a rapid consumption of GSH (approximately 70%). The inability to achieve more extensive depletion of GSH with 30 μM CDNB may be due to its propensity to inactivate the transferase which catalyzes its conjugation with GSH (Pabst et al. 1974).

After 15-22 h of incubation with BSO, P815 and P388 cells contained 2-8% of their initial GSH content and exhibited markedly increased sensitivity to lysis by a flux of H_2O_2. These GSH-depleted tumor cells were effectively lysed by dilutions of glucose oxidase and numbers of PMA-stimulated granulocytes or activated macrophages which were ineffectual against untreated tumors. Treatment of P815 cells with 10-30 μM CDNB for 15 min was equivalent to a 9-h incubation with BSO in terms of the relative increase in sensitivity to lysis by H_2O_2 as well as the extent of GSH depletion. The inclusion of 15 μM CDNB in the 4.5-h cytolysis assay, however, resulted in sensitization to H_2O_2-mediated lysis beyond that obtained with BSO. Perhaps the combination of GSH depletion and inhibition of GR by CDNB, reversible upon

dilution, was responsible for this further enhanced sensitivity to H_2O_2.

Recovery of tumor cell resistance to peroxide after BSO-induced GSH depletion followed the same time course as resynthesis of cellular GSH. Similarly, recovery of resistance to lysis by H_2O_2 after treatment with CDNB was dependent upon de novo GSH synthesis and followed an equivalent time course. The resynthesis of GSH to supernormal levels subsequent to depletion by sulfhydryl-reactive compounds, such as was observed here with CDNB, has been previously reported for rat duodenal mucosa (Pisciotto and Graziano 1980). Tempory release of γ-glutamyl-cysteine synthetase from nonallosteric inhibition by GSH may play a role in this phenomenon (Richman and Meister 1975).

Inhibition of tumor cell GR with BCNU was based on the work of Frischer and Ahmad (1977) and Babson and Reed (1978). Incubation of intact P815 cells with 100 μg/ml of BCNU for 10 min served to inhibit GR by 95% without effect on catalase or GPO activities. Tumor cells in which GR had been inhibited were markedly more susceptible to lysis by H_2O_2. BCNU sensitized tumor cells to lysis by activated macrophages in the presence of PMA or antitumor antibody, but not to allosensitized T cells, nor to lysis by antibody plus complement (Nathan et al. 1981). It has also been reported that GPO-deficient tumor cells, generated by repeated passage of tumor cells in mice fed a selenium-deficient diet, are substantially more sensitive to lysis by glucose oxidase and by PMA-stimulated granulocytes and macrophages (Nathan et al. 1981).

Comparison of results obtained with BSO-induced GSH depletion with those observed with inhibition of GR by BCNU yields informative

differences. BCNU treatment of P388 results in greater sensitization to H_2O_2 than does BCNU treatment of P815 (Nathan et al. 1981 and above), whereas the converse is true with GSH depletion. This is perhaps related to the fact that of the two cell lines, P815 contains more GSH, whereas P388 has more GR. In contrast to the results obtained with P815 and P388, a 95% reduction in the GSH content of the TLX9 lymphoma does not alter its susceptibility to lysis by glucose oxidase. In this regard, it should be noted that GSH depletion sensitizes P815 and P388 cells to amounts of glucose oxidase which will effectively lyse untreated TLX9 cells. Furthermore, of the three cell lines, TLX9 contains the most GR, inhibition of which with BCNU does impair its antioxidant capabilities (Nathan et al. 1981). Thus, the intracellular concentration of GSH appears not to be rate-limiting in the GSH redox cycle of TLX9, even after a 95% depletion. In addition, these tumor cell lines may vary in their content of antioxidants other than GSH. Finally, efforts to demonstrate enhanced macrophage-mediated antibody-dependent cytotoxicity against GSH-depleted tumors, as had been observed with BCNU-treated tumor cells (Nathan et al. 1981), were without success (unpublished observations). The reasons for this difference are not understood.

In erythrocytes, the relative importance of GPO and catalase for the detoxification of H_2O_2 has been shown to depend upon the rate at which H_2O_2 is added to the cells (Cohen and Hochstein 1961, 1963). Similarly, we found the mode of peroxide delivery (as a bolus of pre-formed H_2O_2 versus a flux of nascent H_2O_2) to be an important factor in determining the antioxidant pathways a target cell might effectively

employ to escape injury. In response to nascent H_2O_2, such as that generated by glucose oxidase or leukocytes, the glutathione system, but not catalase, played a major protective role. This may be attributed to the ability of GPO, but not catalase, to detoxify lipid hydroperoxides (Christophersen 1968) or to an affinity of GPO for H_2O_2 3 orders of magnitude greater than that of catalase (Ogura 1955; Flohe and Brand 1969). When H_2O_2 was added as a bolus, however, both catalase and GSH were able to function in a protective capacity, since sensitization was seen only with a combination of GSH depletion and inhibition of catalase. In contrast, it has recently been reported that lysis of human erythrocytes by PMA-stimulated neutrophils can be enhanced by inhibition of erythrocyte catalase with aminotriazole, as well as by inhibition of GR with BCNU (the combination was not tested) (Weiss 1980). Furthermore, erythrocyte lysis was dependent upon methemoglobin formation. Thus, conclusions concerning both the nature of cell-mediated oxidative injury and the relative importance of target cell antioxidant defenses based on studies with erythrocyte targets (Kellog and Fridovich 1977; Borregaard and Kragballe 1980; Weiss and LoBuglio 1980; Weiss et al. 1980; Katz et al. 1980) cannot be generalized to tumor cells without caution.

It should be noted that of the agents commonly used to alter intracellular levels of GSH or its redox cycle, BSO is the least toxic and the most specific. It does not react with cellular nucleophiles. Conclusions as to the importance of GSH for cell division and continued viability which are based on experiments in which cells are depleted of GSH by exposure to such reactive agents as diethyl maleate, phenyl

glyoxal, and 2-cyclohexenone, are weakened by questions of reagent specificity. For murine tumor cells, as with erythrocytes (Kosower et al. 1971), it appears that a temporary reduction in cellular GSH content is compatible with normal viability unless an oxidant challenge is generated concurrently. It is conceivable, therefore, that in situations where potential oxidative injury can be directed to selected target cells, such as by covalent linkage of glucose oxidase to tumor-specific antibody (Philpott et al. 1973), nonspecific GSH depletion might result in marked synergy without loss of selective toxicity.

II. Lysis by Sulfhydryl-reactive Antineoplastics

A. Introduction

In the previous section, the importance of the GSH redox cycle in tumor cell defense against oxidative injury, such as that inflicted by cells of the host immune system or by incubation with glucose oxidase plus glucose, was documented. In a variety of cell types, GSH plays a role in protection against toxic electrophiles by thioether formation. It was conceivable, therefore, that tumor cell GSH might effectively detoxify and thus limit the effectiveness of sulfhydryl-reactive antineoplastic agents. To evaluate this possibility, the role of GSH and its synthesis in tumor cell defense against a class of sulfhydryl-reactive antitumor agents, the sesquiterpene lactones (SLs), was examined.

SLs are among the natural products that have attracted attention recently as prototypes for the development of novel chemotherapeutic agents (Kupchan 1970, 1974; Rodriguez et al. 1976; Douros and Suffness 1978; Cassady and Suffness 1980). Some SLs inhibit cell growth in vitro, and at higher concentrations, lead to cell death (Lee et al. 1971; Rosowsky et al. 1974; Hladon et al. 1975; Woynarowski and Konopa 1981; Dupuis and Brisson 1976). High reactivity towards sulfhydryl groups in aqueous buffer is a characteristic feature of SLs (Kupchan et al. 1970, 1971; Lee et al. 1977; Picman et al. 1979), and has led to the hypothesis that their antitumor activity is the result of S-alkylation of growth-regulatory or otherwise vital macromolecules

(Kupchan et al. 1971; Kupchan 1974, 1976; Fujita and Nagao 1977). Several sulfhydryl-dependent enzymes have been shown to be inhibited by these agents (Hanson et al. 1970; Smith et al. 1972; Lee et al. 1977; Hall et al. 1978). Nonetheless, the cellular targets relevant to their antitumor activity in vitro or in vivo have not been identified. Furthermore, it has not been reported whether SLs are reactive towards sulfhydryl groups in intact cells.

In this section the results of studies with four SLs that are active in vivo are presented (vernolepin, helenalin, elephantopin, and eriofertopin, Fig. 14). Data are provided correlating sulfhydryl reactivity with a role for tumor cell GSH as a determinant of cytolysis by an additional seven unrelated chemotherapeutic compounds.

B. Results

Depletion of Tumor Cell GSH by SLs. The possible interaction of cellular GSH with SLs was first evaluated. Incubation of P815 mastocytoma cells with vernolepin, helenalin, elephantopin, or eriofertopin for 1 h resulted in 70-97% depletion of GSH (Table IV). The time course and dose response of GSH depletion by helenalin was examined in greater detail in the experiments illustrated in Figure 15. Cells were incubated with a range of concentrations of helenalin with or without 0.2 mM BSO, thus allowing for the observation of effects with and without ongoing GSH synthesis. Depletion of GSH by incubation of P815 cells with 25 μg/ml of helenalin was both rapid (90% loss within 15 min) and persistent (up to 7 h of coincubation) (Fig. 15A). Incubation of cells with 1 μg/ml of helenalin did not result in

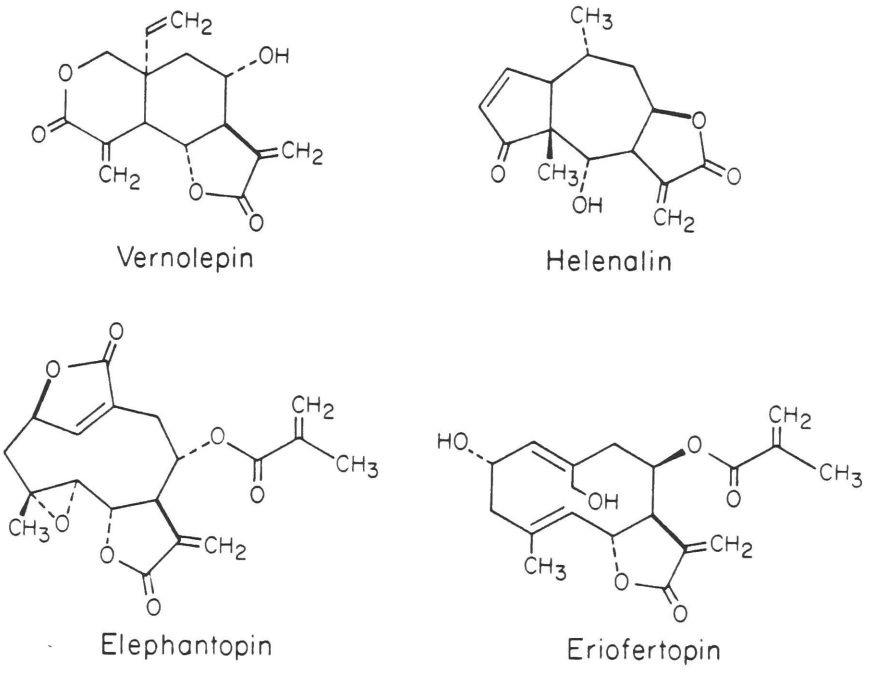

Figure 14. Structures of the sesquiterpene lactones studied.

Table IV

Effect of Cytotoxic SLs on GSH Content of P815 Cells

SL	µg/ml	% GSH remaining*	(N)
Vernolepin	10	6.7 ± 2.7	(5)
	25	2.8 ± 0.5	(2)
Helenalin	5	41 ± 6.8	(2)
	10	23 ± 1.2	(3)
	25	5.7 ± 1.6	(3)
Elephantopin	5	39 ± 2.9	(4)
	15	12 ± 0.2	(2)
Eriofertopin	50	29 ± 3.6	(4)

*P815 cells were incubated in α-MEM, 5% horse serum with the indicated concentrations of sesquiterpene lactones for 1 h. Data are from 10 experiments with initial GSH = 29.6 nmol/mg of protein.

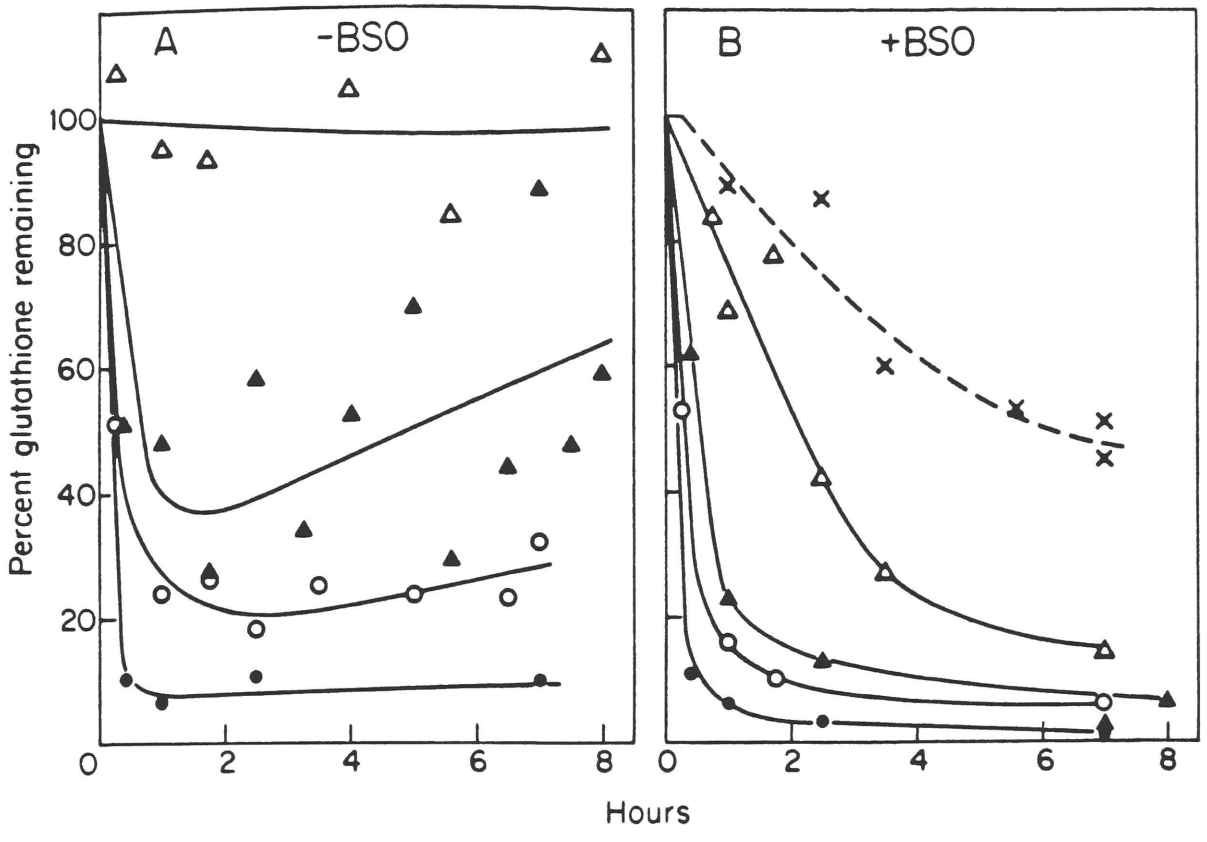

Figure 15. Time course and dose response of GSH depletion by helenalin in the absence (A) and presence (B) of BSO. P815 cells were incubated with helenalin at 1 μg/ml (Δ), 5 μg/ml (▲), 10 μg/ml (o), 25 μg/ml (•), or no helenalin (×). In panel B, the incubation medium also contained 0.2 mM BSO. Data are from five experiments with initial GSH levels of 28.1 ± 6.3 nmol/mg of protein.

detectable GSH depletion. However, the rate of GSH depletion upon incubation with 1 μg/ml of helenalin plus BSO exceeded that observed with BSO alone (Fig. 15B). By inference, 1 μg/ml of helenalin must react with a substantial portion of intracellular GSH, and rapid resynthesis, if unimpeded, maintains GSH content at normal levels. Similarly, the recovery of GSH levels observed with 5 μg/ml of helenalin was abolished by the inclusion of BSO (Fig. 15, closed triangles). By examination of the medium after incubation of cells with helenalin, it was determined that depletion of GSH was not due to its release from the cells (not shown).

Effect of BSO on the Lysis of Tumor Cells by SLs. The sensitivity of ^{51}Cr-labeled tumor cells to lysis by SLs was first evaluated by an 18-h coincubation. Figure 16 illustrates one such experiment in which the lysis of P815 cells by helanalin in the presence or absence of 0.2 mM BSO was measured. Incubation of tumor cells with BSO alone was nontoxic. However, cytolysis by helenalin was greatly enhanced: nonlytic concentrations of helenalin resulted in near-complete lysis when BSO was included (Fig. 16). In Figure 16, BSO reduced the LD_{50} for helenalin by a factor of 4.7. The effect of BSO on the sensitivity of four tumor cell lines to lysis by SLs is documented in Table V. In each experiment, a range of SL concentrations was tested and the LD_{50} was calculated as in Figure 16. In all cases, the presence of BSO reduced the LD_{50} manyfold. BSO provided the most dramatic synergy with vernolepin, leading to an approximately 20-fold decrease in the LD_{50} for P815.

It is of interest to note that for these tumors, the rank order of

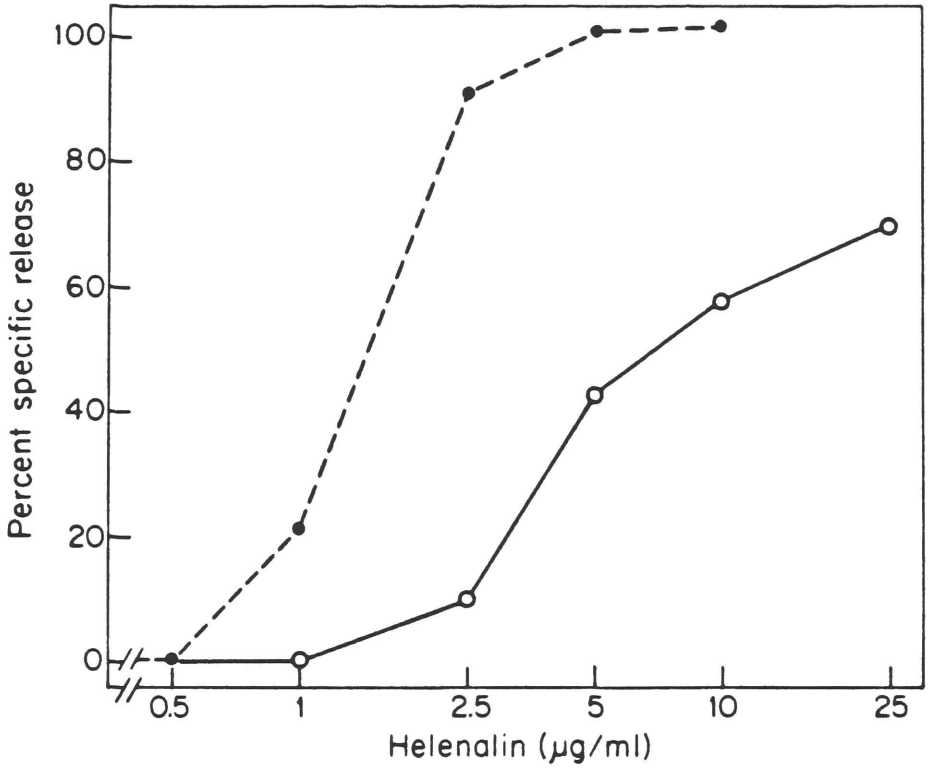

Figure 16. Effect of BSO on the sensitivity of P815 cells to lysis by helenalin. ^{51}Cr release was measured after an 18-h incubation with the indicated concentrations of helenalin in the absence (o) or presence (•) of 0.2 mM BSO. Points are means of triplicate determinations. S.E. averaged 2.4%. Spontaneous release of ^{51}Cr label was 22%.

Table V

Effect of BSO on Sensitivity of Tumor Cells to Lysis by SLs

Tumor	SL	LD_{50}, µg/ml‖ (µM)	BSO-induced relative increase in sensitivity to lysis*	(N)
P815	Vernolepin	21 ± 13 (75)	20 ± 8.3	(13)
P815	Helenalin	12 ± 3.8 (47)	6.4 ± 1.6	(4)
P815	Elephantopin	28 ± 4.5 (79)	5.6 ± 2.5	(4)
P815	Eriofertopin	68 ± 24 (195)	6.8 ± 1.8	(5)
YAC	Vernolepin	4.6 ± 2.0 (17)	8.9 ± 2.4	(4)
TLX9	Vernolepin	16 ± 7.4 (59)	19 ± 10	(4)
J774	Vernolepin	47 ± 16 (169)	34 ± 18	(3)

‖^{51}Cr-labeled tumor cells were incubated for 18 h with the indicated SLs and LD_{50} values were calculated by interpolation from curves like those in Figure 16. Average spontaneous ^{51}Cr release in the presence and absence of BSO, respectively, for the four tumors: P815, 26.6±4.4, 25.7±4.3% (N=26); YAC, 21.3±4.6, 20.0±3.0% (N=4); TLX9, 21.8±1.1, 21.0 ±2.3% (N=4); J774, 20.1±1.7, 19.9±2.4% (N=3).

*The LD_{50} in the absence of BSO divided by the LD_{50} in the presence of 0.2 mM BSO represents the relative increase in sensitivity to lysis by the various SLs.

resistance to lysis by vernolepin (J774 > P815 > TLX9 > YAC) does not correspond to GSH content (J774 > YAC > P815 > TLX9) (Nathan et al. 1981). Furthermore, incubation of P815 cells with 10 μg/ml of vernolepin for 1 h before the 18-h assay did not result in increased cytolysis (not shown), in spite of substantial depletion of GSH (>90% in Table IV). Thus, in contrast to the importance of GSH resynthesis in protecting tumor cells upon exposure to SLs, GSH content prior to exposure to these agents did not influence subsequent lysis.

The synergistic interaction between BSO and vernolepin was further analyzed by considering the kinetics of onset and reversal of their effects. For this, exposure of cells to vernolepin was limited to a 1-h pulse, followed by an 18-h incubation in its absence, at which time lysis was determined. Figure 17 illustrates the results obtained in one of three such experiments in which lysis in the absence of BSO (open triangles, LD_{50} = 63 μg/ml) was compared to lysis observed when BSO was present 30 min before and during the 1-h vernolepin incubation (open circles, LD_{50} = 7.3 μg/ml); during this time as well as the subsequent 18-h incubation (close circles, LD_{50} = 6.8 μg/ml); or during the 18-h incubation only (close triangles, LD_{50} = 13.8 μg/ml). Thus, addition of BSO need not coincide with but can immediately follow exposure to vernolepin for markedly enhanced cytolysis to result.

Time Course and Recovery From the Effects of Vernolepin and BSO. Figure 18 illustrates the correlation between GSH resynthesis and loss of tumor cell sensitivity to the synergistic effect of BSO added after a 1-h pulse of vernolepin. Recovery of GSH content upon subsequent incubation was rapid, reaching control levels by 3.5 h. Recovery of GSH

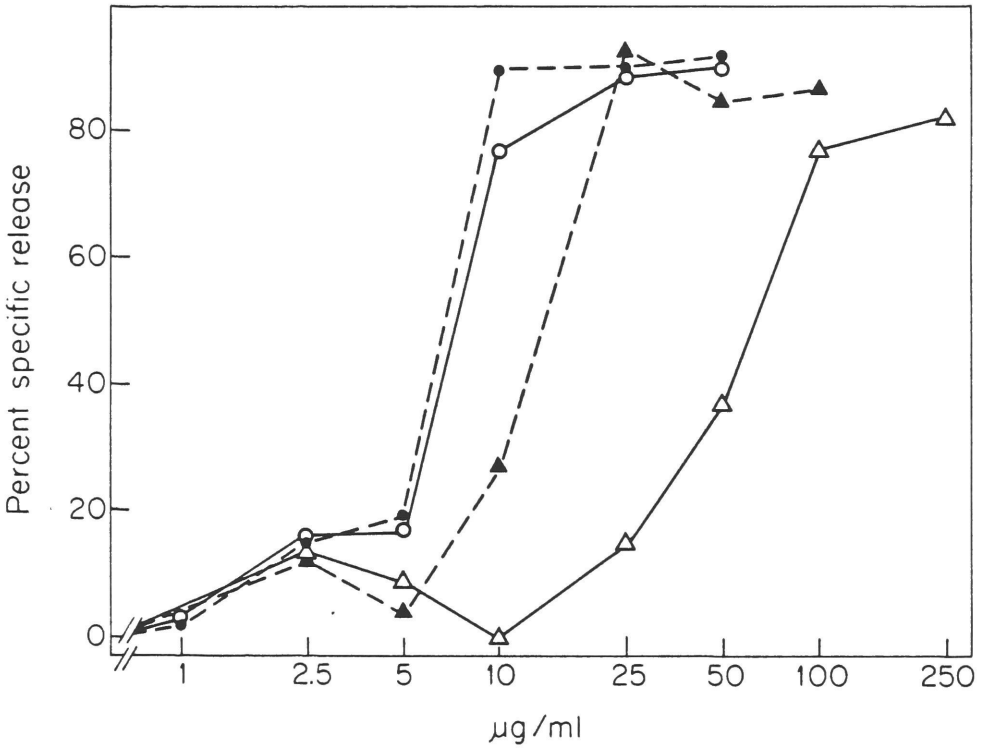

Figure 17. Effect of BSO on sensitivity of P815 cells to lysis at 18 h after a 1-h incubation with vernolepin. Cells were pulsed with the indicated concentrations of vernolepin for 1 h and then incubated for 18 h, at which time ^{51}Cr release was measured. BSO was present at 0.2 mM 30 min before and during the 1-h pulse (o), during this time as well as the subsequent 18-h incubation (•), during the 18-h incubation only (▲), or never (Δ). Points are means of triplicate determinations. S.E. averaged 1.2%. Spontaneous release was 20-22%.

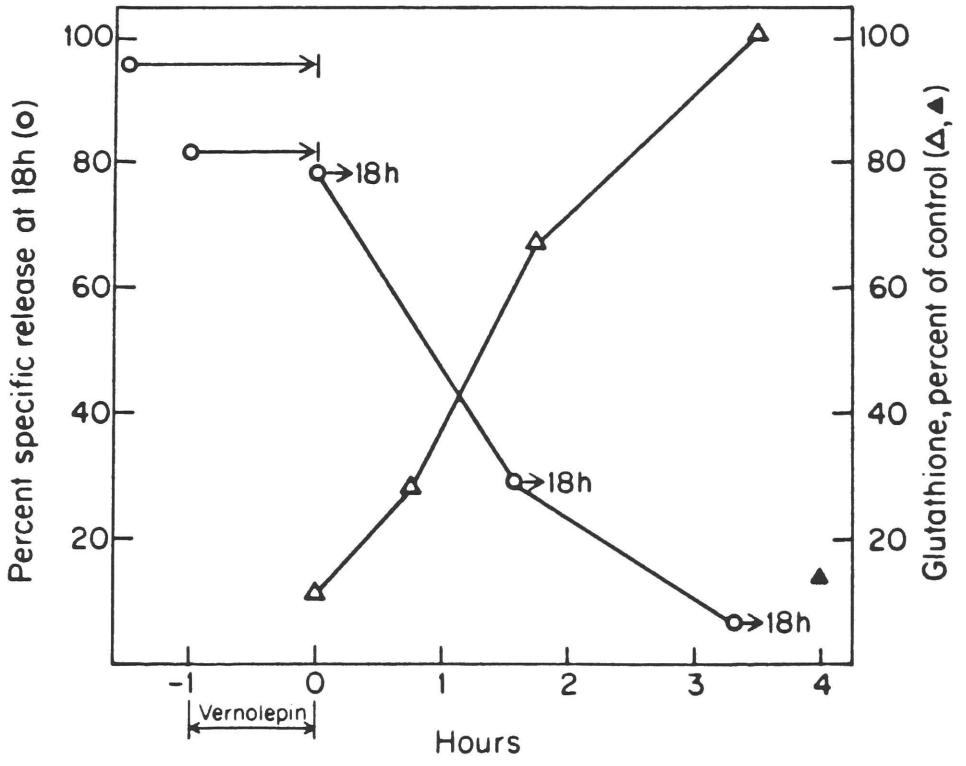

Figure 18. Correlation between the rate of GSH resynthesis after a 1-h incubation with vernolepin and the loss over time of synergistic cytolysis when BSO was added after the vernolepin pulse. In the cytolysis assay, cells were pulsed with 10 μg/ml of vernolepin for 1 h and then incubated for 18 h at which time ^{51}Cr release was determined. BSO was present in the medium for the indicated time spans (o⟶), i.e. either it was present during the vernolepin pulse and then washed out or it was added at the indicated times after the pulse treatment for the remainder of the 18-h incubation. S.E. averaged 1.3% for triplicates. Spontaneous release was <28%. Percent specific release from cells pulsed with vernolepin but never incubated with BSO was <3%. Unlabeled P815 cells were similarly incubated with 10 μg/ml of vernolepin for 1 h, washed, incubated in the presence (▲) or absence (Δ) of BSO for the indicated times, and then assayed for GSH content. Untreated cells contained 24.4 ± 2.4 nmol GSH/mg of protein (N=4).

content could be inhibited by BSO, indicating that de novo resynthesis was occuring. A 1.5-h delay in the addition of BSO to cells previously pulsed for 1 h with 10 μg/ml of vernolepin allowed for resynthesis of GSH to approximately 60% of control, and resulted in roughly a 60% decrease in enhancement of cytolysis, compared to the addition of BSO immediately after the vernolepin pulse. The marked cytolysis observed by treatment of cells with 10 μg/ml of vernolepin followed by an 18-h incubation with BSO was not prevented by the addition of up to 7 mM GSH to the medium shortly after the start of the 18-h incubation (not shown).

The reversal of BSO inhibition of γ-glutamylcysteine synthetase is not immediate (see Table III in Section I of Results). We therefore compared the time course of recovery from the effects of BSO on cellular GSH content and on sensitivity to vernolepin-mediated cytolysis. In the experiment illustrated in Figure 19, P815 cells were incubated with BSO for 1 h, washed, incubated for various additional times, and then assayed both for GSH content and for susceptibility to lysis by vernolepin. Gradual recovery of GSH synthesis was evident within 2 to 3 h of the removal of BSO. A net increase in GSH content did not occur until 4 or more h of incubation in the absence of BSO (Fig. 19A). Similarly, approximately 3 h after a 1-h incubation with BSO, cells began to recover their resistance to the cytolytic effect of vernolepin (Fig. 19B). Thus, augmentation by BSO of susceptibility to lysis by vernolepin disappeared just as the tumor cells regained their capacity to synthesize GSH. Addition of cycloheximide (10 μg/ml) after removal of BSO did not inhibit recovery, indicating that synthesis of

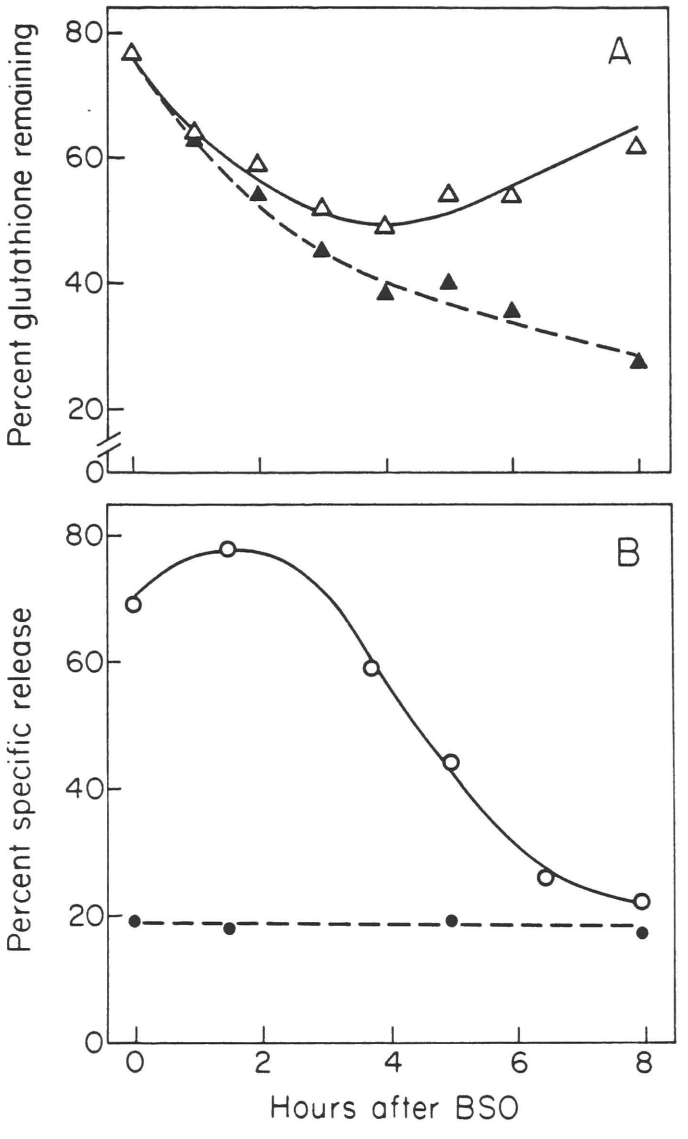

Figure 19. Time course of recovery from the effects of BSO. (A) P815 cells were incubated with 0.2 mM BSO for 1 h, washed, and then incubated in the presence (▲) or absence (△) of BSO. At various times GSH content was determined and is here expressed as percent GSH remaining (initial level, 24.2 nmol/mg of protein). (B) Cells were incubated for 1 h in the presence (o) or absence (•) of BSO, washed, and then incubated in its absence. At the indicated times, vernolepin (20 μg/ml) and ^{51}Cr were added for an additional 1 h, cells were washed, and then incubated for 18 h, at which time specific release was determined. Spontaneous release was 20%. S.E. averaged 1%.

new γ-glutamylcysteine synthetase was not required in order to reverse the effects of BSO (not shown).

GSH Depletion by Other Antitumor Agents and the Effect of BSO on Cytolysis by These Agents. Many antitumor agents recently derived from plants, in addition to the cytotoxic SLs, have been shown to possess high reactivity toward sulfhydryl groups (Hanson et al. 1970; Kupchan and Tsou 1973; Lillehaug et al. 1973; Kupchan and Schubert 1974; Kupchan and Lacadie 1975; Kupchan 1976; Fujita and Nagao 1977). Jatrophone, one such compound, was isolated in 1970 from a plant used in Costa Rica for the treatment of cancer. Incubation of P815 cells with jatrophone resulted in marked depletion of GSH, with only 11% of initial levels remaining after a 1-h incubation with 15 μg/ml (Table VI). P815 cells were incubated for 18 h with various concentrations of jatrophone in the presence or absence of BSO, at which time lysis was measured. One of four such experiments is shown in Figure 20. Inhibition of GSH synthesis resulted in a 21.3 ± 7-fold increase in sensitivity to lysis, relative to cells allowed to synthesize GSH (Table VII). In contrast, prior depletion of GSH by pulsing cells with 10 μg/ml of vernolepin for 1 h did not sensitize the cells to the lytic effects of jatrophone (not shown).

In a similar manner, six other antitumor agents chosen from three major classes of antineoplastics (alkylating agents, anti-metabolites, and natural products) were studied. The effects of these compounds on the GSH content of P815 cells, using a 1-h incubation at concentrations that would be lytic in an 18-h assay, are documented in Table VI. Of these, only BCNU was able significantly to reduce the GSH content of

Table VI

Effect of Various Antineoplastic Agents on GSH Content of P815 Cells

Agent	µg/ml	% GSH remaining*	(N)
Doxorubicin	100	96 ± 10	(2)
	300	101 ± 18	(3)
Mitomycin C	5	91 ± 4.7	(2)
	10	96 ± 0.5	(3)
Vinblastine	100	82 ± 6.0	(2)
Ara-C	300	81 ± 14	(2)
Maytansine	50	88 ± 4.6	(3)
BCNU	50	13 ± 2.9	(2)
	100	10 ± 1.6	(2)
	200	20 ± 9.3	(2)
Jatrophone	5	38 ± 2.7	(3)
	15	11 ± 1.5	(2)

*Data are from 5 experiments with initial GSH = 25.4 ± 10.5 nmol/mg of protein. Cells were incubated with agents at the indicated concentrations for 1 h at 37°C.

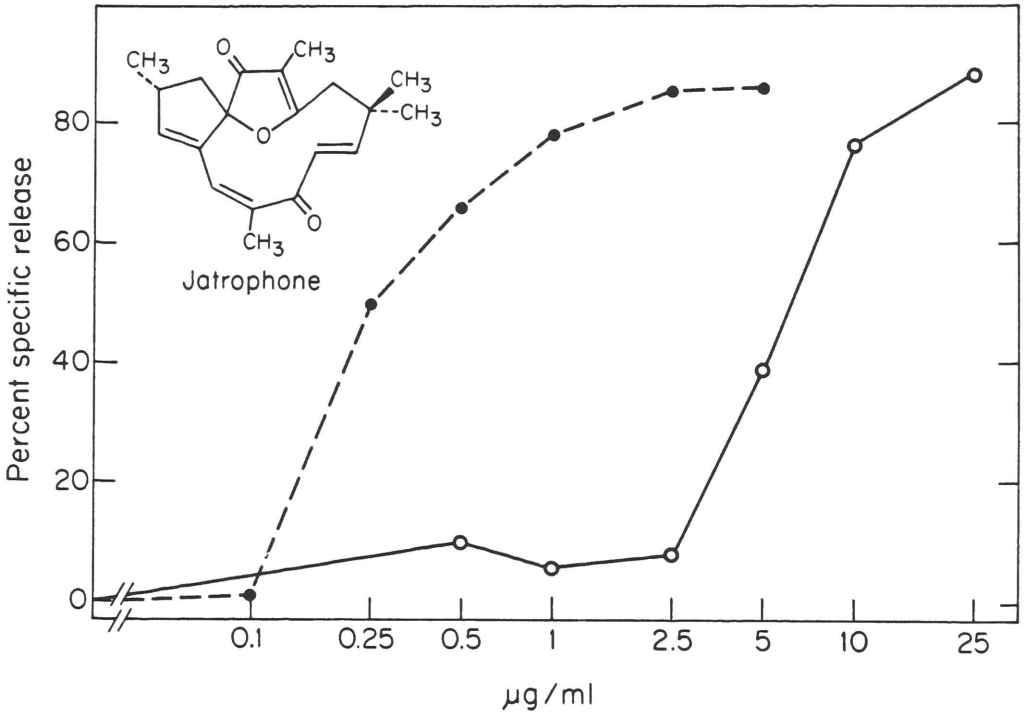

Figure 20. Effect of BSO on sensitivity of P815 cells to lysis by jatrophone. ^{51}Cr release was measured after an 18-h incubation with the indicated concentrations of jatrophone in the absence (o) or presence (•) of 0.2 mM BSO. S.E. averaged 1.9% for triplicates. Spontaneous release was <26%.

Table VII

Effect of BSO on Sensitivity of P815 Cells to Lysis by Various Antineoplastic Agents

Agent	LD_{50}*, µg/ml (µM)	BSO-induced relative increase in sensitivity to lysis	(N)
Doxorubicin	74 ± 14 (127)	1.2 ± 0.8	(3)
Mitomycin C	5.8 ± 0.6 (17)	0.99 ± 0.06	(2)
Vinblastine	30 ± 5.0 (33)	0.74 ± 0.11	(2)
Ara-C	4.9 ± 0.6 (18)	0.71 ± 0.10	(2)
Maytansine	36 ± 4.0 (52)	0.91 ± 0.09	(3)
BCNU	37 ± 7.0 (174)	1.17 ± 0.05	(3)
Jatrophone	6.8 ± 1.0 (22)	21.3 ± 7.0	(4)

*LD_{50} values were calculated by interpolation from dose-response curves. For Ara-C, LD_{25} is reported. S.E. averaged 2.4% for all agents. Average spontaneous ^{51}Cr release in the absence or presence of BSO, respectively, was 23.9 ± 4.2 and 22.3 ± 4.1% (N=19).

the cells. However, unlike jatrophone or SLs, cytolysis by BCNU after an 18-h incubation was not affected by the presence of BSO (Table VII). The lack of an effect of BSO on cytolysis by the other five agents correlated with their inability to deplete cellular GSH (Tables VI and VII).

C. Discussion

Incubation of P815 mastocytoma cells with any of the four SLs studied (vernolepin, helenalin, elephantopin, and eriofertopin) resulted in rapid, dose-dependent depletion of GSH, presumably via adduct formation. Comparison of the rate of GSH depletion by low concentrations of helenalin (1-5 μg/ml) in the presence of BSO, a nontoxic and selective inhibitor of γ-glutamylcysteine synthetase, with that observed with BSO alone or helenalin alone, suggests that a compensatory increase in the rate of GSH synthesis is an early cellular response to these agents.

Extensive GSH depletion of P815 cells by prolonged incubation with BSO was without effect on their viability or even their growth (Section I of Results). In striking contrast, inhibition of GSH synthesis during an 18-h incubation with SLs markedly enhanced the lysis of the same cells. A potent synergistic effect of BSO on cytolysis by vernolepin was also observed with each of the other murine tumors tested (YAC, TLX9, and J774).

To characterize further the role of GSH synthetic capacity as a determinant of tumor cell susceptibility to cytolysis by SLs, P815 cells were pulsed with vernolepin for 1 h, washed, and assayed for ^{51}Cr

release after an additional 18-h incubation in the absence of vernolepin. Addition of BSO to cells promptly after the vernolepin pulse resulted in enhanced lysis. However, as the interval lengthened between the vernolepin pulse and exposure to BSO, the tumor cells rapidly lost this enhanced susceptibility to lysis, with a time course that correlated with the restoration of GSH levels.

BSO probably does not inhibit GSH synthesis by intact cells immediately after it is added to the extracellular medium. This substrate analog must first enter the cell (Griffith 1981a) and be phosphorylated by its target enzyme, γ-glutamylcysteine synthetase (Griffith 1981b, 1982). More cytolysis was evident if BSO was present not only during the 1-h vernolepin treatment, but 30 min before it as well (96% vs. 81%, $p < 0.05$; Fig. 18). Thus, P815 cells appear to be more sensitive to the lytic effects of vernolepin if their GSH synthetic capacity is already inhibited at the onset of vernolepin exposure.

Another aspect of BSO-induced inhibition of GSH synthesis relevant to this analysis is its delayed reversibility. After a 1-h pulse with BSO, GSH synthetic capacity sufficient to replenish cellular GSH was not manifest until 4-5 h of further incubation. Likewise, if a pulse with BSO preceded treatment with vernolepin, a synergistic effect on cytolysis was only seen with the interval between exposures to the two agents was <4-5 h.

The effect of BSO on cytolysis by seven additional antitumor agents was evaluated, and compared to the sulfhydryl-reactivity of the same compounds. With one exception, the relative ability of the

cytotoxic agents to deplete GSH correlated with its ability to interact synergistically with BSO resulting in increased cytolysis. Five of the compounds tested, including the ansa macrolide maytansine, did not significantly affect cellular GSH content and did not cause increased lysis in the presence of BSO. In contrast, the ability of BCNU to deplete GSH, observed by us and others (Babson et al. 1981; McConnell et al. 1979), did not translate into increased lysis in the presence of BSO. One explanation might be that depletion of GSH by BCNU is of no relevance to either its mechanism of injury of the biochemistry of cellular response and repair.

Cytotoxicity of doxorubicin and mitomycin C in the presence of oxygen, such as in these experiments, should be distinguished from their activity against hypoxic cells (Teicher et al. 1981). Under hypoxic conditions, these quinone-containing drugs are believed to undergo reductive metabolic activation to highly reactive quinone methide alkylating agents (Moore 1977; Kennedy et al. 1980b, 1982). It is possible that the quinone methide metabolites of doxorubicin and mitomycin C are detoxified by GSH in a manner analogous to the SLs and jatrophone. Thus, BSO might augment the cytolytic activity of these agents against hypoxic tumor cells while having no effect on their toxicity to oxygenated cells.

As a determinant of cytolytic susceptibility, the importance of functional γ-glutamylcysteine synthetase during and after exposure to SLs surely reflects a critical requirement for GSH synthesis at those times. In contrast, GSH content <u>prior</u> to incubation of cells with vernolepin or jatrophone affords no protection. One possible

interpretation is that the amount of vernolepin consumed by reaction with intracellular GSH at the onset of drug exposure is not significant relative to the amount of vernolepin added to achieve lysis.

One can envisage a number of explanations for the importance of GSH in SL-mediated cytolysis. SLs did not deplete whole-cell GSH to an extent greater than that obtained by prolonged incubation with BSO, which was nontoxic (Section I of Results). However, it is possible that the GSH of particular organelles, such as mitochondria or the nucleus, was depleted more by SLs than by BSO, with toxic consequences. Alternatively, GSH might serve to detoxify SLs, either prior to the alkylation of target molecules, or by preventing cross-linking through reaction with a second sulfhydryl-reactive site, in analogy with the biscysteine adducts of helenalin, vernolepin, and elephantopin (Kupchan et al. 1970; Picman et al. 1979). A role for GSH in the restoration of critical sulfhydryl groups subsequent to alkylation should also be considered. In the next section, data implicating a role for oxidant injury as the basis of vernolepin-mediated toxicity will be presented. Thus, a critical role for GSH and its redox cycle in antioxidant defense is suggested. Finally, the cardinal issue, whether inhibition of tumor cell GSH synthesis will enhance the therapeutic efficacy of SLs and similar sulfhydryl-reactive agents in tumor-bearing animals, is under study. Preliminary findings will be presented in the next section.

III. Cystine-Dependence of Vernolepin-mediated Cytolysis: A Role for Oxidative Injury

A. Introduction

In the previous section, inhibition of GSH synthesis by BSO either during a coincubation with SLs or immediately after a pulse exposure to venolepin markedly augmented cytolysis. As a way of confirming these findings without the use of drugs, we sought to reproduce the synergistic effect of BSO by withholding cystine. Reed and co-workers have reported that two murine lymphoma cell lines (L1210 and L5178Y) require exogenous cystine as a source of cysteine for GSH synthesis, whereas isolated rat hepatocytes are able to utilize the sulfur of methionine for cysteine biosynthesis via the cystathionine trans-sulfuration pathway (Beatty and Reed 1980; Brodie et al. 1981, 1982). In this section, the dependence of P815 cells on exogenous cystine for GSH synthesis is first examined. Then, cytolysis of P815 cells resulting from a 1 h pulse with vernolepin followed by an 18-20 h incubation in medium with or without cystine is evaluated. Contrary to expectation, vernolepin-mediated cytolysis demonstrated a requirement for exogenous cystine. Our experimental approach to this phenomenon is detailed in the remainder of this section.

B. Results

GSH Synthesis in P815 Cells is Dependent upon Exogenous Cystine. To evaluate the dependence of GSH synthesis in P815 cells on the

availability of cystine, medium specifically lacking in L-cystine was prepared (Eagle's MEM minus cystine). P815 cells were incubated with 25 μg/ml of vernolepin for 1 h in cystine-free medium, resulting in >95% depletion of GSH, washed, and then incubated with and without cystine. The time course of GSH resynthesis in the presence of cystine (0.09 mM) is illustrated in Figure 21. GSH content was restored to 90% of control within 5 h. No significant GSH synthesis was observed in cystine-free medium or in medium containing cystine and BSO. The dose-response of cystine-mediated GSH resynthesis is presented in Figure 22. A log-linear relationship is evident for cystine between 9 and 270 μM. Cystine at 2.7 μM was insufficient to provide for detectable GSH synthesis by 3 h.

Effect of Cystine on Vernolepin-mediated Lysis of P815 Cells. With the knowledge that P815 cells require exogenous cystine for GSH synthesis, we expected that vernolepin-mediated lysis of cells in cystine-free medium would mimic that observed in regular medium in the presence of BSO. To our surprise, cytolysis, measured 18-20 h after a 1-h vernolepin pulse, was strongly dependent upon the presence of cystine. In one such experiment, illustrated in Figure 23, P815 cells were pulsed for 1 h with 0, 50, or 250 μg/ml of vernolepin in cystine-free medium, washed, and then incubated for 18 h with various concentrations of cystine, with or without BSO, at which time lysis was measured. There was no significant lysis in the absence of cystine. For cells pulsed with 250 μg/ml of vernolepin, significant lysis was observed with as little as 9 μM cystine with BSO present. Without BSO, equivalent lysis of these cells occurred with 90 μM cystine.

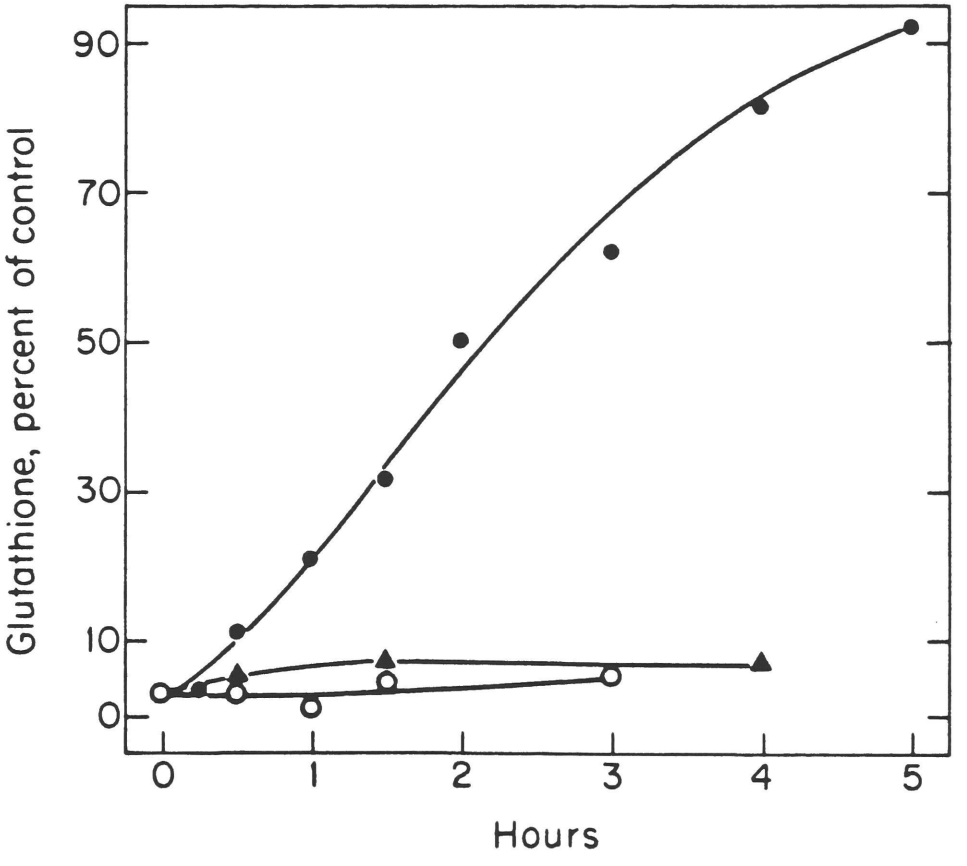

Figure 21. Effect of cystine on resynthesis of GSH after depletion by vernolepin: time course. P815 cells were incubated for 1 h in cystine-free MEM, 5% horse serum with 25 μg/ml of vernolepin, washed, and then incubated in the presence (•,▲) or absence (o) of L-cystine (0.09 mM), in the presence (▲) or absence (•,o) of BSO (0.2 mM). At the indicated times GSH content was determined. Data from two experiments are presented. Untreated cells contained 16.1 ± 1.8 nmol/mg of protein.

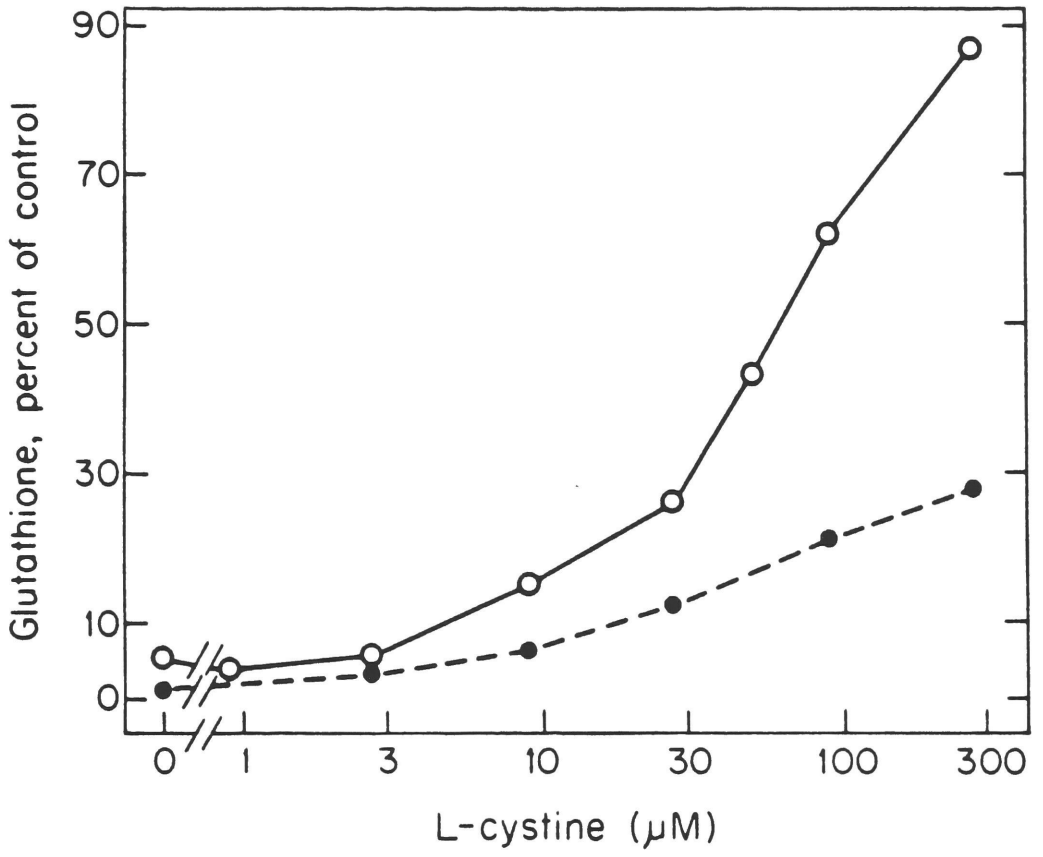

Figure 22. Effect of cystine on resynthesis of GSH after depletion by vernolepin: dose-response. P815 cells were incubated for 1 h in cystine-free MEM, 5% horse serum with 25 μg/ml of vernolepin, washed, and then incubated with the indicated concentrations of L-cystine for 1 h (•) or 3 h (o), at which time GSH content was determined. Data from two experiments are presented. Untreated cells contained 16.1 ± 1.8 nmol GSH/mg of protein.

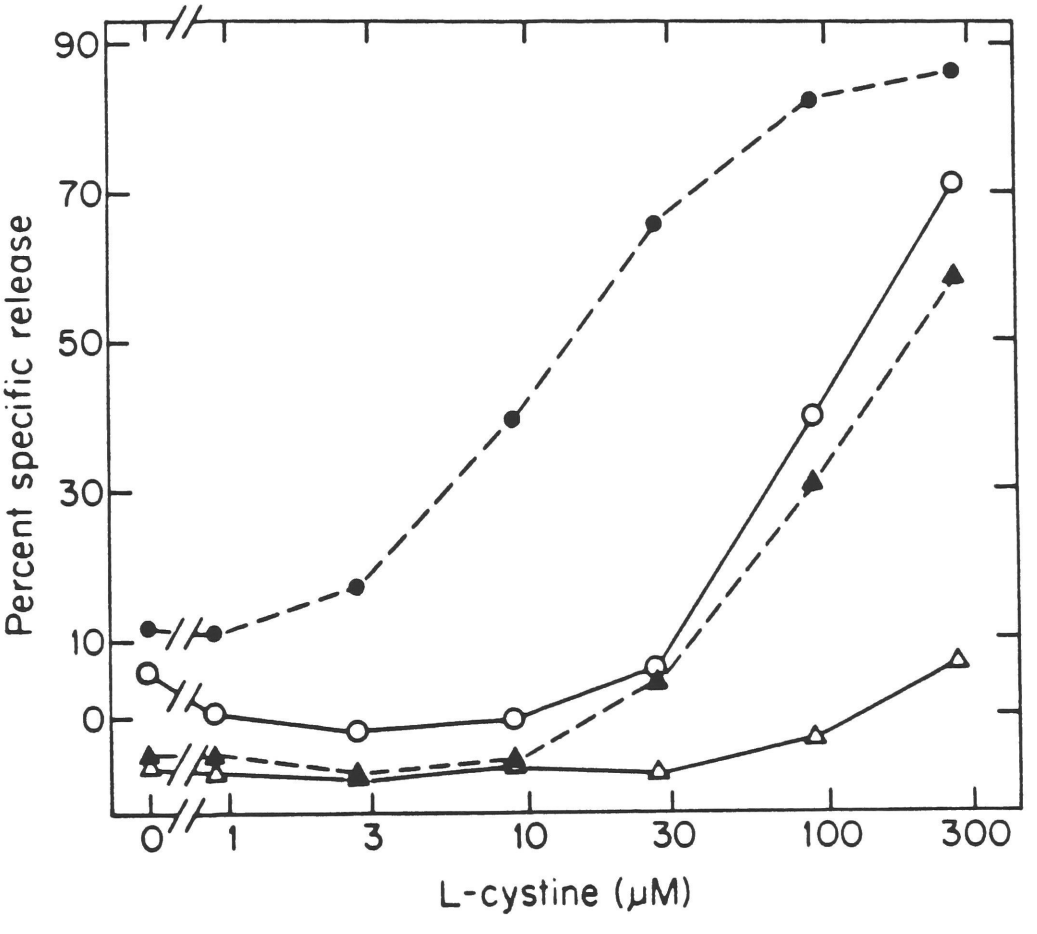

Figure 23. Effect of cystine on vernolepin-mediated lysis of P815 cells. P815 cells were pulsed for 1 h in cystine-free MEM, 5% horse serum with 50 μg/ml (Δ,▲) or 250 μg/ml (o,•) of vernolepin, washed, and then incubated for 18 h with the indicated concentrations of cystine, with (•,▲) or without (o,Δ) BSO (0.2 mM), at which time release of label was determined. Points are means of triplicates. S.E. averaged 1%. Spontaneous release after 18 h with the indicated concentrations of cystine from cells not exposed to venolepin was 18-20%.

Incubation of P815 cells in leucine- or tryptophan-free medium after the vernolepin pulse did not abolish cytolysis (Table VIII). Similarly, the addition of 25 μg/ml of cycloheximide to the post-vernolepin incubation in cystine-containing medium did not inhibit lysis. Thus, this cytolytic requirement for cystine is not shared by other essential amino acids, nor does it reflect a need for protein synthesis.

Role of Oxidative Injury in Lysis of P815 Cells by Vernolepin. A number of investigators have described cysteine-associated toxicity, both in vivo and in vitro. The addition of cysteine to mammalian cell cultures was cytotoxic (Nishiuch et al. 1976) in a manner inhibitable by catalase (Higuchi 1963). Carlsson et al. (1979) have found the bactericidal effect of cysteine under aerobic conditions to be enhanced by superoxide dismutase and abolished by catalase or metal ion chelating agents. They further demonstrated metal-catalyzed production of H_2O_2 by cysteine, as have Cavallini et al. (1968). The generation of superoxide anion as a consequence of thiol autoxidation has been reported by Misra (1974).

The remainder of this section will be organized about the hypothesis that intracellular cysteine, arising in our case from the uptake and reduction of exogenous cystine, generates H_2O_2 intracellularly as it autoxidizes. Such an oxidant stress may be easily managed by a GSH-containing cell, though fatal to a severely GSH-depleted cell.

Recall from Section I of Results that depletion of GSH by prolonged incubation with BSO or exposure to CDNB markedly enhanced susceptibility of P815 cells to oxidant injury. It was therefore of

Table VIII

Vernolepin-mediated Lysis of P815 cells: Role of Exogenous * Cystine, Leucine, or Tryptophan, and Effect of Cycloheximide

Incubation conditions	BSO	Percent specific release at 20 h: vernolepin (1-h pulse) 50 µg/ml	150 µg/ml
Complete medium‖	–	3	22
	+	17	71
– cystine	–	-4	0
	+	-2	4
– leucine	–	1	31
	+	32	76
– tryptophan	–	1	24
	+	24	78
+ cycloheximide#	–	0	28
	+	20	74

*P815 cells were labeled with ^{51}Cr for 1 h with vernolepin in MEM lacking cystine, leucine, and tryptophan (plus 5% horse serum), washed, and then incubated for 20 h in the indicated media, with or without BSO (0.2 mM). Under any of the 20-h incubation conditions, there was no lysis of cells which had not been exposed to vernolepin. Spontaneous ^{51}Cr release in complete medium was 24%. Data are means of duplicates (S.E. averaged 1.9%).

‖Complete medium is Eagle's MEM, 5% horse serum, with cystine, leucine, and tryptophan present at 0.09, 0.4, and 0.05 mM, respectively.

#25 µg/ml

interest to evaluate the effect of vernolepin on lysis of P815 cells by a flux of H_2O_2 generated by the addition of glucose oxidase to the glucose-containing medium. In one such experiment (Fig. 24A), H_2O_2-mediated lysis at 5 h was markedly enhanced in the presence of 10 μg/ml of vernolepin. Figure 24B illustrates the dose-response of vernolepin-induced sensitization to glucose oxidase-mediated cytolysis. A >6.5-fold increase in susceptibility to lysis by H_2O_2 was achieved with 20 μg/ml of vernolepin (Fig. 24B). Addition of BSO together with the indicated concentrations of vernolepin served to enhance even further the oxidative lysis of these cells (Fig. 24B), presumably due to more extensive GSH depletion (see Fig. 15). Inclusion of BSO alone was without effect (Fig. 24A). Cytolysis of vernolepin-treated cells by the addition of cysteine was also observed and was inhibited by catalase. (not shown).

Near-complete GSH depletion does not automatically result in cytolysis in cystine-containing media. P815 cells in α-MEM will remain viable and continue to divide in the presence of BSO for at least 3 weeks. Yet, after 24 h of incubation with BSO, GSH content is undetectable by our assay method (less than 3-5% of control). Vernolepin exposure which results in GSH depletion on a whole-cell basis equivalent to prolonged incubation with BSO leads to cytolysis in the presence of cystine. One possible explanation for this difference is that gradual GSH depletion, such as with BSO, allows for cellular adaptation so that intracellular cysteine autoxidation does not occur. If this were the case, one might expect that after prolonged BSO incubation, cells would be refractory to cytolysis by a pulse exposure

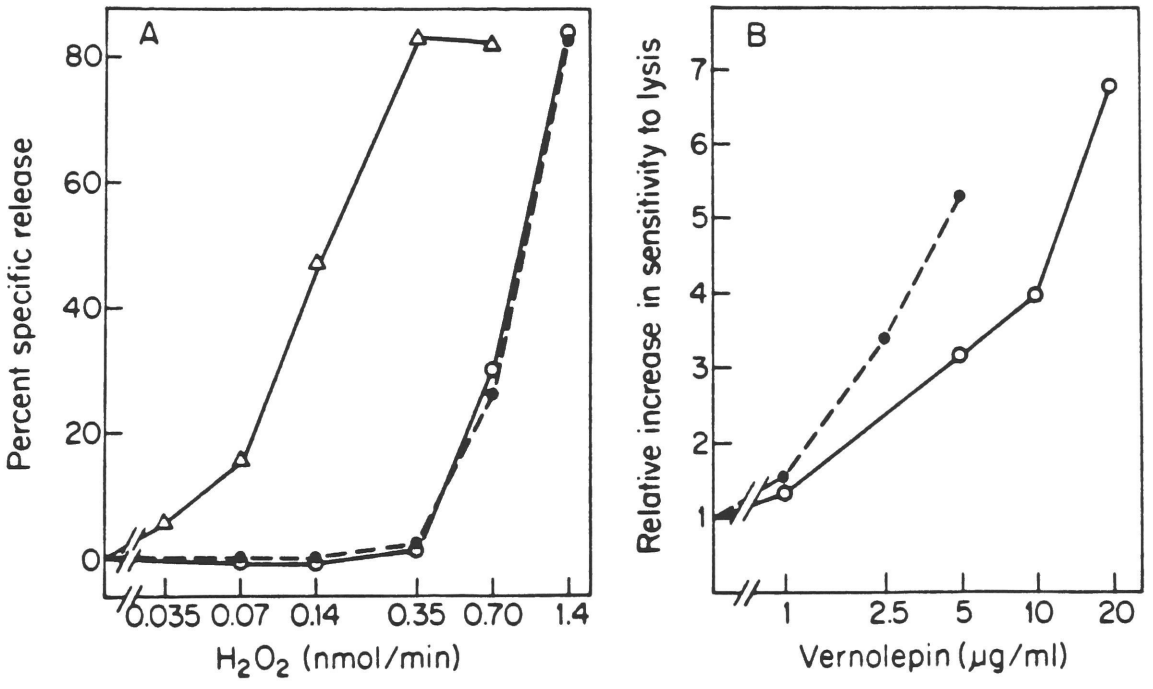

Figure 24. Effect of vernolepin on sensitivity of P815 cells to lysis by a flux of H_2O_2. ^{51}Cr release was measured after a 5-h incubation with dilutions with glucose oxidase. (A) Present during the cytolysis assay was 10 μg/ml of vernolepin (Δ), 0.2 mM BSO (•), or neither (o). Points are means of triplicates. S.E. averaged 1.4%. Spontaneous release of ^{51}Cr was <10%. (B) Vernolepin at the indicated concentrations was included in the 5-h incubation with glucose oxidase in the presence (•) or absence (o) of BSO. LD_{50} were calculated by interpolation from curves like those in panel A, and the relative increase in sensitivity to lysis (LD_{50}(control)/LD_{50}(treated)) was determined. LD_{50}(control) = 0.79 nmol of H_2O_2/min. Spontaneous release averaged 13.1 ± 0.8% (N=8). Addition of vernolepin at concentrations greater that those indicated resulted in significantly elevated spontaneous releases (i.e. lysis in the absence of glucose oxidase).

to vernolepin. As illustrated in Table IX, the opposite proved to be the case. Incubation of P815 cells with BSO for 72 h prior to vernolepin exposure resulted in substantially greater cytolysis compared to cells not incubated with BSO. Recall from Section I of Results (Fig. 8) that pre-depletion of GSH by incubation with BSO abolished the ability of CDNB to further sensitize the cells to oxidative cytolysis. Thus, either vernolepin gains access to a BSO-resistant pool of GSH, quantitatively small though crucial for cell survival, or cellular GSH is not its only relevant target.

Inhibition of GR or Catalase Enhances Lysis of P815 Cells by Vernolepin. Inhibition of tumor cell antioxidant defenses, in particular the GSH redox cycle, markedly augments oxidative cytolysis (Section I of Results). The hypothesis that vernolepin-mediated cytolysis is due to the intracellular generation of H_2O_2 from the autoxidation of cysteine suggests that interference with tumor cell antioxidant pathways, beyond the GSH depletion brought about by vernolepin, might increase cytolysis even further.

Table X presents data from 2 experiments in which P815 cells were pulsed with vernolepin, with or without aminotriazole, for 1 h in cystine-free medium. Treatment with BCNU was for 15 min, either prior to or during the end of the vernolepin pulse. Cells were then washed, and incubated for 20 h with or without cystine, aminotriazole, or BSO, at which time lysis was determined. Cells exposed to BCNU alone (10 µg/ml) were not lysed. The combination of BCNU and vernolepin was especially potent, resulting in near-complete cytolysis at doses of vernolepin which were minimally toxic without BCNU. This synergistic

Table IX

Effect of Prior Incubation with BSO on Lysis of P815 Cells by Vernolepin With and Without Cystine

Treatment*		Percent specific release at 20 h‖		
72 h prior incubation with BSO	1-h pulse with vernolepin	BSO#	− cystine	+ cystine$^{\beta}$
−	0	−	[22]	−5
		+	−5	−4
−	50	−	16	3
		+	22	53
+	0	−	[20]	−2
		+	−1	−2
+	50	−	32	87
		+	45	87

*P815 cells were incubated with or without BSO (0.2 mM) for 72 h prior to vernolepin exposure, as indicated. Cells were labeled with ^{51}Cr during the 1-h pulse with vernolepin.

‖Release of ^{51}Cr from labeled cells was determined after a 20-h incubation under various conditions, as indicated. Spontaneous release values for the two cell populations are shown in the brackets. S.E. averaged 1.7% for duplicates.

#0.2 mM

$^{\beta}$0.09 mM

Table X

Effect of BCNU and AT on Lysis of P815 Cells by Vernolepin*

1-h pulse			Percent specific release at 20 h					
			without cystine		with cystine			
					-AT		+AT	
vernolepin (μg/ml)	AT# (mM)	BCNU (μg/ml)	-BSO	+BSO	-BSO	+BSO	-BSO	+BSO
Experiment 1								
100	0	0	-4	-3	-1	62	-	-
0	0	10	7	5	2	2	-	-
100	0	10	-2	3	92	90	-	-
Experiment 2								
50	0	0	-10	-14	-11	6	-7	40
0	50	0	6	1	-1	-1	5	4
50	50	0	-13	-10	-11	6	-4	39
50	0	10	0	-2	9	85	22	94

*P815 cells were labeled with ^{51}Cr for 1 h in cystine-free medium with or without vernolepin and AT, as indicated. Treatment with BCNU was for 15 min, either prior to (experiment 1) or during the last 15 min of (experiment 2) the 1-h vernolepin pulse. Cells were then washed and incubated for 20 h with or without cystine (0.09 mM), AT (50 mM), or BSO (0.2 mM), as indicated, at which time cytolysis was determined. In experiment 1, S.E. averaged 1.2% for duplicates and spontaneous release was 26%. In experiment 2, S.E. averaged 1.5% for duplicates and spontaneous release was 22%.

#AT, aminotriazole

cytolysis was dependent upon the presence of cystine. Cytolysis due to BCNU, observed at substantially higher concentrations (≥100 μg/ml), was not dependent upon the addition of cystine (not shown). Aminotriazole alone was not toxic, even when present during both the vernolepin pulse and the 20 h post-vernolepin incubation. Modest enhancement of lysis by vernolepin was evident if aminotriazole was present during the 20 h incubation with cystine. The combination of aminotriazole and BCNU, as indicated in the last line of Table X, augmented vernolepin-mediated cystine-dependent cytolysis to a greater extent than either agent alone.

Synergy Between BCNU and Vernolepin Plus BSO in the Treatment of Mice Inoculated with P815 Mastocytoma. Much remains to be learned concerning the mechanism of action of vernolepin and the role therein of oxidative injury. Application of our findings to the treatment of tumor-bearing animals, especially as regards synergy between vernolepin and inhibitors of antioxidant metabolism, is currently under study. I will present preliminary results of one such experiment.

In vivo, P815 mastocytoma is a highly metastatic tumor. Dividing approximately every 20 h, an 8.5-day prolongation of survival as a result of treatment is equivalent to a 3-log kill of the tumor inoculum. (C. Nathan, unpublished observations). In the experiment presented in Table XI, mice were inocluated i.p. with 1×10^5 P815 cells ($>10^4$ the lethal inoculum), followed by injection of the indicated drugs. Mice treated with vehicle alone died 16-20 days later (average survival = 18.2 ± 1.5 days). BCNU alone had no effect on 3 mice, prolonged the lifetime of 1 mouse by 7 days, and produced 1

Table XI

Effect of Vernolepin and BSO, With or Without BCNU, on Survival of Mice Inoculated with P815 Mastocytoma.

Treatment*				
vernolepin (mg/kg)	BSO	BCNU	Days to death	Number surviving‖ / number injected
0	-	-	16, 17, 19, 19, 20	0/5
0	-	+	17, 19, 19, 25	1/5
5	+	-	19, 20, 28, 30, 61	0/5
10	+	-	2, 18, 30, 41, 45	0/5
2	+	+	28, 41, 70	2/5
5	+	+	25, 65	3/5

*Five mice per group were inoculated i.p. with 1 x 10^5 P815 cells in 0.3 ml α-MEM with antibiotics. Within 15 min, vernolepin and BSO (1 mmol/kg), or vehicle alone, were administered i.p. as a single injection in 1 ml 0.9% saline, 0.36% DMSO. BCNU (30 mg/kg) was then (within 15 min) administered i.p. in 0.5 ml saline, 2% ethanol, as indicated.

‖ >90 days

long-term survivor (>90 days). This dose of BCNU has been shown to inhibit GR of P815 ascites cells >80% when injected i.p. into mice with advanced ascites (Nathan and Cohn 1981). Without BCNU, vernolepin (5 or 10 mg/kg) plus BSO did not result in any long-term survivors, though prolongation of survival was evident for a few mice. The combination of BCNU with vernolepin plus BSO demonstrated dramatic antitumor activity, even with as little as 2 mg/kg of vernolepin. Additional experiments are required to establish this point. Furthermore, the role of BSO in this setting, and the effectiveness of this therapeutic approach for the treatment of established tumors, must be addressed.

C. Discussion

P815 mastocytoma cells in tissue culture are dependent upon exogenous cystine for GSH synthesis. Incubation of P815 cells in cystine-free medium for up to 22 h was not toxic, as judged by trypan blue exclusion or release of ^{51}Cr label. The effect of cystine-free medium on cell division or long-term viability was not evaluated. Presumably, these cells are also dependent upon cystine for protein synthesis. Three additional murine tumor cell lines in tissue culture also required extracellular cystine for GSH resynthesis after depletion by vernolepin (TLX9, P388, and WEHI-164) (not shown). Studying a series of human lymphoid cell lines from leukemic and normal individuals, Inglehart et al. (1977) have noted a correlation between cystine auxotrophy and leukemic origin; lymphoid cell lines from normals did not require cystine and contained detectable cystathionase activity, unlike cell lines of leukemic origin. Exploitation of this difference

by cyst(e)ine depletion therapy has been suggested (Uren et al. 1978; Uren and Lazarus 1979; Glode et al. 1979).

It was expected that P815 cells incubated in cystine-free medium would exhibit a level of susceptibility to lysis by a pulse of vernolepin equivalent to that observed in the presence of BSO in medium containing cystine. In contrast, and to our great surprise, in the absence of cystine cells appeared resistant to vernolepin-mediated lysis. In these experiments, as observed previously, lysis in the presence of cystine was markedly enhanced by the addition of BSO after the vernolepin pulse. It should be noted that the vernolepin pulse itself was in cystine-free medium, and thus cystine need not be present during vernolepin exposure for cytolysis to occur. In fact, addition of cystine 8.5 h after the vernolepin pulse resulted in significant cytolysis measured 11.5 h later (not shown).

Vernolepin-mediated cytolysis was not dependent upon two other essential amino acids (leucine or tryptophan) and was not inhibited by the addition of cycloheximide. Thus, a role for protein synthesis in the manifestation of toxicity was not evident. It is possible that the action of some cyst(e)ine-dependent enzyme is required for cell death resulting from vernolepin exposure.

The hypothesis that cystine-dependent toxicity of vernolepin-treated cells is the result of intracellular generation of H_2O_2 as a result of cysteine autoxidation arose from two observations. First, cysteine-associated toxicity to cells in culture, inhibitable by catalase, has been reported. Second, as demonstrated in Section I of Results, GSH-depleted P815 cells are very susceptible to lysis by H_2O_2

Thus, vernolepin-mediated GSH depletion might sensitize cells to the oxidant stress associated with intracellular cysteine oxidation. As noted, however, depletion of GSH may not be the only relevant action of vernolepin in this setting (Table IX).

Two experimental approaches are suggested by this hypothesis. First, cytolysis should be dependent upon oxygen. We are just beginning to learn how to perform the necessary manipulations under conditions of anoxia. Future experiments will address this question. The second approach, reminiscent of the work presented in Section I of Results, involves inhibition of tumor cell antioxidant defenses, beyond the GSH depletion brought about by vernolepin, as a way of further augmenting cytolysis. Inhibition of GR with BCNU was found to dramatically enhance vernolepin-mediated cystine-dependent cytolysis; inhibition of catalase with aminotriazole demonstrated less synergy (Table X). While these results are not direct evidence for an oxidative basis of vernolepin toxicity, they are supportive of the hypothesis.

Intracellular production of H_2O_2 via cysteine autoxidation may not be restricted to vernolepin-treated cells. Thus the susceptibility of such GSH-deficient cells to oxidative injury may have allowed for the dramatic manifestation of an aspect of cyst(e)ne metabolism occuring in untreated cells as well. The potential significance of cysteine as a source of intracellular oxidant stress should be considered. Avoidance of such toxicity may be a factor in the maintenance of remarkably low levels of intracellular cysteine. An analysis of the intra- and extracellular concentrations of free amino acids in HeLa cells grown in vitro has been provided by Piez and Eagle (1958). Intracellular cystine

was below the level of detection, specifically <0.05 mM, (the concentration of cystine in the medium was 0.023 mM). Other essential amino acids were present at higher levels. For instance, intracellular concentrations of threonine, valine, and histidine were 0.96, 0.79, and 0.26 mM, respectively, representing a 5- to 8-fold increase relative to extracellular concentrations. Intracellular GSH was present at a concentration of 4.42 mM. Our hypothesis concerning intracellular cysteine autoxidation, and the role of antioxidant pathways, is illustrated in Figure 25.

The rapid mobilization of the cysteine within the GSH molecule for protein synthesis in rat liver suggests that GSH can function as a storage form of cysteine (Higashi et al. 1977), thus allowing a cell quick access to this amino acid without the need to maintain a potentially toxic intracellular pool of the free amino acid. Cellular GSH can also serve to detoxify the H_2O_2 that may be generated by the cysteine that is present. Thus, as regards the potential for oxygen-dependent toxicity of intracellular cysteine, GSH metabolism may be of critical importance.

Other thiols readily autoxidize with the concomitant formation of H_2O_2. For instance, Trotta et al. (1974) have reported that inhibition of the glutaminase activity of glutamine-dependent carbamyl phosphate synthetase by dithiothreitol is due to formation of H_2O_2. Another thiol of considerable biological interest, diethyldithiocarbamate (DDC), has been found to induce the production of H_2O_2 when incubated with human erythrocytes (Sinet et al. 1982), perhaps as a result of oxidation to the disulfide disulfiram. Disulfiram is taken up by erythrocytes and

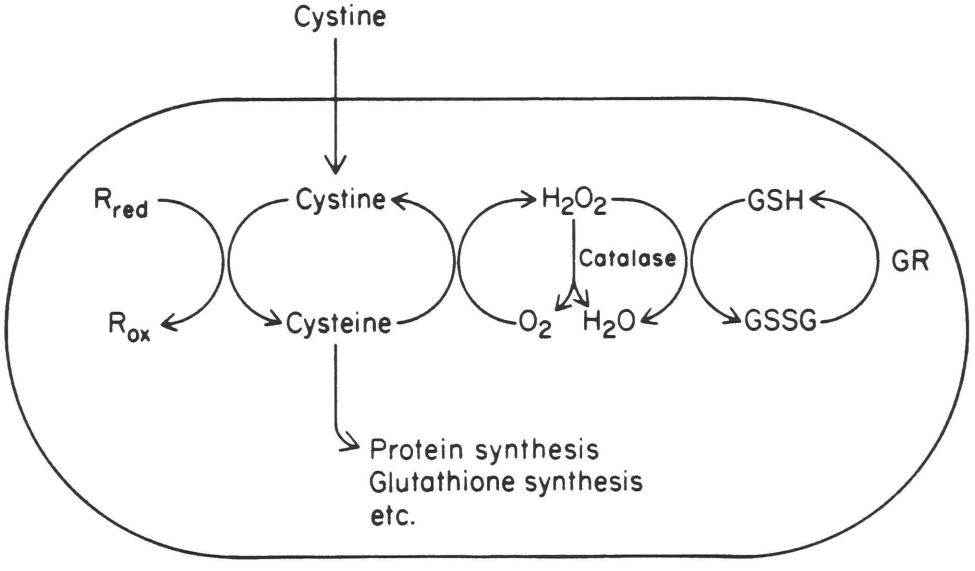

Figure 25. Cysteine autoxidation as a source of intracellular oxidant stress: a hypothetical scheme.

reduced to DDC (Stromme 1963). Thus, a cyclic oxidation-reduction of disulfiram, as proposed above for cysteine, may be a potent source of intracellular H_2O_2. The relevance of such a mechanism to the inhibitory activity of disulfiram toward the malaria parasite P. falciparum (Scheibel et al. 1979) is suggested by the existence of other antimalarial drugs which generate an intraerythrocytic oxidant stress, notably primaquine (Pinder 1973).

SUMMARY AND DISCUSSION

In response to oxidative injury, murine tumors depend upon the GSH oxidation-reduction cycle. The susceptibility of tumor cells to lysis by a flux of H_2O_2, such as generated by granulocytes or activated macrophages in the presence of the secretagogue PMA, or by the enzyme glucose oxidase, was significantly augmented by interference with the GSH redox cycle. Various approaches to the inhibition of antioxidant metabolism of tumor cells were tested (Section I of Results). First, GSH biosynthesis was inhibited by incubation of cells with BSO, a selective inhibitor of γ-glutamylcysteine synthetase. In the presence of BSO, cells remained viable and continued to divide, while GSH content gradually declined, such that 90% depletion was achieved by 15 h. As an alternative method for GSH depletion, cells were treated with CDNB, a reactive electrophile and substrate for endogenous GSH S-transferase. Sensitization to oxidative cytolysis correlated with depletion of GSH by either method by the critera of both dose-response and the time course of onset and recovery. A third approach involved inhibition of GR, thereby interrupting the redox cycle. The anti-neoplastic agent BCNU, an inhibitor of GR at nontoxic doses, was used in this regard. Here too, interference in tumor cell GSH metabolism enhanced cytolysis by H_2O_2. Similar sensitization to oxidative cytolysis as a result of selenium-deprivation-induced deficiency in tumor cell GPO has been reported by Nathan et al. (1981). In contrast, inhibition of tumor cell catalase with aminotriazole had no effect on

susceptibility to lysis by a flux of H_2O_2.

In Section II of Results, this theme was extended into another arena of GSH metabolism, the detoxification of reactive electrophiles by the formation of thioether conjugates with GSH. The interaction of tumor cell GSH with four sesquiterpene lactones (SLs), a class of sulfhydryl-reactive natural products with in vivo antitumor activity in animal models, was evaluated. SLs are potent depletors of cellular GSH. Prompt resynthesis of GSH is a critically important component of tumor cell recovery following exposure to SLs. SL-mediated cytolysis was dramatically augmented by inhibition of GSH synthesis with BSO. Potent synergy between BSO and the SL vernolepin was evident even if the two agents were not present together; inhibition of GSH synthesis by BSO during or immediately after a 1-h exposure to vernolepin resulted in equivalent cytolysis. For six additional, unrelated antineoplastics, sulfhydryl reactivity, in terms of their ability to deplete GSH, correlated with synergistic lysis in the presence of BSO. Cytolysis by BCNU, however, was not enhanced by BSO, in spite of significant GSH depletion.

Vernolepin-mediated cytolysis of P815 cells was dependent upon exogenous cystine, as was GSH synthesis. This dependence upon cystine for cytolysis was not due to a requirement for protein synthesis or GSH synthesis. In view of the tendency of cysteine to autoxidize and thereby generate H_2O_2, it seemed reasonable to suspect that in the absence of GSH, intracellular cysteine, derived from extracellular cystine, is a source of H_2O_2 sufficient to cause cytolysis. Thus, vernolepin-mediated cytotoxicity may have a basis in oxidative injury.

In accord with this hypothesis, inhibition of GR by BCNU significantly enhanced the toxicity of vernolepin. Inhibition of catalase by amino-triazole resulted in less dramatic augmentation of vernolepin-mediated lysis. Future experiments will evaluate the oxygen dependence of vernolepin toxicity.

Application of these findings to the treatment of tumor-bearing animals is underway. It is felt that the crucial issues of host toxicity and therapeutic efficacy are best addressed in the in vivo setting.

An understanding of the biochemical pathways which function within the target cell to escape or repair injury can guide the development of effective anti-microbial therapy as well. Recent findings with the African trypanosome T. b. brucei illustrate this point. Meshnick et al. (1977) have reported an unusually high intracellular H_2O_2 concentration in bloodstream forms of this organism, presumably due to a lack of catalase. This aspect of trypanosome biochemistry was exploited by the development of a class of anti-trypanosomal compounds which promote cleavage of H_2O_2 into highly reactive radical species (Meshnick et al. 1977, 1978a). Reasoning that trypanosomes would be more susceptible to this oxidative damage if their antioxidant defenses were compromised, they have demonstrated that arsenicals decreased trypanosome GSH content, increased susceptibility to heme lysis in vitro, and interacted synergistically with hematoporphyrin D in vivo (Meshnick et al. 1978b). Indeed, even without exposure to H_2O_2-cleaving agents, these organisms appear to be highly dependent upon their GSH for protection against the toxicity of self-generated H_2O_2. Inhibition of

GSH synthesis by administration of BSO to infected mice for a length of time sufficient to result in >95% depletion of trypanosome GSH (based on the observed half-life of 5 h) resulted in significantly prolonged survival (2 out of 6 mice were considered cured) (Arrick et al. 1981).

In summary, the importance of tumor cell GSH metabolism as a determinant of susceptibility to lysis by H_2O_2 or sulfhydryl-reactive antineoplastic agents has been demonstrated. In addition, these findings serve to illustrate a potentially useful concept for the treatment of neoplastic diseases: Elucidation of the critical defense mechanisms employed by tumor cells in response to therapy could be of use in the design of synergistic therapeutic combinations, and in the analysis of drug resistance.

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Table IV

Effect of Cytotoxic SLs on GSH Content of P815 Cells

SL	µg/ml	% GSH remaining*	(N)
Vernolepin	10	6.7 ± 2.7	(5)
	25	2.8 ± 0.5	(2)
Helenalin	5	41 ± 6.8	(2)
	10	23 ± 1.2	(3)
	25	5.7 ± 1.6	(3)
Elephantopin	5	39 ± 2.9	(4)
	15	12 ± 0.2	(2)
Eriofertopin	50	29 ± 3.6	(4)

*P815 cells were incubated in α-MEM, 5% horse serum with the indicated concentrations of sesquiterpene lactones for 1 h. Data are from 10 experiments with initial GSH = 29.6 nmol/mg of protein.

End