MECHANISMS OF TUMOR CELL DRUG RESISTANCE: THE ROLE OF GLUTATHIONE-S-TRANSFERASE IN MAMMARY ADENOCARCINOMA CELLS

by

Robyn L. Schecter

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Department of Medicine, Division of Experimental Medicine
McGill University
Montreal, Quebec

ABSTRACT

The development of resistance of malignant tumors to the chemotherapeutic agents used in the treatment of neoplastic disease is a major factor responsible for treatment failure. Rat mammary adenocarcinoma cells (MatB) which model the human disease in their pattern of growth were studied to elucidate mechanisms of drug resistance. Cell lines that have acquired drug resistance *in vitro* as a result of continuous exposure to increasing concentrations of drug have been utilized to this effect. Two separate cell lines were selected for resistance to either a "natural product" (Adr^R) or an alkylating (Mln^R) antineoplastic drug. Each line displayed phenotypic changes that were stereotypic for the selecting agent. Adriamycin selected cells maintained the multidrug resistant phenotype *in vitro* and *in vivo*. In cells selected for primary resistance to an alkylating agent (melphalan), overexpression of the phase II conjugating enzyme glutathione-S-transferase (GST) was a dominant feature. The GSTs play a central role in the protection of cells from cytotoxic and carcinogenic compounds either by catalyzing the conjugation of glutathione (GSH) with reactive electrophiles or by reducing reactive organic peroxides. The former reaction prevents electrophiles from reacting with macromolecules that possess critical cellular functions and the latter reaction protects against oxidative stress. Using DNA transfer techniques, it was possible to demonstrate that resistance to alkylating agents in particular could be conferred to drug sensitive MatB tumor cells following the introduction of a detoxifying GST gene into these cells.

RÉSUMÉ

Le développement de la résistance des tumeurs aux médicaments anti-cancéreux est l'une des causes majeures des échecs thérapeutiques en clinique. Afin d'étudier les mécanismes de la résistance, des cellules isolées d'adénocarcinome mammaire de rat (MatB) ont été utilisées comme modèle expérimental. En traitant ces cellules avec des concentrations croissantes d'agents anti-cancéreux, deux lignées cellulaires résistantes ont été sélectionnées: l'une à l'adriamycine (antibiotique naturel) et l'autre au melphalan (agent alkylant). Les cellules résistantes à l'adriamycine possèdent les caractéristiques classiques du phénotype de "résistance multiple". Les cellules résistantes au melphalan (Mln^R) présentent essentiellement une surexpression des glutathione-S-transférases (GST). Cette dernière classe d'enzymes joue un rôle très important dans les mécanismes de défense (cellulaires) contre les agents cytotoxiques et cancérigènes. D'une part, les glutathione-S-transférases catalysent la conjugaison du glutathione (GSH) aux réactifs électrophiles des agents cytotoxiques. D'autre part, les enzymes GST réduisent la formation des réactifs peroxydes organiques. Par conséquent, ces deux mécanismes protègent les cellules des effets cytotoxiques et génotoxiques des médicaments anti-cancéreux. En utilisant les technologies de transfert de l'ADN, nous avons démontré que lorsque des cellules sensibles sont transfectées par le gène qui code pour la GST, elles acquièrent une résistance à de nombreux agents alkylants.

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NOMENCLATURE

Adr^R	Adriamycin resistant
AuBMT	Autologous bone marrow transplantation
BCNU	1,3 - Bis (2-chloroethyl) -1-nitrosourea
BSA	Bovine serum albumin
BSO	L-Buthionine-(S,R)-sulfoximine
CDDP	Cisplatinum
cDNA	Complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
CLB	Chlorambucil
CLL	Chronic lymphocytic leukemia
DNA	Deoxyribonucleic acid
γ-GCS	γ-glutamyl cysteine synthetase
γ-GGT	γ-glutamyl transpeptidase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthetase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
GST-π	Acidic or anionic glutathione-S-transferase
GST-α	Basic or cationic glutathione-S-transferase
GST-μ	Neutral glutathione-S-transferase
HN2	Nitrogen mustard
Kb	Kilobase
Kd	Kilodalton
M Abs	Monoclonal antibodies

3-MC	3-methylcholanthrene
MDR	Multidrug-resistance
MDR-1	Multidrug resistant gene
MEM	Minimal essential medium
MLN^R	Melphalan resistant
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PB	Phenobarbitol
PBS	Phosphate-buffered saline
SDS	Sodium dodecyl sulfate
SSC	Salt saturated citrate
WT	Wild type

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PREFACE

In accordance with the Guidelines Concerning Thesis Preparation I have taken the option, according to section (7), of writing the experimental part of the thesis (Chapters II to IV inclusive) in the form of original papers already published in learned journals or submitted for publication. This provision reads as follows:

> The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.
>
> It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationery and bound as an integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary explanatory material is almost always necessary.
>
> The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims, e.g. before the Oral Committee. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review.

Thus, each chapter of this thesis bears its own Summary, Introduction, Methods, Results, Discussion and Bibliography. Also as required by the Guidelines I have a common abstract, Chapter I is a General Introduction, Chapter V summarizes the conclusions as well as claims to originality and suggestions for further research. A nomenclature is placed at the beginning of Chapter I; it serves to define the symbols used therein, and in subsequent chapters of the thesis.

The published and submitted manuscripts are as follows:

Chapter II — Schecter RL, Woo A, Duong M, Batist G.
In vivo and *in vitro* mechanisms of drug resistance in a rat mammary carcinoma model. Cancer Res 51: 1434-1442, 1991.

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In Chapter II, the candidate was responsible for all experiments and the preparation of manuscripts. A. Woo and M. Duong helped the candidate with laboratory technique regarding biochemical assays and molecular biology.

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Dedicated to my mother,
with respect for her love of learning

CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

A major limitation in the treatment of human cancer is the relatively narrow "therapeutic margin" which exists between the dosage of drug which is effective and the one that results in intolerable toxicity. This margin often narrows or even disappears with the frequent development of resistance to chemotherapy drugs.

Clinical drug resistance may be a result of pharmacokinetic factors leading to inadequate drug delivery to the tumor cells (Sobrero and Bertino, 1986). After optimization of the dose, route of administration, and treatment schedule, the drug may not reach the tumor cells in adequate concentrations due to impaired blood circulation (McVie, 1984) or localization of the target cells in "sanctuaries", e.g., the central nervous system (Freireich, 1984). Additionally, a tumor may be insensitive because of cell kinetic factors, e.g., the presence of a large fraction of growth-arrested cells (Drevinko and Barlogie, 1984). Tumor cell susceptibility to chemotherapeutic agents can be divided into three categories: i) tumors which can be cured with anticancer drugs; ii) tumors which are intrinsically resistant to therapy; and iii) tumors which are initially sensitive to therapy but, after an initial response, become resistant to the drugs, i.e., acquired resistance. In the case of acquired drug resistance, the generation of drug resistant cells is a major cause for unresponsiveness of a tumor and involves selection processes. One mechanism requires the existence of a heterogeneous cell population, within the tumor, that exhibit differential sensitivities to cytotoxic drugs. In this case, initial treatment with an anticancer drug results in the destruction of sensitive cells leaving predominantly viable drug-resistant cells. The drug resistant cells become the major cell population within the tumor. Alternatively, administration of the drug may cause phenotypic changes either in enzymes or membrane structure within the tumor (possibly also by selection) that makes the cell resistant to further drug treatment. In this case, adaptive changes account for the acquired drug resistance.

Cellular drug resistance may arise by a variety of mechanisms that can usually be classified in one of the following categories: (a) decreased transport of the drug into the

cell, (b) enhanced efflux out of the cell, (c) defective metabolism of the drug to its active species, (d) altered intracellular nucleotide pools, (e) increased drug inactivation, (f) enhanced cellular repair processes, and (g) altered target molecules (Curt et al., 1984). These alterations may be associated with distinct genetic abnormalities such as spontaneous or drug-induced mutations, or gene amplification (Henderson, 1984). It appears likely that primary and acquired drug resistance may share at least some of their mechanisms and that several mechanisms may be concomitantly operative. Because of this complexity, the development of rational strategies for preventing or overcoming resistance to an agent is seriously hampered by the difficulty in establishing which type of resistance is operative in the malignant cells of cancer patients.

Only recently has the spectrum of mechanisms by which cells in culture may become resistant begun to unfold.

2. BREAST CANCER

Drug resistance has emerged as one of the most important problems in the treatment of cancer. Although approximately 75% to 80% of patients with metastatic breast cancer will respond to combination chemotherapy, a large number will eventually develop drug resistance and die of their disease (Dalton, 1990). Acquired resistance is the pattern of resistance seen in tumors that are initially responsive to chemotherapy, such as adult breast cancer, and the correlate of the clinical pattern of initial remission followed by the untreatable relapse.

The origins of drug resistance appear to occur primarily at the cellular level. There, changes are likely to be under genetic control and spontaneous genetic mutations have been demonstrated to confer drug resistance (Goldie and Coldman, 1979). In most cases, the smaller the tumor burden, the less likely it is to contain cells that have mutated to a drug-resistant phenotype. As the tumor burden increases, the likelihood of resistant clones increases and the probability of a cure decreases.

This is best demonstrated in the treatment of breast cancer, in which the use of adjuvant chemotherapy in primary breast carcinoma is able to cure a subset of women with low tumor burden. Those patients who have a small tumor mass and minimal lymph node involvement (< 4 nodes) at the time of diagnosis appear to benefit most from the use of adjuvant chemotherapy. In contrast, patients with larger tumor masses and greater lymph node involvement benefit less from the use of adjuvant chemotherapy.

Attempts have been made to improve chemotherapeutic results by treating patients earlier in the course of their disease, by using combinations of chemotherapeutic drugs that may differ in their mechanisms of drug resistance, and by increasing the dose intensity of drugs used. Studies indicate that a better response rate may be achieved with higher doses of drug/unit time (Hryniuk and Bush, 1984; Hyrniuk, 1987).

The clinical course of carcinoma of the breast is quite variable. Patients can develop recurrences either soon after their primary therapy or many years later. The principle risk factors are clinical staging (tumor size, lymph node status), pathologic grading and the expression of hormone receptors (Harris and Henderson, 1987). However, even within a group of patients of the same stage and hormonal receptor status, the course of disease and response to therapy can be quite variable. Investigators have sought to resolve the factors involved through the study of additional markers by measuring, for example, expression of specific genes (eg. c-*erb* B) (Slamon et al., 1987).

One type of drug resistance that develops in experimental systems following repeated exposure to natural chemotherapeutic agents such as doxorubicin is multidrug resistance (MDR). The most important finding in the study of the MDR phenotype is the discovery of P-glycoprotein. Whether P-glycoprotein plays a role in conferring drug resistance in breast cancer is debatable and is currently under intense investigation. Initial studies demonstrate that primary breast tumors have a very low expression of P-glycoprotein; however, this may increase as the tumor metastasizes and becomes drug-resistant.

Another form of resistance that develops following treatment with alkylating drugs such as melphalan is that associated with the overexpression of glutathione-S-transferases.

The glutathione transferases were selected for study because of evidence from *in vitro* studies that they might be associated with the development of drug resistance and might be linked to other tumor markers such as estrogen receptor proteins (Moscow and Cowan, 1988). Primary breast cancers are quite heterogeneous with regard to their GST activity.

Estrogen receptor-poor tumors have been shown to have high levels of the GST pi subunit. No relationship was found, however, between GST expression and/or tumor stage or lymph node involvement.

Identifying the changes in the GST profile following the onset of drug resistance may provide information about drug action and ways to improve treatment.

3. THE GENETIC BASIS FOR DRUG RESISTANCE

A fundamental property of DNA is spontaneous mutation; there is also evidence that tumor cells may be more genetically "unstable" than normal cells. In 1943, Luria and Delbruck described a principle in bacterial genetics important to our understanding of the development of genetically determined resistance to cancer chemotherapy (Luria and Delbruck, 1943). They noted that E. coli developed resistance to bacteriophage, not by surviving exposure, but by expanding clones of bacteria that had spontaneously mutated to a type inherently resistant to phage infection. In 1979, Goldie and Coldman applied this principle to the development of resistance by cancer cells to anti-cancer drugs (Goldie and Coldman, 1979). They proposed that the non-random cytogenic changes now known to be associated with most human cancers was very likely tightly associated with the development of the capacity to resist the action of certain types of anti-cancer drugs (Yunis, 1989). They developed a mathematical model that predicted that tumor cells mutate to drug resistance at a rate that is intrinsic to the genetic instability of a particular tumor, and that these events would occur at population sizes between 10^3 and 10^6 tumor cells, below

clinically detectable levels. Thus, the probability that a given tumor will contain resistant clones would be a function of the mutation rate and the size of the tumor.

In the clinic, such tumors would appear to respond initially to treatment with a complete or partial remission, but then reappear as the resistance clone(s) expanded. Such a pattern is seen with the use of chemotherapy in many cancers in the clinic.

Goldie and Coldman have proposed some of the most noteable models to describe the emergence of drug resistance. Selection operates on pre-existing variants and the frequency of cross-resistance should be the product of the individual, underlying, single mutation frequencies. The increased genetic instability of tumor cells, compared with non-transformed cells, has been documented (Nowell, 1986), and suggests that the cross-resistance frequency is higher than a simple mutation model would predict (Kuczek and Chan, 1988). When malignant cell lines are made resistant to a single chemotherapeutic agent by stepwise incubation in increasing amount of drug, some lines are curiously found to be resistant to structurally unrelated cytotoxic compounds. This finding has been repeated for many different cell lines, initially exposed to many different drugs and has been termed multi-drug resistance (MDR). Investigation of MDR has not focused on specific enzymes but rather on the cells' basic defense mechanism against toxic agents found naturally in the environment.

Drug resistance may be present before exposure to any cytotoxic drugs, as in colon cancer, non small cell lung cancer and malignant melanoma. Here, environmental, including dietary factors, may have selected for several phenotypic alterations during tumor development, leading to a highly resistant tumor which responds poorly to any chemotherapy.

In vitro studies using cells selected for resistance to a single cytotoxic drug have documented the biochemical changes accompanying resistance. When the active forms of anti-cancer drugs reach their target, specific and permanent biochemical resistance to anti-cancer drugs develops. The study of drug resistance in cultured cells has revealed a

complicated set of changes at the genetic and biochemical level. Many specific mechanisms of primary drug resistance have been revealed. Resistance is stable when the underlying changes are preserved in the absence of active selection, whereas unstable resistance is characterized by the loss of resistance mediating changes under the same conditions. Stable resistance is often associated with chromosomal changes such as the generation of homogeneously staining regions or gene deletions, whereas unstably resistant cells often show extrachromosomal alterations, such as the appearance of double minute chromosomes (Shimke et al., 1981).

4. DRUG DETOXIFICATION

Over the past 30 years, several pathways of xenobiotic metabolism have been elucidated which enable cells of higher organisms to excrete unwanted foreign chemicals. One of the most general and basic forms of adaptation to xenobiotic chemicals, including the majority of drugs, is induction of enzyme patterns that relate to the metabolism and possible detoxification of the chemical. This type of response is seen in many organisms, including the simplest bacteria. In mammals, this often includes two major aspects of detoxification.

Termination of drug effect is usually by biotransformation and excretion. Important characteristics of a drug are its molecular weight, solubility, shape, site of absorption, degree of ionization and lipid solubility of ionized and non-ionized forms. Non-ionized drugs are usually lipid soluble. Many drugs are lipid soluble, weak acids or bases that are not readily eliminated from the body. Drug metabolites usually are more polar and less lipid soluble than the parent molecule, and this enhances their excretion and reduces their volume of distribution. Biotransformation not only promotes drug elimination but also often results in inactivation of the compound.

4.1 Biotransformation by Phase I and Phase II Reactions

The chemical reactions concerned in the biotransformation of drugs are classified as phase I and phase II reactions. Phase I reactions usually convert the parent drug to a more polar metabolite by oxidation, reduction or hydrolysis. Phase II reactions are conjugation reactions.

Xenobiotics are metabolized by mammalian and lower organisms by different classes of enzyme systems. These include the microsomal cytochromes P-450 containing mixed-function oxidases, flavoprotein-linked reductases, esterases catalyzing the hydrolysis of esters and amides, hydrolases catalyzing the hydrolysis of epoxides and various transferases (including the glutathione-S-transferases) catalyzing synthetic reactions which conjugate xenobiotic substrates or their metabolites with glucuronic acid, sulfate, glutathione, acetyl CoA or methyl donors (Jakoby, 1980). In many cases, the enzymes involved in xenobiotic metabolism may exist in multiple isozymic forms which may direct the flow of substrates into alternate metabolic pathways.

The cytochromes P-450 are a multi-isozymic system in which the distribution of isozymes may govern substrate processing into different products and thus into alternate metabolic pathways. This multi-isozymic system is also responsible for the metabolism of certain classes of endobiotics which include steroids, fatty acids, bile acids and prostaglandins (Sato and Kato, 1982). Cytochrome P-450 catalyzed metabolism may be either beneficial or hazardous, leading to either detoxified metabolites that may be conjugated and safely excreted or to metabolites that are toxic, mutagenic or carcinogenic (Goldstein et al., 1974). There are at least 15 to 20 forms of cytochromes P-450 (Sato et al., 1980). The diversity of enzymes having different but sometimes overlapping specificities helps the organism to deal with many different foreign chemicals.

The enzyme systems concerned in the biotransformation of many drugs are primarily located in the hepatic smooth endoplasmic reticulum. Fragments of this network are isolated by centrifugation of liver homogenates in the microsome fraction. Kidney,

gastrointestinal and lung epithelium also contain these enzymes. Microsomal enzymes catalyze glucuronide conjugations and most of the oxidation of drugs. Reduction and hydrolysis of drugs are catalyzed by both microsomal and non-microsomal enzymes (Levine, 1983).

All conjugations other than glucuronide formation and some oxidation reduction and hydrolysis of drugs are catalyzed by non-microsomal enzymes. Non-microsomal biotransformation of drugs mediated by the glutathione-S-transferases, for example, occurs primarily in the liver but also in plasma and other tissues.

5. MECHANISM OF DRUG ACTION

5.1 Adriamycin

Anthracyclines such as adriamycin alter DNA structure by intercalating into dGdC sequences of DNA and cause DNA unwinding, stiffening and elongation and formation of covalent DNA adducts. They cause extensive chromosome damage and these DNA rearrangements may be involved in doxorubicin induced cytotoxicity. Additionally, adriamycin stimulates the production of reactive oxygen, can stabilize the topoisomerase II-DNA cleavable complex and induce apoptosis (Ross, 1985; Potmesil and Ross, 1987; Zhang et al., 1990). Adriamycin toxicity is not dictated solely by uptake, in contradiction with mechanisms requiring an intracellular target; cytotoxicity is also caused by membrane damage from outside the cell by binding to a site that is structure-dependent, since cytotoxicity can be lost when membrane temperature is decreased (Tritton and Yee, 1982).

The generation of oxy-radicals has been proposed to represent one mechanism of tumor cell kill by doxorubicin (Doroshow, 1986a). NADPH-dependent flavin reductases reduce doxorubicin to a semiquinone radical, which under aerobic conditions can donate its free electron to molecular oxygen, generating a superoxide radical (Bachur et al., 1978). Superoxide dismutase catalyzes production of hydrogen peroxide from superoxide. Glutathione peroxidase is able to detoxify hydrogen peroxide to water and organic

peroxides to the corresponding alcohol. Increased activity of such detoxifying pathways may make tumor cells less susceptible to doxorubicin-mediated damage. Addition of radical scavengers and compounds with peroxidase activity to the extracellular medium can reduce the cytotoxic effect of doxorubicin in cytotoxicity assays *in vitro* (Doroshow, 1986b). The therapeutic application of doxorubicin is severely restricted by its dose-dependent cardiotoxicity (Hederson and Frei, 1980). The inability of cardiac mitochondria to effectively inactivate hydroxyl radical formation by adriamycin has been shown to be a factor involved in adriamycin-induced cardiotoxicity (Ogura et al., 1991).

ADR resistant cells have demonstrated a higher degree of structural order in the lipid phase of the plasma membrane in association with P-glycoprotein expression, and their larger intracellular lipid content may account for the decreased rate of intracellular accumulation of anthracyclic drugs.

5.2 Alkylating Agents

Alkylating drugs are cell cycle phase nonspecific in cytotoxic action but are generally more toxic toward proliferative cells and show enhanced cytotoxicity toward cells in the S phase of the cell cycle (Frei et al., 1988).

Despite the structural similarity in the alkylating moiety of the various members of the bifunctional nitrogen mustard class of drugs, different substitutions on the amine can result in marked differences in chemical reactivity and clinical utility as well as differences in rates and mechanisms of nucleophilic attack (Williamson and Witten, 1974). The lethal event following exposure of cells to these agents is thought to involve some form of DNA damage, in particular, DNA crosslinking (Kohn et al., 1966; Lawley and Baskes, 1967).

The formation of interstrand crosslinks may be the most relevant cytotoxic lesion, particularly with nitrogen mustards and nitrosoureas. Such lesions are formed by an initial covalent reaction of drug with an electrophilic site on the DNA to form a monoadduct, which can be converted to a crosslink by a second reaction with the other DNA strand. Not

all monoadducts are converted to crosslinks and the "second-arm" reaction is generally assumed to be slow compared to initial monoadduct formation (with melphalan, nitrosoureas and platinum coordination complexes).

The dichloroethyl-amino nitrogen mustards appear to alkylate through an aziridium ion intermediate (Colvin et al., 1976) which has strong electrophilic properties. The formation of the conjugated adduct proceeds through the aziridinium intermediate and not through direct nucleophilic substitution of the chlorine atom with the thiolate of GSH (Gamcsik et al., 1990).

The major site of base alkylation for most chemotherapeutic alkylating agents, such as nitrogen mustards, is the N7 position of guanine and these agents have been shown to react with DNA in a sequence selective manner showing a general preference for guanines in sequence (Hartley et al., 1986; Mattes et al., 1986). This is thought to be due in part to preferential reaction of positively charged intermediates (such as the aziridinium group of activated nitrogen mustards) with the strongly negative molecular electrostatic potential in the interior of G clusters (Kohn et al., 1987).

Bifunctional alkylating agents cause inactivation of the DNA template as a result of crosslinking. This results in inhibition of DNA synthesis. Repair of the crosslinks and attempted DNA synthesis are in effect two competing processes such that if the former occurs before the latter, the cells may replicate normally. Repair of the crosslinks has been described and appears to be important in determining resistance to the drugs (Roberts et al., 1971; Yin et al., 1973; Richon et al., 1987; Xue et al., 1988).

There are multiple potential cellular mechanisms which could result in decreased crosslinking in alkylating agent resistant cells, including 1) a decrease in uptake of the alkylating agent, 2) metabolism of the active drug to a less cytotoxic intermediate, 3) interaction of the electrophilic alkylating agent with a non-critical nucleophile, thereby decreasing the amount of active species available for interaction with DNA, 4) increased repair of interstrand DNA crosslinks and 5) a quenching (or repair) of monoalkylated DNA

prior to formation of cytotoxic interstrand crosslinks by bifunctional alkylating agents. It is also possible that a combination of these mechanisms could account for alkylating agent resistance.

Resistance to nitrogen mustards has been correlated with a) an alteration in the transport of these agents (Rutman et al., 1968), b) cytoplasmic metabolism of the chloroethyl alkylating moiety to the inactive hydroxyethyl derivative associated with an increase in the intracellular GSH concentration (Suzukake et al., 1983), c) alterations in the kinetics of DNA crosslinks formed by these agents (Zwelling et al., 1981) and d) elevated topoisomerase II activity associated with enhanced monoadduct repair (Tan et al., 1987).

Cancer chemotherapeutic agents, such as the bifunctional alkylating agents, react with GSH in a reaction catalyzed by glutathione-S-transferase. The sulfur of GSH provides electrons for nucleophilic attack on an electrophilic substrate, with the formation of a thioether. The nitrogen mustard melphalan (bis-(2-chloroethyl)-phenyl) gives three adducts with GSH due to substitution of either one or both of the chlorine substitutents and the formation of p-(glutathione-S-yl) phenyl-alanine (Dulik and Fenselau, 1987). Melphalan also undergoes rapid hydrolysis *in vitro* and *in vivo*, forming mono- and dihydroxy degradation products which are inactive. Degradation of melphalan is similar *in vitro* and *in vivo* (Evans et al., 1982).

It is possible that the increased levels of GSH may protect from alkylating agent cytotoxicity at numerous sites within the cell. The alkylating agent ethylmethanesulfonate was one of the first genotoxic electrophiles shown to be a substrate for rat hepatic GSH *in vitro* (Booth et al., 1961). The mercapturic acid 5-ethyl-N-acetylcysteine had been isolated previously from the urine of rats given ethylmethanesulfonate, indicating that GSH conjugation is an *in vivo* pathway of detoxication (Roberts and Warwick, 1958).

Melphalan resistance in L1210 leukemia cells was accompanied by an elevation in GSH. In the same cells, melphalan was shown to be metabolized to a less cytotoxic intermediate (dihydroxy melphalan) and at a greater rate than observed in drug-sensitive

L1210 cells (Suzukake et al., 1983). In a human ovarian cancer cell line with acquired resistance to melphalan there is also an increased formation of the inactive dihydroxy derivative of melphalan (Green et al., 1984).

It is also possible that GSH conjugation of drug mediated by glutathione-S-transferase can decrease DNA alkylation and crosslink formation or facilitate repair of cytotoxic crosslinks once they have been formed. Consistent with this hypothesis is the observation in CHO cells that GSH depletion by misonidazole or diethylmaleate increases both the binding of drug to macromolecules and the formation of crosslinks (Taylor et al., 1983).

The direct assessment of the role of GSH in crosslink formation and repair in human cancer cells has not been determined.

6. MULTIDRUG RESISTANCE

The results of treatment of metastatic carcinoma on patient survival has been discouraging. A number of experimental systems have been developed to determine mechanisms of drug resistance in order to inhibit or overcome them so that efficacy is maintained or even improved. The investigation of resistance to chemotherapeutic agents has demonstrated that different potential mechanisms of resistance exist. Some are due to specific changes in tumor cell phenotype which renders them more resistant to drugs. Much of this work has been carried out using models of drug resistance derived from *in vitro* manipulation of cell culture conditions to achieve a resistant subline to compare with the existant sensitive cell line. These resistant cell lines have provided useful model systems for investigations into the mechanisms of drug resistance. However, the factors which lead to clinically important tumor resistance *in vivo* to therapy have not yet been well characterized. Of several mechanisms of drug resistance that have been identified, one of the best characterized is termed multidrug resistance (MDR).

Cell lines selected for high levels of resistance to one of several "natural" lipophilic drugs are found to be cross-resistant to other drugs which may be unrelated in their structure or mechanism of action. The cross-resistance usually includes anthracyclines, vinca alkaloids, actinomycin D, colchicine, epipodophyllotoxins, and taxol.

Drug resistance appears to involve cellular selection, processes analogous to emerging antibiotic resistance in bacteria. Populations of drug-resistant cells may be inherently produced by clonal evolution (Nowell, 1976), or mutation, perhaps under the influence of a mutagenic cytotoxic agent. The selection process then occurs in the presence of a cytotoxic agent and creates a higher proportion of drug-resistant cells. At the same time, it provides them with a growth advantage. At the molecular level, there are a number of major mechanisms by which drug resistance can be generated.

Biedler and Riehm were the first to describe the phenomenon of MDR. P388 leukemia cells and Chinese hamster lung cells made resistant to dactinomycin by serial incubation in increasing concentrations of the drug displayed the cross-resistance now known as typical of MDR, with resistance to a broad range of structurally dissimilar drugs including daunomycin and vinblastine (Biedler and Riehm, 1970). It was later shown that MDR was associated with decreased intracellular drug accumulation (Juliano and Ling, 1976). The presence of an approximately 170 Kd plasma membrane associated glycoprotein (P-glycoprotein, P170) in these MDR cells was not detectable in the parental drug-sensitive cell line (Bech-Hansen et al., 1976). It was also demonstrated that P-glycoprotein content directly correlated with both the degree of decrease in intracellular accumulation of the toxins as well as the degree of drug resistance exhibited by these cells (Ling and Thompson, 1973). These observations were the first to suggest that the P-glycoprotein conferred resistance by regulating transport of toxins in or out of the cell.

6.1 Structural Features of the Protein

Predicted structural features of P-glycoprotein (Chen et al., 1986; Gros et al., 1986) derived from the amino acid sequence deduced from cloned cDNAs, as well as photo-affinity crosslinking studies (Cornwell et al., 1986a; Safa et al., 1986a), have led to a proposal suggesting that these proteins function in MDR cells by forming pores in the membrane through which they actively expel intracellular antineoplastic compounds.

The amino acid and domain organization of P-glycoprotein is typical of the ABC (ATP-binding cassette) superfamily of active transporters (Hyde et al., 1990) that are found in bacteria (Ames, 1986) and also includes the cystic fibrosis transmembrane conductance regulator (CFTR). The protein has two almost identical ATP-binding domains and a large hydrophobic domain consisting of several transmembrane regions (Abramson et al., 1989). Current evidence suggests that the ATP-binding domains are required for energizing transport and that the hydrophobic domain anchors P-glycoprotein in the cell membrane and operates, perhaps as a pore, in facilitating extrusion of various cytotoxic agents (Endicott and Ling, 1989).

6.2 P-Glycoprotein Expression in Normal Tissue

It has been suggested that P-glycoproteins play an important role in the excretory systems of normal animals. *In situ* hybridization (Mukhopadhyay et al., 1988) and immunocytochemical analyses (Thiebault et al., 1987; Georges et al., 1990) have demonstrated high levels of P-glycoprotein transcripts on the lumenal surface of several epithelial cell types, including hepatocytes, intestinal mucosal cells and kidney proximal tubule cells. The physiological function(s) of P-glycoprotein is so far unknown, although its tissue distribution is suggestive of a role in cellular transport of specific metabolites. Since colon, kidney and liver tissues are exposed to naturally occurring environmental toxins, the role of P-glycoproteins in these tissues may be one of protecting cells by

facilitating the efflux of these toxins. Expression in the adrenal cells suggests that the protein may be involved in the transport of hormones.

6.3 P-Glycoprotein Expression in Drug-Selected Cell Lines

Apart from P-glycoprotein's demonstrated role in drug efflux, the presence of this molecule in tumor cells is associated with another phenomenon. The promoter of the human MDR 1 gene was shown to be a target for the c-Ha-*ras* oncogene and the p53 tumor suppressor gene products (Chin et al., 1992), both of which are associated with tumor progression (Bishop, 1991; Feinstein, 1991; Kedor, 1990). A mutant p53 specifically stimulated the MDR 1 promoter and wild-type p53 exerted specific inhibition. These results imply that the MDR 1 gene could be activated during tumor progression associated with modulation in *ras* and p53.

Selection for resistance to natural products demonstrates the presence of the MDR phenotype in a number of human tumor cell lines (Riordan and Ling, 1985; Cornwell et al., 1986b; Goldstein et al., 1989). Most cell lines with the MDR phenotype that have been established show expression of the gene encoding P-glycoprotein, the MDR gene (Fojo et al., 1985; Scotto et al., 1986; Fairchild et al., 1987). The expression of MDR in conjunction with decreased intracellular accumulation has suggested that these two observations are related. The ability of P-glycoprotein to bind drugs has been shown using photoaffinity analogues of vinblastine, a reaction that is competitively inhibited by vinblastine as well as anthracyclines (Cornwell et al., 1986a; Safa et al., 1986b). Calcium channel blockers can also bind and compete with the vinblastine analogues for binding with P-glycoprotein. Other binding studies raise questions about its precise role in drug transport. The binding constants of various drugs with P-glycoprotein are estimated to be in the micromolar range (Cornwell et al., 1986a), levels higher than are clinically achievable, raising initial questions about the actual role of P-glycoprotein in clinical drug resistance.

Recently, expression of this gene at the mRNA level has been found in a number of clinically resistant tumor specimens, although there is not an unequivocally consistent pattern. Despite the overexpression of the MDR gene observed in drug-resistant cell lines, there have been few reports relating P-glycoprotein expression *in vivo* to clinical drug resistance. In some cases, P-glycoprotein overexpression was detected upon relapse from initial response to combination chemotherapy. However, in other cases, P-glycoprotein overexpression occurred without previous exposure to chemotherapy (Chan, 1991; Gerlach, 1987; Moscow et al., 1989; Goldstein et al., 1989; Weinstein, 1991). P-glycoprotein positivity is a factor indicating poor prognosis in a variety of malignancies. However, presence of P-glycoprotein does not necessarily mean that chemoresistance is present, since P-glycoprotein could be, for example, a marker of a more aggressive tumor phenotype. P-glycoprotein positive invasive colon cancer cells were found to have increased potential for dissemination. This finding suggests that P-glycoprotein may influence cell adhesion and, as a result of this, the processes involved in cancer dissemination (Weinstein et al., 1991). No study has demonstrated a clear role for the MDR gene in clinical drug resistance.

The mechanisms by which natural product anti-cancer drugs cross cell membranes in drug-sensitive or MDR tumor cells has not been resolved. Kinetic analysis for uptake is consistent with a non-saturating uptake system. Although little is known about how the drugs involved in the multidrug resistance phenotype are taken up, all these drugs are relatively hydrophobic and may be assumed to pass through the membrane by virtue of their lipid solubility. Once within the cell, most of the drugs will be trapped by interaction with their target sites (i.e., microtubules for colchicine and vinca alkaloids, DNA for daunomycin and actinomycin D). Decreased drug toxicity in MDR most likely stems from a decrease in net drug accumulation ascribed to either decreased uptake (Ling and Thompson, 1974) or reduced drug retention (Skovsgaard, 1978). Several laboratories have attributed this decreased retention to the action of an "active efflux pump" of broad

specificity. It appears that the diminished steady-state drug levels are due primarily to decreased drug retention. This conclusion comes from two types of experiments. When resistant cells were depleted of ATP energy, either by removing glucose from the incubation buffer or by adding a metabolic inhibitor (azide), the steady-state level of drug increased by comparison with that in metabolically intact cells. Conversely, when glucose was added back to the poisoned cells, the cell-associated drug rapidly decreased to a lower steady-state level. These results have been used by many to argue for the existence of an "active efflux pump" with broad specificity (Dano, 1973; Skovsgaard, 1978; Inaba and Sakurai, 1979).

A number of studies have shown that in MDR cell lines, whether P-glycoprotein positive or negative, altered drug distribution is seen in resistant cells. Drug-sensitive cells predominantly localize drug into the plasma membrane and into the perinuclear region. The resistant counterparts distribute drug in a punctate pattern with considerably less nuclear uptake (Keizer et al., 1989; Gervasoni et al., 1991).

6.4 Transfection of the MDR cDNA into Sensitive Cells

Direct evidence for P-glycoprotein acting as an energy dependent efflux pump was studied in human ovarian carcinoma cells where daunomycin efflux against a concentration gradient was shown to be an active process (Lankelman et al., 1990). The most dramatic evidence for the role of P-glycoprotein in MDR has come from studies that have demonstrated that the MDR phenotype can be conferred through transfer of genetic material encoding the P-glycoprotein. The first experiments involved transfer of genomic DNA from MDR hamster cells into drug-sensitive mouse cells. Using species-specific anti-P-glycoprotein antibodies, the mouse cells that subsequently developed the MDR phenotype showed the presence of hamster and not mouse P-glycoprotein (Deuchars et al., 1987). A similar experiment conferred drug resistance to mouse NIH3T3 cells by transfection of genomic DNA from MDR human KB cells (Shen et al., 1986). Recently, full length

cDNAs coding for both the mouse and human P-glycoprotein have been subcloned into expression vectors and transfected into drug-sensitive cells (Ueda et al., 1987a). These transformed cells displayed the MDR phenotype, again demonstrating the acquisition of drug resistance by genetic transfer.

Three MDR genes have been reported in rodents, while only two have been identified in humans. Mouse MDR-3 gene is the homolog of human MDR-1 gene. Transfection studies have shown that mouse MDR-1 (Gros et al., 1986) and MDR-3 (De Vault and Gros, 1990) and human MDR-1 (Ueda et al., 1987b) genes can confer the multidrug resistance phenotype to drug-sensitive cells, whereas the MDR-2 genes cannot (Gros et al., 1988; Van der Bliek et al., 1988). These observations indicate that expression of the MDR genes is necessary for the MDR phenotype and that different MDR isoforms may have different physiological roles in normal tissues. The levels of the three MDR gene transcripts differ among normal mouse tissues, confirming a previous report that the expression of these genes is tissue specific (Croop et al., 1989).

In studies of transgenic mice it has been shown that the insertion of a human MDR gene leads to the resistance of mouse bone marrow cells to drugs which are normally toxic to these cells (MDR-responsive drugs) (Pastan and Gottesman, 1991; Galski et al., 1989). Normal bone marrow cells usually have low levels of P-glycoprotein and thus are particularly sensitive to MDR-responsive drugs. MDR gene insertion may be a way of providing normal bone marrow cells with a high-level of MDR expression, which could lead to i) the resistance of these cells to the toxic effects of subsequent chemotherapy and ii) the generation of an enriched population of P-glycoprotein-expressing cells, which eventually might be further increased in number by exposure to MDR-responsive chemotherapeutic agents. Transfer of the human MDR gene, using retroviral vectors, into mice demonstrated expression is possible using MDR compounds (Podda et al., 1992).

Although it is clear that P-glycoprotein is found in many tumor cell types, considerable uncertainty remains regarding a) the extent of heterogeneity in the level of

expression among tumor cells, b) its function in stromal cells and c) the level of expression that is therapeutically significant. If P-glycoprotein is a single protein with one set of properties, one would expect all MDR cell lines, regardless of the selecting agent, to exhibit the same level of cross-resistance. The detailed molecular basis for different patterns of cross-resistance to drugs is not known but could involve mutational alteration of P-glycoprotein structure (Choi et al., 1988) and differential overexpression of other MDR genes.

Differences in cross-resistant patterns may be due to alternative splicing of the MDR-1 pre-mRNA or changes in the MDR-1 gene itself, suggesting differences in gene expression in different cells or differences in post-transcription modification of the P-glycoprotein gene product in different cell lines.

Overexpression of P-glycoprotein has been observed without amplification of the gene (Fuqua et al., 1987). The transcriptional control mechanisms which activate expression of the genes involved in MDR in human tumors remain largely unknown. Mutations in the untranslated region of the MDR gene may lead to greater mRNA stability (half-life) or rate of synthesis, as well as additional mutations in the protein coding regions. Mutation in the 5' regulatory sequences of a human MDR gene was suggested as a cause for the appearance of a new transcription initiation site in a vinblastine-resistant derivative of the human KB carcinoma cell line (Ueda et al., 1987b). Expression of other genes may also be necessary to turn on the MDR phenotype in the clinical setting.

6.5 Inhibition of P-Glycoprotein Function

Because drug resistance is such an important aspect of treatment failure, the means of circumvention have taken on a great deal of importance. Reversal of multidrug resistance has been noted by various classes of drugs which are not themselves anti-tumor drugs. Drugs such as calcium channel blockers (eg. verapamil) (Tsuruo et al., 1982; Bellamy et

al., 1982) are known to modulate the MDR phenotype by increasing cellular sensitivity to drugs of the MDR family.

Calmodulin inhibitors (eg. trifluoroperazine (Ganapath and Grabowski, 1983), and cyclosporine (Slater et al., 1986) have also been shown to modulate the MDR phenotype. It is clear that cyclosporine can bind to the P170 drug efflux pump (Goldberg et al., 1988). It has also been shown that cyclosporine can modulate doxorubicin cytotoxicity by means other than interference with the P-glycoprotein system (Shoji et al., 1991). Clinical application of these agents have met with limited success, mainly because of the side effects of these agents when used at concentrations required to overcome multidrug resistance (Ozols et al., 1987a; Thiessen et al., 1992).

Another approach to overcoming multidrug resistance is by encapsulating cytotoxic drugs in liposomes. Liposomes have been shown to be excellent carriers of drugs such as doxorubicin (Treat et al., 1988). The combination of verapamil and doxorubicin-encapsulated liposomes does enhance circumvention of multidrug resistance beyond the effect of each agent alone, implying a synergistic effect (Sadasiron et al., 1992).

Recently, expression of P-glycoprotein has been associated with a cell volume-regulated chloride channel (Valverde et al., 1992). The electrophysiological characteristics of this chloride channel are similar to those of volume-regulated chloride channels of epithelial cells. Epithelial cells swollen in hypotonic medium can regulate their volume by activation of chloride and potassium channels (Diaz et al., 1992) and inhibitors of the P-glycoprotein-associated chloride channel, such as verapamil and quinine (Valverde et al., 1992) can block this cell volume regulation. Thus, the cell volume-activated chloride channel probably reflects a physiological role for P-glycoprotein in the regulation of epithelial cell volume. The transport and channel functions of P-glycoprotein have been shown to be separate by directed mutagenesis of its nucleotide binding domains. While drug transport requires ATP hydrolysis by the protein, ATP binding is sufficient to enable channel activation. These studies provide evidence that P-glycoprotein is bifunctional with

both transport and channel activities (Gill et al., 1992). Agents such as verapamil, which reverse multidrug resistance by inhibiting drug transport by P-glycoprotein, also inhibit the channel activity of this protein (Valverde et al., 1992). These studies suggest that the identification of agents that can block drug transport but do not affect the function of the chloride channel might have clinical utility with less severe side effects.

6.6 Non P-Glycoprotein Mediated Multidrug Resistance

Other mechanisms have been described in both P-glycoprotein positive and negative MDR cell lines, including altered glutathione levels and glutathione enzyme systems (Russo and Mitchell, 1985; Batist et al., 1986), alterations in cytochrome P-450 reductase and superoxide dismutase (Mimnaugh et al., 1984) and altered levels and activity of topoisomerase II, an enzyme involved in replication, recombination and DNA repair (Ross et al., 1988). In some cells, two or more mechanisms may operate simultaneously, suggesting that resistance may be multifactorial (Moscow and Cowan, 1988).

Some *in vivo* MDR lines have no P-glycoprotein expression but have increased GSH and GSH Px (Se-dependent) and form less $OH^{\bullet}$ radicals after drug exposure. If the cells are treated with L-buthionine-(S,R)-sulfoximine (BSO), doxorubicin sensitivity can be restored, suggesting that P-glycoprotein-mediated drug efflux is not the only mechanism of doxorubicin resistance (Samuells et al., 1991).

P-glycoprotein-mediated MDR can be preceded by a non-P-glycoprotein related mechanism conferring an MDR-like phenotype (Kuiper et al., 1990). These findings indicate that early steps of pleiotropic resistance to anti-cancer drugs, induced by doxorubicin selection, can be mediated by mechanisms other than a gradual increase in P-glycoprotein expression. Many cell lines have been described with MDR profiles but no P-glycoprotein overexpression (Slapek and Levy, 1989; Taylor and Dalton, 1989; Mirski et al., 1987). The relevance of P-glycoprotein related MDR and other mechanisms for tumor cell drug resistance in cancer patients is still unknown. In a clinically derived ovarian

carcinoma cell line which was anthracycline resistant, treatment with verapamil had no effect on overcoming the resistance, further questioning the clinical relevance of P-glycoprotein (Rogan et al., 1984). Interestingly, accumulation of drugs in non-P-glycoprotein MDR human lung carcinoma cell lines has been shown to be reduced by an energy-dependent drug export mechanism which prevents efficient transport of drug to the target (Versantvoort et al., 1992). Recently, a multidrug resistance-associated protein (MRP) (Cole et al., 1992) has been identified in a doxorubicin-resistant human lung cancer cell line which does not express P170. MRP mRNA is detectable in normal tissue of the lung, testis and peripheral blood mononuclear cells. MRP is a member of the (ATP)-binding cassette superfamily of transport systems. Unlike MDR, MRP overexpression does not affect drug accumulation. It may, however, alter cytoplasmic or intra-organelle pH such that, in an acidic environment, protonated drugs such as the anthracyclines and vinca alkaloids would be sequestered. The overexpression of MRP may play a role in resistance to drugs in other malignancies as well.

7. GLUTATHIONE

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) occurs in animal cells and also in most plants and bacteria. GSH is synthesized intracellularly, and it is effluxed across cell membranes. This process is connected with a transport system for γ-glutamyl amino acids, reactions that involve the cell membrane and its immediate environment, and inter-organ transport of amino acid sulfur (Meister, 1983). The finding of inhibitors and compounds that increase GSH synthesis make it possible to effectively manipulate the metabolism of this compound to achieve potential therapeutic effects.

7.1 Biological Function of Glutathione

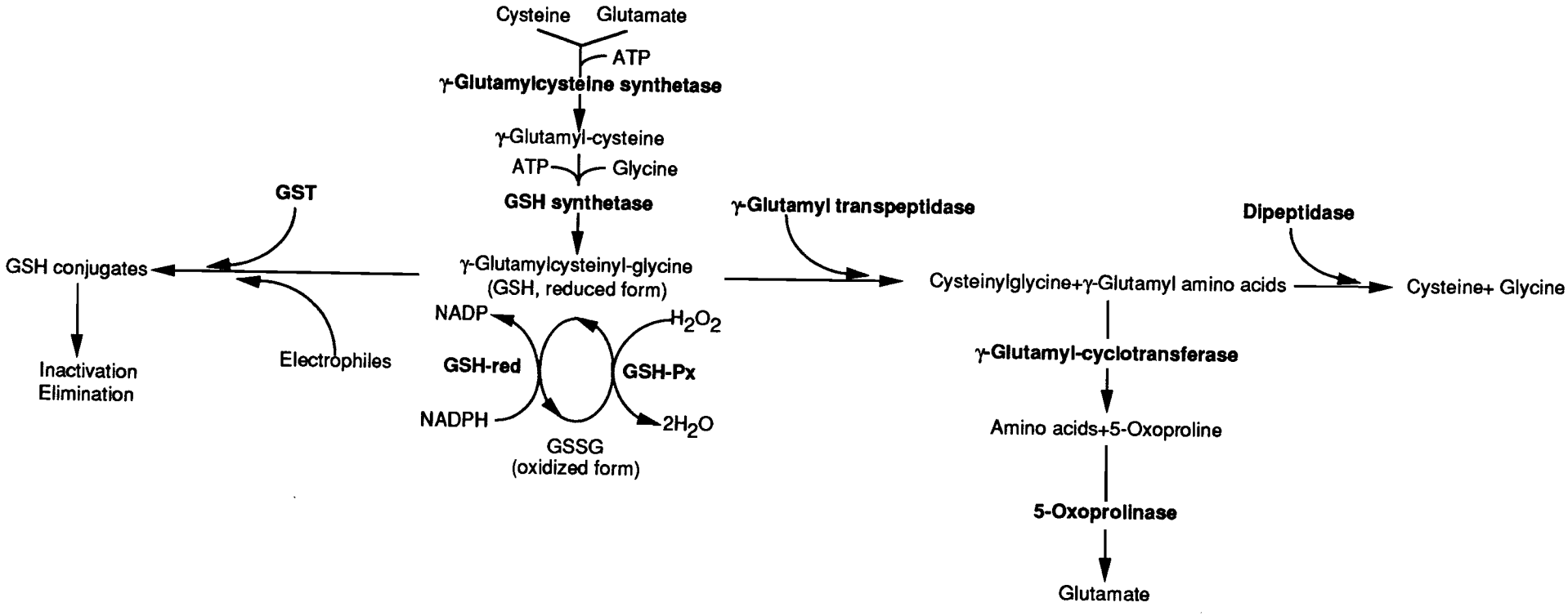
GSH serves many important biological functions. Crucial among these is protection from the toxic effects of free radicals and xenobiotics which can be conjugated with the

sulfhydryl residue of GSH (Gillette, 1972). In addition, GSH has a critical role in many normal cellular activities including: acting as a coenzyme in several reactions, including the two-step conversions of methylglyoxal to lactate and of formaldehyde to formate (Meister, 1983), control of ion permeability at the plasma membrane (Jevell et al., 1982), amino acid transport (Meister, 1973), maintenance of enzymes in active states (Racker, 1955), maintenance of a biologically favorable sulfhydryl-disulfide oxidation-reduction balance, presumably by a nonenzymatic reaction; however, sulfhydryl-disulfide interchanges between glutathione and proteins can be mediated by protein-disulfide reductase (Kosower and Kosower, 1983), reduction of oxidized membrane proteins, such as spectrin (Kosower and Kosower, 1978), control of synthesis of macromolecules, including proteins, DNA and RNA, where GSH serves as a reduced carrier for the reduction of glutaredoxin which is a hydrogen donor for nucleotide reductase (Kosower and Kosower, 1978) and the assembly and disassembly of tubulin during the formation of the mitotic apparatus (Kimura, 1973). The significance of this tripeptide to homeostasis is evident from the extensive pathology associated with inborn errors in its metabolism; these include brain dysfunction, acidosis, peripheral neuropathy, myopathy, and hemolysis (Meister, 1983).

7.2 Biosynthesis of GSH

Biosynthesis of GSH occurs in two successive ATP-requiring steps (Fig. 1). GSH is synthesized intracellularly from glutamate, cysteine, and glycine. 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-glutamylcysteinyl-glycine. Under normal steady-state conditions, the majority of GSH exists in the reduced form. Oxidation of reduced GSH, either nonenzymatically or by the action of glutathione peroxidase, yields glutathione disulfide (GSSG). The high ratio (about 100:1) of GSH to GSSG found intracellularly is maintained by the activity of reduced NADPH-dependent

Figure 1. Sulfhydryl-Dependent GSH Metabolism



GSSG reductase. Efflux of GSSG also maintains its very low intracellular level. The rate-limiting precursor of GSH synthesis is cystine and the GSH level is regulated by the transport of cystine (Bannai and Ishii, 1982; Watanabe and Bannai, 1987). Cystine is taken up by the cells through a unique transport system that mediates an exchange of cystine and glutamate across the plasma membrane (Bannai, 1986). Once transported into cells, cystine is rapidly reduced to cysteine. Entrance of cystine in the anionic form into the cell accompanies the exit of glutamate from the cell. This transport activity is induced by some electrophilic agents (Bannai, 1984). Membrane bound γ-glutamyl transpeptidase contributes to GSH regulation by functioning as a salvage pathway for cysteine moieties (by cleaving cysteinylglycine and γ-glutamyl which gets linked to amino acids) and has been reported to be elevated in some drug resistant cell lines (Ahmad et al., 1987; Lewis et al., 1988a). Cleavage of cysteinylglycine to cysteine and glycine may be catalyzed by membrane bound dipeptidase followed by transport of the free amino acids or may occur intracellularly after transport of the dipeptide. Transported γ-glutamyl amino acids are converted by γ-glutamyl cyclotransferase to amino acids and 5-oxoproline; the latter is decyclized by 5-oxoprolinase to glutamate.

7.3 Glutathione as a Nucleophile

In order to react with weakly nucleophilic positions of DNA bases, genotoxic electrophiles must have a high chemical reactivity. Since GSH (the thiolate anion) is a highly polarizable nucleophile, the spontaneous reaction of GSH with genotoxic electrophiles is not very rapid. There is a range of nucleophilic species *in vivo*. At one end are protein thiols, which are similar in their susceptibility to electrophiles as GSH itself, while at the other end are the nucleophilic sites on DNA, which are toward the polarized end of the nucleophilicity scale, that are susceptible to carcinogenic electrophiles.

Because GSH detoxifies genotoxic electrophiles poorly by the spontaneous reaction, catalysis of GSH conjugation by the glutathione-S-transferases (GSTs) is particularly

important and can lead to efficient detoxification. The net negative charge and overall hydrophilicity of GSH greatly increases the aqueous solubility of the lipophilic moieties with which it becomes conjugated. The rates of non-catalytic reactions of electrophiles with GSH will depend on the thiol concentration and pH. However, the enzyme-catalyzed reaction is less dependent on GSH concentration, since the Km for GSH is of the order of 0.1 mM (Ketterer and Coles, 1990) and reaction rates will fall only when GSH levels approach this value.

Glutathione-S-transferases catalyze reactions between GSH and a wide variety of electrophilic compounds of exogenous origin to form GSH conjugates. Compounds formed endogenously also form GSH conjugates. A wide variety of chemical compounds bearing electrophilic carbon, sulfur, nitrogen, or oxygen atoms have been found to react with the sulfur atom of GSH under the influence of GSTs (Chasseaud, 1979).

In erythrocytes and hepatocytes, it is well established that excretion of GSH and glutathione conjugates is the result of an active extrusion pump with ATPase activity (Kondo et al., 1982; LaBelle et al., 1986). Oxidized glutathione (GSSG) is also excreted at a slow rate from many cell types and appears to be a substrate for this transport system (Ishikawa, 1989). A single pump that recognizes the glutathionyl cysteinyl group common to glutathione conjugates and mercapturic acids would suffice. Mutual competitive inhibition has been demonstrated for oxidized glutathione and a number of hydrophobic glutathione conjugates, including leukotriene C4 (but no inhibition from reduced glutathione) (Nicotera et al., 1985; Sies, 1989). This is evidence for a shared pump or a shared binding site. The glutathione conjugate GS-R may be disposed of via bile and faeces or degraded enzymatically to an S-cysteine derivative cys-S-R. The latter derivative yields a corresponding mercapturate by N-acetylation with acetylcoenzyme A. Glutathione conjugates are transported to the kidney where they are transformed into the corresponding mercapturic acids by the action of (1) γ-glutamyl-transpeptidase, which removes the γ-glutamic acid, (2) cysteinyl-glycine dipeptidase, which splits off the glycine moiety, and

(3) N-acetyl transferase, which acetylates the residual cysteine derivative. The mercapturates are the chemical urinary excretion products of the glutathione conjugates. Mercaptans can be further converted to excretion products by glucuronosylation or methylation. The mercaptan retains only the sulfur atom of glutathione. Degradation of the unacetylated cysteine derivative can also take place. Although little is known of the hormonal regulation of GSH, evidence suggests that regulation of hepatic GSH efflux by hormones acting through cAMP-dependent signaling which enhance sinusoidal efflux of GSH in the perfused rat liver (Sies and Graf, 1985). During fasting and other stressful conditions, mobilization of hepatic GSH may serve as a reservoir for cysteine (Lu et al., 1990).

7.4 Influence of Glutathione on Cellular Protection

The anti cancer therapies where GSH may play an important role in detoxification can be split into two major categories: drugs with electrophilic centers which are detoxified by direct conjugation with GSH to form a thioether, and drugs where formation of reactive oxygen intermediates are involved. Peroxides and free radicals are produced in increased amounts after irradiation, after the administration of drugs and in the presence of increased oxygen tension. They are also formed in normal metabolism and have physiological function (for example, in phagocytosis and in NK cell tumor destruction).

The mechanism(s) by which GSH protects against radiation and chemotherapy toxicity include:

i) Scavenging of free radicals. The free radicals, particularly the hydroxyl radical, produced by ionizing radiation or chemotherapeutic agents, can cause macromolecular damage. Sulfhydryl scavengers can combine with the free radicals before DNA damage is produced (Arrick and Nathan, 1984).

ii) Hydrogen donation. In the process of repair of DNA radical-induced damage, hydrogen atom transfer is necessary to restore the DNA to its native form (Wilson, 1983).

iii) Enhancement of biochemical repair processes (Yuhas, 1980).

iv) Thiols may react with either chloride atom of aqueous species to prevent crosslinking (Chabner, 1982).

Whenever organisms live under aerobic conditions or gain their bioenergy from the reduction of molecular oxygen to water, the possible formation of highly reactive intermediates of oxygen are produced. These reactive oxygen species include the superoxide radical $\bullet O_2^-$, hydrogen peroxide, H_2O_2, and the most potent oxidant, the hydroxyl radical $\bullet OH$. These active oxygen intermediates are also generated in cells exposed to a variety of environmental insults, including radiation, and various other redox active compounds, including chemotherapeutic agents. H_2O_2 is produced by a variety of enzymes, not only within peroxisomes, but also in the microsomal, mitochondrial and soluble fractions of the cell. Hydrogen peroxide results from dismutation of superoxide anions. This favors the formation of the additional oxygen species, $\bullet OH$ and singlet oxygen, which in turn may attack a variety of organic compounds. Evolution has favored enzymes regulating the steady-state levels of H_2O_2, other hydro-peroxides, and $\bullet O_2^-$ in cells which live under aerobic conditions. H_2O_2, $\bullet O_2^-$, and other free radicals are toxic to cells and can cause tissue damage by oxidizing DNA, proteins, and lipids (Halliwel, 1987; Machlin and Bendich, 1987).

Antioxidant defenses include enzymatic systems such as superoxide dismutase, catalase, GST and GSH Px and non-enzymatic lipid-free radical scavengers such as vitamin C, vitamin E and β carotene (Halliwel, 1987). $\bullet O_2^-$ produced mainly by phagocytic cells and endothelial cells is also secreted into the extracellular fluids and might be a source of either $\bullet O_2^-$ derived free radicals or H_2O_2 (Thomas et al., 1988; Matsubara and Ziff, 1986).

GSH Px has anti-oxidant function. The Km of peroxides for GSH Px are much lower than for catalase. Catalase, which is restricted to peroxisomes, metabolizes inorganic peroxides such as H_2O_2. Hydrogen peroxide, either of cytoplasmic or mitochondrial origin, is probably metabolized by GSH Px (Boveris et al., 1972; Loschen et al., 1974). It is a selenium-dependent enzyme with Se at the active site of four identical subunits.

Therapeutic irradiation releases electrons from the target tissues. Subsequently, many very short-lived free radicals are formed. These radicals are formed in water, DNA, as well as other cellular molecules. The ionization itself can produce DNA double strand breaks which are lethal if they accumulate and overwhelm repair processes. One mechanism of protection could be restoration of DNA radicals to undamaged DNA by hydrogen donation from reducing species such as thiols (-SH groups) or damage can be prevented by reduction of the initial free radical. Radicals produced by X-rays may react with oxygen, producing toxic intermediates. GSH can react with hydroxyl radicals (•OH), organic radicals (-R) and hydroperoxides (ROOH). GSH reacts with these to produce thiol radicals that self-associate to produce oxidized glutathione (GSSG). GSH is then regenerated by reduction with NADPH generated from the pentose cycle. Alternatively, the DNA• radical may interact with oxygen, which contains unpaired electrons, to produce a peroxy-DNA radical, DNAOO•.

7.5 Expression of GSH in Tumor Cell Lines

As noted above, GSH is a major component of the cellular defense mechanism against toxic challenges such as ionizing radiation and cytotoxic drugs. The finding that human tumor cells contain very high levels of GSH has led to the suggestion that it is an important factor limiting the therapeutic efficiency of conventional cancer treatment.

Intracellular GSH is highest in cell lines derived from clinically resistant tumors, while GSH levels are comparatively much lower in cell lines derived from tumors that are clinically sensitive to cytotoxic chemotherapeutic agents (Green et al., 1984; Kramer et al.,

1987). Alkylating agents, such as nitrogen mustards can react with a variety of cellular nucleophiles, including sulfhydryl groups. Numerous cell lines selected for resistance to alkylating drugs have demonstrated elevated GSH content (Suzukake et al., 1982; Begleiter et al., 1983). Moreover, depletion of GSH has been shown to sensitize resistant cell lines.

In some tumors, clinical resistance may be correlated to the ability of tumor cells to detoxify the cytotoxic moiety of the antineoplastic agent (Fojo et al., 1987a). Hepatic metastases arising from intraperitoneal innoculation of L1210 cells had elevated GSH levels and were more resistant to melphalan compared to their counterparts found in the peritoneal cavity (Ahmad et al., 1986).

The interest of GSH in cancer therapy is its ability to scavenge free radicals from radiation-induced lesions, and its detoxification of electrophiles, such as alkylating agents, via glutathione-S-transferase. Thus, there is currently an interest in glutathione depletion as a means of sensitizing cells to the effects of radiation and chemotherapy. The extent of GSH depletion varies among different tissues. The therapeutic ratio achieved by GSH depletion greatly depends on the endogenous concentration of GSH and on the extent of depletion in the target tissue. Depletion of tissue GSH to less than about 30% of normal values, particularly in liver, can result in altered xenobiotic metabolism and increased toxicity of electrophilic metabolites (Plummer et al., 1984). A widely used method for lowering tissue GSH levels is the administration of compounds that react enzymically with GSH to form conjugates. Inhibition of GSH synthesis using buthionine sulfoximine (L-BSO) can also result in depletion of this tripeptide in those organs with a sufficient turnover rate.

There is a relationship between intracellular GSH levels and cytotoxicity to melphalan, cisplatin and irradiation in ovarian cancer cell lines (Green et al., 1984; Louie et al., 1986). Cell lines with resistance induced *in vitro* to either melphalan or cisplatin have a 2-3-fold elevation in intracellular GSH levels compared to the parental sensitive cell lines. GSH depletion with L-BSO (a synthetic amino acid analog which irreversibly binds and

inactivates γ-glutamylcysteine synthetase) has been reported to increase the cytotoxicity of a number of chemotherapeutic agents, particularly the alkylating agents (Andrew et al., 1985; Crook et al., 1986; Green et al., 1984; Kramer et al., 1987). In addition, depletion of GSH is associated with the reversal or cross-resistance to irradiation and some chemotherapeutic agents present in cell lines with acquired resistance to either melphalan or cisplatin (Hamilton et al., 1985). BSO treatment was found to partially inhibit DNA repair following cisplatin treatment, and the addition of aphidicolin caused nearly a 100% inhibition in DNA repair activity, suggesting that GSH may be involved in DNA repair. It is generally considered that the BSO effects on alkylating agents and irradiation sensitivity were related to GSH reduction, which in turn "facilitated" drug interaction with DNA or led to more available free radicals to cause DNA damage (Lai et al., 1989). GSH has been shown to mediate the formation and subsequent repair of DNA interstrand crosslinks by regulating the activity of both DNA ligase I and II (Rairkar and Ali-Osman, 1992). The dependence of DNA polymerase activity on cellular GSH content has also been demonstrated (Ali-Osman and Rairkar, 1992). These findings may represent other mechanisms where GSH mediates tumor cell resistance.

It has previously been demonstrated that BSO administration to mice leads to a transient reduction in GSH levels in many normal tissues, including the liver and kidney (Griffith, 1982). However, for BSO to increase the therapeutic index of alkylating agents requires that toxicity to tumor cells be greater than to normal tissues. In non tumor-bearing animals, the combination of melphalan and BSO is not lethal. The mechanism responsible for the preferential effect of L-BSO plus melphalan on cytotoxicity in tumor cells compared to normal tissues has not been determined. It is possible that tumor cells require higher levels of GSH for their growth and that a reduction in GSH makes these cells more vulnerable to the cytotoxic effects of chemotherapy compared to normal tissues. It is also possible that there are alternate mechanisms not associated with GSH levels which protect

normal cells from the effects of alkylating agents which are absent or less functional in tumor cells.

The precise mechanism by which glutathione depletion enhances the cytotoxicity of melphalan is not yet known; it is likely that the extent of melphalan inactivation by reaction with the glutathione sulfhydryl is decreased in glutathione-depleted tissues (Arrick and Nathan, 1984). It has been shown that BSO-mediated depletion of glutathione in L1210 leukemia cells results in an increase in DNA-DNA crosslinking following exposure to melphalan, with no change in the rate of repair of crosslinks following drug exposure (Dorr, 1987). Additionally, Dulik et al. (1986) have shown that GST catalyzes formation of melphalan-glutathione adducts in L1210 leukemia cells. These observations suggest that melphalan is directly detoxified by glutathione. It is not yet clear if the percentage of depletion of initial GSH content or the absolute GSH concentration following exposure to BSO is the most important relevant value influencing sensitization to melphalan. To address this directly, specific cell lines or tissues of variable initial GSH content will require study.

8. GLUTATHIONE-S-TRANSFERASE

In 1965, Ketterer (Ketterer, 1967) isolated a protein from rat liver that bound azo dye carcinogens. Subsequently, another group (Litwack and Morey, 1969) described proteins from the same source which bound cortisol and bilirubin, as well as several drugs. It was later shown that the cortisol-binding and the carcinogen-binding proteins were identical (Litwack and Morey, 1970). Litwack (Litwack et al., 1971) later coined the term "ligandin" to represent these hepatic proteins. Ligandin was subsequently established to be identical with glutathione-S-transferase alpha, which was later found to be the same as β-3-ketosteroid isomerase of rat liver (Benson et al., 1977)..

Glutathione-S-transferases (GSTs) comprise a family of enzymes with a broad substrate specificity which participate in a variety of detoxication processes leading to

excretion of xenobiotic agents or mercapturic acids. Many toxic xenobiotics are strongly lipophilic and the GST mediated conjugation of reduced GSH with such compounds facilitates their elimination from the body since the resulting metabolites are more water-soluble. Cytosolic, microsomal and nuclear GSTs exist and the different forms appear to serve distinct detoxification roles.

Although conjugation with glutathione as a rule is a detoxification pathway, some chemicals are actually activated, i.e., more reactive or more toxic products are formed through conjugation; examples include vicinal dihalogenoalkenes (Van Bladeren et al., 1981) and bromobenzene (Monks et al., 1985).

The glutathione transferases are known as enzymes that catalyze the nucleophilic attack of the sulfur atom of glutathione on electrophilic groups in a second substrate. The enzymes occur abundantly in most cells and tissues investigated and are generally considered to serve in the intracellular detoxication of mutagens, carcinogens and other noxious chemical substances. Multiple forms of glutathione transferase have been discovered in virtually every organism in which glutathione transferase activity has been found. In most cases, the variant forms are distinguished by differences in catalytic properties, suggesting separate or complementary activities. In addition to their enzymatic function, it has been suggested that GSTs serve as intracellular carrier proteins of certain organic molecules, acting as an intracellular equivalent to albumin in blood plasma, i.e., ligandin (Tipping and Ketterer, 1978).

The expression of the multiple forms of glutathione transferase differs from one tissue to another. The occurrence of the different forms changes dramatically in an organ-specific manner, during the transition from the fetal to the adult state (Fryer et al., 1986). In the mouse, a sex-related difference in the hepatic expression of a specific enzyme form, apparently under testosterone control, has been noted (Hatayama et al., 1986). The structure and control of the genes of glutathione transferase are currently under study.

The GSTs have been referred to as multi-functional proteins. The fact that these enzymes represent as much as 5% of the cytosolic protein in the liver, and 1% of the protein in kidney and intestine, indicates that they play important house-keeping functions in cells. Further, the different GSH isoenzymes appear to have distinct functions as they exhibit different catalytic activities (Hayes, 1984; Hayes, 1986). Differences in the location of the isoenzymes within tissues have been noted (Redick et al., 1982) as well as differences in the subcellular localization of individual GSTs (Bennett et al., 1986). The reason for the differences in expression of GST isozymes between tissues is not clear but one can speculate that it reflects an adaptation to the varying roles in the biosynthesis of endogenous compounds.

8.1 The Classification of Glutathione-S-Transferases

The soluble GSTs are composed of two subunits and exist in either homo- or heterodimeric forms (23- to 29-Kd subunits, Mannervik, 1985). Early classification of cytosolic GSTs was based on differences in substrate utilization (Boyland and Chasseaud, 1969). However, further studies with purified enzymes demonstrated considerable overlap in substrate specificity between different isozymes. Subsequent nomenclature has relied on physical structural properties, and has been adapted to reflect subunit composition (Jakoby et al., 1984).

Although a considerable number of different isoenzymes have been described in rat, man and mouse, the same multigene families are seen and there is significant identity in primary structure across the three species (Coles and Ketterer, 1990). Amino acid sequence homology and immunological cross-reactivity indicates considerable similarity between isozymes of one class belonging to different species (Mannervik and Danielson, 1988). In the rat, more than 13 subunits have been characterized, which are designated by arabic numerals and as Y proteins. Heterodimeric and homodimeric combinations of these subunits occur, but only polypeptides with extensive sequence homology (> 65%) can

hybridize, i.e., within each class (Hayes, 1984). The cytosolic subunits all possess a minimum of about 25% homology with each other (Tuc and Qian, 1987).

GSTs have been divided in mammals into five gene families termed alpha (α) (subunits 1, 2, 8, 10), mu (μ) (subunits 3, 4, 6, 9, 11), pi (π) (subunit 7) and theta (θ) (subunits 5, 5*, 12) (cytosolic and nuclear forms) or microsomal. Rat GST θ differs from the α, μ or π classes by its lack of activity towards the model substrate CDNB. The number of genes within each family varies considerably and ranges between 5-10 for the alpha and mu class GST and one (or two) genes for the microsomal and pi class GST. Although substrate specificities of the GST isozymes are broad and overlapping, major differences exist; some subunit's activity are specific enough to be used for the classification of the isozymes. Class α GSTs are highly reactive with cumene hydroperoxide, the non-selenium glutathione peroxidase activity (Wendel, 1980). Class π isozymes are highly reactive towards ethacrynic acid while trans-stilbene oxide is used for the classification of class μ isozymes. Class μ transferases exhibit by far the highest rates of conversion of polycyclic aromatic hydrocarbon epoxides and diolepoxides (Warholm et al., 1983). Although the substrate specificity of θ has not been characterized, these isozymes do have peroxidase activity (Meyer et al., 1991).

8.2 The Catalytic Mechanism of Glutathione Conjugation

Each subunit of the dimeric enzyme has a complete active site, which does not appear to contribute to the catalytic properties of the active site of the neighboring subunit (Danielson and Mannervik, 1985). Each active site has a binding site for GSH and an adjacent, partly hydrophobic binding site for the electrophilic substrate. These subsites of the active-site cavity have been referred to as the G- and H-site, respectively (Mannervik et al., 1978). The specificity of the G-site is high and the binding of GSH appears to involve ionic binding (Schasteen et al., 1983). The determination of the crystal structure of the class π isozyme from human plancenta has helped to specify the amino acids lining the G

and H sites (Reinemer et al., 1992). Site-directed mutagenesis studies have implicated highly conserved amino acid residues among several GST isozymes involved in binding GSH (Manoharan et al., 1992). Modification of the glutathione molecule includes altering the γ-glutamyl moiety and the sulfhydryl moiety. The γ-glutamate portion of GSH is important for GST binding since synthetic GSH analogues modified in these residues are poor substrates for GST (Adang et al., 1989). A number of modifications may be introduced without major effects on the catalytic activity of the rat GST, however (Adang et al., 1990).

GSH binding may cause a conformational stabilization for proper orientation for catalysis to occur, which may explain the high specificity for GSH (Mannervik and Danielson, 1988). Each isozyme generally has a unique spectrum of substrate selectivity which depends on the type of electrophilic functional group as well as the overall topology of the substrate. On the other hand, the basic mechanism for activation of the physiologic substrate GSH is essentially the same among the cytosolic isozymes, presumably by a base-assisted deprotonation.

The transferases specifically bind reduced glutathione and probably render its thiol group more nucleophilic by lowering the pKa two units. At neutral pH, the sulfhydryl group of enzyme-bound glutathione is predominantly in the thiolate ionization state. One aspect of the catalytic mechanism is the ability of GST to lower the pKa of the thiol of GSH, which is about 9 in aqueous solutions, to less than 7 on the surface of the protein. It is likely that destabilization of the thiol is achieved by positioning it in a positively charged electrostatic field in the active site of the enzyme (Zhang et al., 1991). By site-directed mutagenesis, three conserved arginines in an isozyme from the alpha gene class are involved in either the binding of GSH or in stabilization of conformational states of the enzyme (Stenberg et al., 1991). It has been shown that Tyr is conserved in the active site of all GSTs and that a hydrogen bond between Tyr and the enzyme bound nucleophile helps to lower the pKa of GSH (Liu et al., 1992). If the bound compound is inert, nothing

happens. But if it contains an electrophilic center it will react with this activated thiolate anion. One common feature of the electrophiles that are conjugated by these enzymes is the presence of an α,β-unsaturated bond adjacent to an electron withdrawing group, such as a nitro, carbonyl or diester (West, 1990). Thiols react, in any case, with such groups and with other electrophilic centers; for example, by nucleophilic substitution of a halide leaving-group in iodoacetate, chlorodinitrobenzene or by nucleophilic addition to a carbonyl carbon. The transferases, however, serve to accelerate the spontaneous reactions.

8.3 GST Peroxidase

The name GST refers to only one of serveral enzymatic activities which these enzymes possess; others include GSH-dependent isomerase activity, which is important in prostaglandin biosynthesis and Se-independent GSH peroxidase activity.

The Se-dependent GSH peroxidase is a tetramer of Mr 84,000 with activity towards both H_2O_2 and organic hydroperoxides. It contains one residue of seleno-cysteine per mole at each of the active sites. Se-independent GSH peroxidase activity is mediated by the glutathione-S-transferases. The Km for organic peroxides (cumene hydroperoxide, for example) is approximately twenty times higher for GST than it is for Se-dependent GSH peroxidase (Sies et al., 1981). GSTs have relatively low activity towards organic hydroperoxides, but none at all towards H_2O_2. What they lack in specific activity they tend to make up for in quantity, being 0.1-0.2 mM in the cytosol of hepatocytes (Ketterer and Meyer, 1989).

The distribution of Se-dependent and Se-independent GSH peroxidase varies with the species and the cell type and within the cell type, the cell compartment. In human liver, GST transferase accounts for 84% of total GSH peroxidase activity (Laurence and Burk, 1978). In rat liver cytosol GST peroxidase accounts for 35% of overall peroxidase activity; in the nucleus 86% of total peroxidase activity is GST mediated (Tan et al., 1988).

8.4 Biological Function

Most of the chemical compounds studied as substrates for glutathione transferases do not occur naturally and may be unrelated to the true physiologic substrate of the enzymes. It has been stressed, however, that oxidative metabolism of a variety of endogenous substances gives rise to reactive electrophiles that should be considered possible "natural substrates" (Mannervik, 1987). In an evolutionary perspective, it appears that GSH emerged as an important biomolecule when oxygen became an abundant compound of the atmosphere (Fahey, 1977). Consequently, it has been proposed that glutathione dependent enzymes, including the transferases, evolved in aerobic organisms in response to the requirements of inactivation of toxic products of oxygen metabolism (Mannervik, 1986). Substances containing carbon-carbon double bonds may be particularly prone to yield reactive oxidation products. Aromatic compounds and polyunsaturated fatty acids are abundant biomolecules of this kind. In addition to its endogenous compounds, an organism is also exposed to a wide range of potentially toxic compounds produced by other species in the environment. Some of the possible types of substrates that may be biologically important are given below.

The GSTs are involved in the formation of several important classes of endogenous compounds. The alpha class Ya isozymes possess Δ^5-3-ketosteroid-isomerase activity and catalyze the isomerization of Δ^5-3- to Δ^4-3-ketosteroids (Benson et al., 1978). The conversion of prostaglandin H_2 into prostaglandin E_2, D_2 and F_2 is primarily catalyzed by a μ-class GST acting as a reductase and isomerase (Ujihara et al., 1988). The conversion of the peroxide leukotriene A_4 (LTA_4) to the glutathionyl derivative LTC_4 is also catalyzed by a μ-class GST (Bach et al., 1984). No LTC_4 synthesis can occur in the absence of enzyme. A number of non-substrate ligands, including steroids, bilirubin and bile acids are bound noncovalently by the GSTs (Boyer and Versly, 1987).

Quinones represent one class of reactive compounds which may be detoxified by glutathione conjugation. Organic hydroperoxides are substrates for glutathione transferases

(eg. arachidonate hydroperoxide, linoleate hydroperoxide, DNA hydroperoxide). Arachidonic acid is a polyunsaturated fatty acid that gives rise to several epoxide derivatives or GST substrates (Soderstrom et al., 1985). Hydroxyalkenals produced during lipid peroxidation have also been shown to be substrates for these enzymes (Alin et al., 1985). These examples lead to the conclusion that several types of toxic electrophiles which are produced intracellularly may function as "natural" substrates for the glutathione transferases. The variety of functional groups and the carbon skeleton on which they are attached may be one of the causes why so many different forms of glutathione transferase have evolved.

8.5 Microsomal Activity

Microsomal GST is most abundant in the liver, although it has been detected in extra-hepatic tissues (Morgenstern and DePierre, 1987). This form has also been observed in mitochondria and membrane fractions from other cells (Morgenstern and DePierre, 1988). Although a significant variation exists in the cytosolic content of different livers, microsomal GST appears to be expressed in all individuals.

Microsomal GST has a calculated Mr of 54 Kd and is composed of three identical subunits of 17 Kd (Morgenstern et al., 1985). This microsomal GST polypeptide is unique, and does not appear to combine with the the cytosolic GST subunits.

Of the compounds known to be substrates for various cytosolic glutathione transferases, the majority of those tested are also conjugated with glutathione by the microsomal enzyme. This enzyme is activated by reagents that react with thiols, including disulfides and quinones and has CDNB conjugating activity. Most xenobiotics are quite hydrophobic and thus are expected to accumulate in hydrophobic compartments of the cell, including membranes and binding sites on certain soluble proteins. The endoplasmic reticulum in rat hepatocytes contains almost 50% of the total cellular GST molecules. Thus, there is a great potential for enrichment of hydrophobic, electrophilic xenobiotics in

the endoplasmic reticululm (Boyer et al., 1983). Microsomal GSTs function in the same way as cytosolic GSTs, but may have certain unique functional characteristics as well due to its localization in a membrane. The peroxidase activity that these enzymes possess affords them the ability to reduce lipid peroxides which are normally generated from the electron transport chain. Immobilized microsomal fractions have also been used to catalyze GSH-dependent detoxification of the genotoxic compound, melphalan (Dulik et al., 1986).

8.6 Nuclear Activity

Glutathione-S-transferases have been shown to inhibit lipid peroxidation *in vitro* and reduce peroxidized DNA (Tan, 1988). The substrates involved appear to be free fatty acid hydroperoxides and pyrimidine hydroperoxide residues, respectively. The overall GST content of the nucleus is about 3 orders of magnitude lower than that of the soluble cytoplasm. Se-dependent GSH peroxidase from rat liver is also active towards DNA hydroperoxide, however, since this enzyme accounts for only 14% of the GSH peroxidase activity detectable in the nucleus, GSTs may be the more important source of this activity. Nuclear GST are composed of subunits 1, 2, 3, 4, 5 and 5*, in the proportion 40:25:5:5:25. Subunits 1 and 2 can detoxify lipid hydroperoxides, whereas subunits 3 and 4 detoxify DNA hydroperoxides. Subunits 5 and 5*, which are assigned to class θ, have the highest specific activity toward peroxidized DNA. These subunits do not cross-react with antisera prepared against members of either the alpha, mu or pi class.

Although the GST content of the rat nucleus was found to be much lower than that of the soluble supernatant, nuclear GSTs are likely to be more important in the detoxification of DNA hydroperoxide produced *in vivo* (Tan et al., 1988). DNA peroxidation free radicals are produced by ionizing radiation. Most of the DNA peroxides reside in the pyrimidine fraction and involve thymine. The same radicals and presumably the same hydroperoxides are produced during radiolysis of single and double-stranded DNA *in vitro* and double-stranded DNA *in vivo* (RotiRoti and Cerutti, 1974). GSH has been shown to

be oxidized by both DNA and thymine hydroperoxide in the presence of rat liver-soluble supernatant; this implies the action of a GSH peroxidase (Christophersen, 1969). Both Se-dependent GSH Px and purified GST from the soluble supernatant catalyze the reduction of S-hydroperoxymethyluracil (Tan et al., 1986). Recently, treatment of rat hepatocytes with aflatoxin or the antioxidant ethoxyquin was shown to induce a 2-4-fold increase in GST conjugating activity in isolated nuclei, further suggesting a role for nuclear GST in preventing genotoxicity (Green et al., 1993).

Nonhistone protein BA has been shown, by immunoblotting and peptide mapping techniques, to be two glutathione-S-transferase Yb subunits. These subunits were demonstrated to be both nuclear and cytoplasmic proteins by immunolocalization on rat liver cryosections (Bennett et al., 1986). The most obvious function of the GSTs in the nucleus is the biotransformation of electrophilic compounds that have escaped detoxification in the cytoplasm, thus preventing their interaction with DNA or other macromolecules. GST, for example, has been shown to inhibit the formation of covalent adducts between benzo[a]pyrene and DNA *in vitro* and *in vivo* (Hesse et al., 1982; Jernstrom et al., 1982) Alternatively, GSTs may modulate gene expression in view of their localization in regions where processing of RNA takes place (taking into account their low levels) (Bennett et al., 1986). It has been reported that actin, a major cellular protein, is involved in the transcription of chromosome loops in Pleurodeles oocytes, suggesting that cellular proteins may play a role in the transcriptional process (Van Omman et al., 1990).

8.7 Tissue Selective Expression of the GSTs

The existence of a relatively large number of different GST isozymes with strongly varying substrate selectivities affords an organism the potential to detoxify a wide range of electrophilic xenobiotics. However, the extent to which a given tissue or cell type will be able to detoxify these compounds, and thus its sensitivity towards adverse effects, will depend on the number and amount of specific isozymes present, i.e., the actual isozyme

pattern. Factors such as age and exposure to inducing and inhibiting agents are known to cause changes in isozyme patterns and activities.

In the rat and in humans, the highest amount of tested GST protein is present in the liver (up to 5% of total soluble protein) (Van Omman et al., 1990). However, the major portion consists of the α (subunits 1 and 2) and μ families (subunits 3 and 4); only trace amounts of the π family (subunit 7) are present. Most extrahepatic organs express class π (Mannervik, 1985). Similarly, in kidney, the expression of subunit 3 is extremely low, although relatively high levels of subunit 7 are present (Van Bladeren and Van Omman, 1991). Subunit 1 is lacking from rat lung.

In humans, studies on tissue distribution are complicated by the large variation observed for all classes of isozymes, but a number of similarities to the rat are apparent. For example, the α form is found in all liver but the μ form is detected in only 60% of human livers (Warholm et al., 1981). The liver contains only minor amounts of π class isozyme, which is found in numerous other organs, notably placenta, kidney and intestine. The lung contains relatively low amounts of α class subunits (Fryer et al., 1986).

8.8 Drug Metabolism Mediated by GSTs

Many chemical carcinogens and their electrophilic metabolites are detoxified by conjugation with glutathione (Table 1). For instance, AFB_1-8,9-oxide, the hepatocarcinogenic derivative of aflatoxin B_1 (AFB_1) is detoxified by the formation of a glutathionyl-AFB_1 conjugate. Studies with purified rat isozymes demonstrate that this reaction is catalyzed by enzymes containing the alpha class subunits 1 and 2 (Coles et al, 1985). GSTs have also been shown to catalyze the conjugation with glutathione of activated electrophilic polycyclic hydrocarbon carcinogens. An important member of this group of carcinogens, benzo[a] pyrene is activated by P450 mixed-function oxidases to a more potent carcinogen (anti-BPDE). This compound, along with some related diol

Table 1. Biologically Important Substrates of the GSTs

Arachidonic acid derivatives	
leukotriene A4	
prostaglandin H2	
Carcinogens	
aflotoxin B_1-8,9-oxide	
anti-benzo[a] pyrene-7,8,diol-9,10-epoxide	
Antineoplastic agents	
nitrogen mustards -	chlorambucil
	melphalan
	cyclophosphamide
nitrosoureas -	1,3-bis(2-chloroethyl)-1-nitrosourea
anthroquinone -	mitoxantrone
Products of membrane and DNA oxidation	
fatty acid hydroperoxides	
4-hydroxy alkenals	
DNA hydroperoxides	

epoxide isomers, is a good substrate for the human mu and pi class isozymes (Robertson et al., 1986).

Several clinically active antineoplastic drugs are detoxified in reactions catalyzed by GST. Reactions involving alkylating agents have been well described. For example, GSTs catalyze the formation of three glutathionyl conjugates with melphalan (Dulik and Fenselau, 1987). Additionally, GST mediates the conjugation of glutathione with the nitrogen mustards chlorambucil (Ciaccio et al., 1990) and the cyclophosphamide metabolite acrolein (Berhane and Mannervik, 1990). Further support for a role of GSTs in anticancer drug inactivation is provided by the finding that 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) can be activated by a GSH/GST-dependent denitrosation reaction catalyzed by a purified class μ GST (Smith et al., 1989).

In addition to direct inactivation, GSTs may lessen the toxicity of nitrosoureas by yet another mechanism. The antitumor effects of nitrosoureas are believed to depend ultimately on the formation of DNA interstrand crosslinks. Chloroethylation of one DNA strand at an O^6 position of guanine is presumed to produce the reactive intermediate with which the second DNA strand forms a covalent linkage. Treatment, in cell-free systems, of these chloroethylated DNA intermediates with glutathione inhibits the formation of DNA strand crosslinks (Ali-Osman, 1989). This suggests the possibility that nuclear GSTs may catalyze the same reaction *in vivo* and thus provide a second mechanism of defense against nitrosourea toxicity. Finally, glutathione is known to bind cisplatin and, therefore, to compete with DNA for drug binding (Eastman et al., 1988). GSH has also been shown to quench platinum-DNA adducts (Eastman and Richon, 1986). Although GSTs have not been shown to catalyze this reaction, treatment of adult rats with cisplatin increased liver expression of the Yc mRNA with a corresponding change in Yc protein levels (Waxman et al., 1992).

Nonalkylating anticancer drugs are also metabolized by GSTs. Mitoxantrone, an anthraquinone drug related to doxorubicin, is detoxified by at least two conjugation

pathways. One of these pathways, which apparently requires prior metabolism by P450 mixed-function oxidases, involves enzymatic conjugation with glutathione. For many drugs there is no evidence to support a direct role of GST in detoxification. GST-catalyzed metabolic transformation or conjugation with glutathione has not been demonstrated for most drugs associated with the classical multidrug resistant phenotype - drugs including anthracyclines, vinca alkaloids, epipodophyllotoxins. It remains possible that the GSTs function in the detoxification of as-yet unrecognized metabolites or other toxins generated secondarily to the action of these drugs. Indeed, secondary toxins, such as lipid hydroperoxides and hydroxy alkenals, may be produced in response to adriamycin-generated free radicals (Kappus, 1985), and these are GST substrates.

8.9 GST and Drug Resistance

Living organisms encounter a wide range of chemical structures among the xenobiotics in their environment. A characteristic of many enzyme systems that metabolize them is multiplicity (eg. cyto P-450, UDP glucuronyl transferases, sulfotransferases, GSH transferases). However, as far as the GSH transferases are concerned, the multiplicity that exists is not sufficient to utilize electrophiles derived from all xenobiotics as substrates for detoxification, i.e., cytotoxic or genotoxic damage cannot always be avoided. This is well illustrated by aflatoxin 8,9-oxide which is a poor substrate for the GSH transferases in humans, and by the aromatic amines, which are not substrates and which escape detoxification by the abundant GSH transferase activity of the liver. Similarly, the polycyclic aromatic hydroxides which, though effectively metabolized in the liver, may not encounter appropriate isoenzymes in sufficient quantity in mouse skin or rat mammary gland for effective detoxication. Although these enzymes do not provide complete protection, they are significant for the cell, as shown by their abundance in tumor cells that acquire selective advantages over normal cells and their increased concentration in cells that become resistant to anti-cancer drugs.

Evidence for a role of GST in drug resistance in mammalian cells is provided by the following:

i) Cell lines selected for resistance to chemotherapy agents often have elevated GST isozyme content (Batist et al., 1986; Robson et al., 1987; Smith et al., 1989).

ii) GST and GSH are increased when normal cell populations (eg, bone marrow) are primed with a cytotoxic agent (Millar et al., 1975; Carmichael et al., 1986).

iii) In rat hepatocarcinogenesis, increases in GST levels coincide with the appearance of drug resistant hyperplastic pre-cancerous nodules (Sato et al., 1984).

iv) Modulation of GST *in vivo* with the antioxidant butylated hydroxyanisole can cause a profound change in susceptibility to toxins and carcinogens (Hayes et al., 1991a).

v) Inhibition of GST sensitizes cells to cytotoxicity (Clapper et al., 1990).

vi) Elevated GST levels have been observed in human tumor cell lines after the onset of clinical drug resistance (Wolf et al., 1987).

vii) Expression of transfected GST genes into cells can result in increased resistance to cytotoxic agents (Black et al., 1990; Puchalski and Fahl, 1990).

Some of the most compelling evidence indicating that GST overexpression may be part of a stress response, and as a consequence be involved in drug resistance, has come from the selection of drug resistant cell lines *in vitro*. The changes in GST expression have been most marked in cell lines selected for resistance to alkylating agents such as chlorambucil, melphalan, nitrosoureas or to redox cycling drugs such as adriamycin. In the above studies all three classes of cytosolic GST have been shown to be overexpressed in cell lines resistant to cytotoxic agents.

The initial studies demonstrating elevation in GST expression in drug resistant cell lines were performed by Tew and coworkers. The study of a rat mammary carcinoma cell line selected for resistance to bifunctional nitrogen mustards (Tew and Wang, 1982) showed that the alpha class GST Yc was overexpressed (Buller et al., 1987).

Many intrinsically resistant human tumors have elevated levels of GST relative to the surrounding normal tissue. For example GST π has been shown to be expressed at high levels in tumors of the colon, stomach, pancreas, uterine cervix and lung (Kodate et al., 1986; Sato et al., 1987; Carmichael et al., 1988; Peter et al., 1989; Moorghen et al., 1990). Immunoblotting and mRNA determinations have shown that GST π is also increased in adenoma of the breast and lung, as well as lymphoma and CLL (Shea and Henner, 1987; Moscow et al., 1989; Forrester et al., 1990; Schisselbauer et al., 1990).

It has been suggested that the elevated GST π levels are a marker for drug resistance. However, direct evidence that GST π overexpression is a contributing factor in the failure of chemotherapy in cancer patients remains elusive. In addition to GST π, many human tumors express significant levels of the α subunit (Tew et al., 1987; Carmichael, 1988; Forrester et al., 1990).

GST π elevations are a frequent event in cells selected for alkylating agent resistance. However, the lack of cross-resistance among different cell lines selected for resistance to different alkylating agents, all of which have elevated GST π levels, indicates that increased levels of GST π cannot be the predominant mechanism for resistance to the tested drugs.

There is currently significant debate as to whether the GSTs are indeed part of the mechanism of resistance towards anti-cancer drugs or whether their overexpression is a consequence of a drug-involved stress. The fact that GSTs appear to have evolved as part of an adaptive response to environmental stress provides strong evidence that they confer protection against some toxic environmental stimulus. The detection of amplified GST genes in a cell line resistant to chlorambucil (Lewis et al., 1988a) provides strong evidence for a role in the resistance mechanism, as such amplification events only arise and become fixed in the population as a consequence of selective pressure.

8.10 Inhibitors of GST Activity

Most GST inhibitors display their effect through reversible interaction with the GST active site, either competing with the electrophile-GST interaction, or else by interfering with glutathione binding. The largest range of GST inhibitors is found in the compounds that bind to the electrophilic substrate site. As with the substrates, specificities towards certain isozymes are observed. All substrates, due to their binding capacity, will act as competitive inhibitors for other substrates. A good example in this respect is ethacrynic acid (EA), both used as an inhibitor of GST (Ahokas et al., 1985) and as a substrate. EA is a reversible inhibitor (competitive towards CDNB and non-competitive towards GSH) toward all major rat and human hepatic isozymes (Ploeman, 1990), with strongest activity against the μ class isozymes. The glutathione conjugate of this compound is also a strong competitive inhibitor of GST isozymes (Ploeman et al., 1990). A large number of phenols have been shown to effectively inhibit GST, eg., quercitin, ellagic acid and caffeic acid. Quinones such as vitamin K and tetrachloro-1,4-benzoquinone also inhibit GST activity.

In view of the two major functions of the GSTs, i.e., the scavenging of alkylating agents and the biosynthesis of endogenous mediators such as prostaglandins and leukotrienes, the effects of inhibition of GST may be complex. For example, the anti-inflammatory drug sulfasalazine inhibits the formation of leukotriene C4 from leukotriene A4, by inhibiting both the enzyme leukotriene C synthase and several GST isozymes. Similarly, the prostaglandin derivative piriprost, known as an inhibitor of leukotriene formation, also inhibits GST. Inhibiting GST might have toxic consequences due to inhibition of prostaglandin synthesis. Increased toxicity may result from GST inhibition to normal tissues. An important, but so far largely unknown aspect in that regard, is the time course of the inhibition, and particularly, whether there is a post-inhibition rebound in GST expression.

8.11 Gene Transfer Studies

Cell-free incubation of several antineoplastic agents or their metabolites with glutathione can result in the generation of less toxic products. It has been argued that GSTs are poor catalysts because purified enzymes exhibit relatively low specific activities toward many of their xenobiotic substrates (Mannervik and Danielson, 1988) and because in some instances glutathione and electrophiles can react nonenzymatically. The importance of GSTs in the detoxification reactions can be assessed by analysis of drug resistance in cultured cells.

Manipulation of intracellular glutathione levels and GST activity have been shown to influence the sensitivity of cultured cells to alkylating agents. Depletion of glutathione with agents such as BSO can potentiate the cytotoxicity of cyclophosphamide (Tomushefsky et al., 1985) and melphalan (Somfai-Relle et al., 1987). A role for GSTs in drug detoxification is suggested more directly by experiments that show potentiation of chlorambucil toxicity by treatment of cells with inhibitors of GST (Tew et al., 1988). These data taken together offer suggestive correlation but provide no direct evidence for the role of GSTs in antineoplastic drug resistance.

Interpretation of the studies described above is complicated by the observation that resistant cells selected by prolonged drug exposure often exhibit multiple genetic and biochemical differences from the parental cell line. Other direct evidence indicating the capacity of the GSTs to protect against the toxic effects of anti-cancer drugs comes from studies involving the transfection of GST cDNAs into a variety of different cell lines.

Transfection of genes encoding human pi and alpha class GSTs confers some resistance to chlorambucil in yeast (Black et al., 1990). The influence of human GST π or rat Ya and Yb genes on resistance to alkylating agents was studied by transient (π, Ya and Yb) or stable (π, Ya) transfection of these genes into mammalian cells (Puchalski and Fahl, 1990). Expression of transfected π, α or μ class GST genes provided a modest but statistically significant decrease in sensitivity to nitrogen mustards. These preliminary

experiments support a role for certain isozymes of GST in the protection of cells against these alkylating agents.

Transfection of the human π cDNA can confer resistance to adriamycin, but not alkylators (Nakagawa et al., 1990). Transfection of a human α class cDNA into NIH-3T3 cells was shown to confer low level resistance to a range of cytotoxic drugs (Lewis et al., 1992). On the other hand, with MCF-7 as the recipient cell line, neither π nor a human liver αGST, nor rat liver Yc cDNAs confer any resistance to any chemotherapy drug (Moscow et al., 1989; Leyland-Jones et al., 1991; Bailey et al., 1992). This data may be important. However, it is also possible that the MCF-7 cells, which have amongst the lowest baseline GST in cell lines tested, represent a target that may have absent some other features required for the enzymes to function.

8.12 Chemical Induction of the Various GST Subunits

Like other drug metabolizing enzymes, GST activity can be induced by the administration of chemicals such as phenobarbitol and 3-methylcholanthrene (3-MC) or by the ingestion of food additives like butylated hydroxyanisole (Benson et al., 1978; Hayes et al., 1979; Ding et al., 1986). Recently, treatment of mice *in vivo* with the chemopreventive agent, oltipraz, was shown to induce the overexpression of GST μ and π mRNA (Clapper et al., 1993).

Relatively few studies to date have addressed the central issue of the mechanism of GST overexpression both in preneoplasia and cell lines resistant to cancer chemotherapy agents. In one case, the high level of resistance to bifunctional alkylating agents was shown to be due to amplification of alpha class GST genes (Lewis et al., 1988a). There are now several examples where the elevation in GST expression is related to increased mRNA levels. Whether this is due to message stabilization or an increased rate of transcription has not been determined. In the case of a cell line resistant to CDNB, it appears that neither transcriptional activation nor mRNA stabilization will explain the

increasd expression of the alpha class GST, indicating that protein stabilization may be involved (Wareing et al., 1992). One intriguing aspect of the studies in drug resistance both in cell lines, preneoplastic lesions and in drug priming is the concomitant overexpression of a cluster of other proteins and cofactors. This indicates that a single transcription factor or group of related factors, may be involved in the regulation of a number of genes. The identification of such a transcription factor or regulating protein and determination of whether it acts directly on the genes or whether it acts indirectly on other transcription factors remains a critical area for further study.

The variation in the distribution of GSTs, both between and within organs, suggests that complex regulating mechanisms control their expression. Little is known about the physiological control of GST. The levels of hepatic GST increase rapidly postnatally and, in the rat, reach adult levels after about 7 weeks (Hales and Neims, 1976a). Some differences in both hepatic and renal GST activity have been described (Daby et al., 1985) and limited literature exists describing the hypothalamic and pituitary modulation of the gonadal control of GST in rat liver (Hales and Neims, 1976b; Lamartinier, 1981).

Several promoter elements have been identified in the 5' noncoding region of GST and MDR genes, including phorbol ester response elements (Sakai et al., 1988) and antioxidant responsive elements (ARE). The recent identification of the ARE is of particular interest as it appears that this element is activated under conditions of oxidative stress (Rushmore et al., 1990).

Differential increases in mRNA levels coding for α subunit 1 (5-10 fold) and μ subunits 3 and 4 (5-6 fold) by phenobarbitol and 3-MC have been demonstrated (Pickett et al., 1984; Ding et al., 1986). However, at the protein level, induction is considerably smaller, and displays a different subunit selectivity, indicating that differences in translational efficiency and/or turnover rates between mRNAs coding for individual subunits may exist *in vivo* (Vos et al., 1988).

8.12.1 Alpha Class Enzymes

The differences in the tissue distribution of expression and inducibility by various drugs is distinct for Ya and Yc genes. This may be explained in part by distinct regulatory regions. Classical inducers such as phenobarbitol, 3-MC and benzo[a] pyrene enhance transcriptional rates of the Ya gene; however, the Yc gene is only minimally affected (Pickett et al., 1984). The two subunits have different gene structures. Both the 5' and 3' untranslated region nucleotide sequences are highly divergent, as opposed to the protein coding region which is highly conserved. Within the protein coding region there is an overall 75% sequence homology. In the 5' coding region, there is 85% sequence identity between the Ya and Yc cDNAs. This may represent the corresponding protein domain bearing the site of the common function of GSH binding. At the 3' end it declines to 68% (Telakowski-Hopkins et al., 1985). Whether this degree of divergence in sequence is important in alkylating agent resistance is not known, but when the cloned cDNAs are used to generate pure homodimeric Ya/Ya and Yc/Yc, significant differences in some substrate specificity are found (Huskey and Wang, 1990).

Based upon the nucleotide differences in the coding region of the Ya and Yc mRNAs along with divergent 5' and 3'-untranslated regions, the Ya and Yc subunits of the rat liver GST are derived from two different genes rather than by post-transcriptional processing of a single gene. In the rat, two Ya forms (Lai et al., 1984) in addition to the Yc form (Telakowski-Hopkins et al., 1985) have been defined by cDNA sequencing. It is estimated that there are at least two genes for Yc and perhaps 5 genes for Ya (Rothkopf et al., 1986). In Walker 256 rat mammary carcinoma cells, selective induction of Yc and not Ya was demonstrated following exposure to chlorambucil (Clapper, 1991). The Yc induction was not seen after 12-24 hr, as is seen for Ya induction with phenobarbitol, but occurred after 7 days following chlorambucil exposure. This suggests an alternate mechanism of induction of the Yc gene(s) by some drugs.

A rat liver Ya cDNA was used to clone and characterize a Ya structural gene (Telakowski-Hopkins et al., 1986). It was shown to consist of 7 exons and 6 introns and is approximately 11 Kb in length. Exons 2 and 4 of the Ya gene encode a sequence of Ya subunit that are highly conserved in the Yc subunit, suggesting similar structural and functional properties (i.e., GSH binding site). Exons 3 and 5 encode amino acids that are diverged in the Yc subunit, suggesting that they may have unique structural or functional properties (e.g., substrate binding site) (Telakowski-Hopkins et al., 1986). The regulating function has been analyzed in some detail. One region, which is required for basal level expression, has a DNA sequence with identity to the hepatocyte nuclear factor 1 (HNF1) recognition sequence. Two distinct regulatory regions have been identified in the 5' flanking region of the Ya gene that were found to be responsible for inducible expression by planar aromatic compounds and phenolic antioxidants. One of the regions has sequence identity to the xenobiotic responsive element (XRE) found in the 5' region of P450 genes. The second region is shown to be a high affinity recognition motif for a *trans*-acting factor(s) (Nguyen and Pickett, 1992). ARE was previously identified as the planar aromatic and phenolic antioxidant element. Activation through the ARE by phenolic antioxidants does not require the presence of a functional Ah receptor (Rushmore et al., 1991). The ARE is also responsive to H_2O_2, suggesting a mechanism of gene activation by reactive oxygen species (Rushmore et al., 1991).

The ARE contains a recognition motif similar to the AP-1 binding site which suggests the possible involvement of c-*jun* (which codes for AP-1) in the ARE regulatory protein complex. c-*jun* is inducible in response to H_2O_2 (Friling et al., 1990; Friling and Bergelson, 1992).

c-*jun* and c-*fos* produce *trans*-acting factors which interact with AP-1 binding sites in phorbol ester inducible genes. A pathway proposed for the alteration of gene expression by phorbol esters involves a cascade of events triggered by the activation of protein kinase C (PKC) which, by specific phosphorylation, may then modify the AP-1 DNA binding

activities of jun and fos proteins. PKC is considered the receptor protein and the major cellular protein target for the action of tumor-promoting phorbol esters.

The presence of multiple alpha-class GSTs in liver raises the possibility that each performs a unique function within the tissue. Several examples of differences in substrate specificity between alpha-class forms have been documented. Rat Ya and Yc differ greatly in their reactivity toward several substrates (Mannervik and Danielson, 1988). The human alpha-class GST subunits Ha_1 and Ha_2 expressed from cDNA clones in E. Coli had overlapping but distinct specificities even though the forms differed by only 11 amino acids (Chow et al., 1988).

The higher percentage of replacement site divergence between the rat Ya and Yc genes is consistent with the notion that rates of nucleotide substitution is higher in rodents than in man (Wu and Li, 1985). There is likely a duplication of the Ya/Yc ancestral gene in the rat genome that did not occur in the human genome. The duplicated genes of rat have diverged from each other at a faster rate than in man.

Human liver GSTs lack a mobility class equivalent to the rat liver GST Yc subunit (Mr = 28,000) yet Yc hybridizes to the Ha subunit cDNA. Human alpha class subunits are 80% identical base for base with Ya and Yc. Comparison of amino acid replacement mutations in the coding sequence reveal that the percent divergence between Ya and Yc rat genes is more than that between the Ha and Ya or Ha and Yc genes (Tu and Qian, 1986).

Southern blot analysis of the murine genome suggested the presence of at least four or five α-class GST genes (Czoshek et al., 1984). Analysis of GST protein has revealed the presence of at least two different α-class proteins in mouse liver (McLellan and Hayes, 1989; Ramsdell and Eaton, 1990). The homology between rat and mouse Yc isoforms proved to be significantly lower than for mouse and rat Ya isoforms (85% vs 95% homology) (Buetler and Eaton, 1992). An ethyoxyquin-inducible rat Yc_2 subunit with 92% sequence homology to the constitutively expressed Yc subunit has been demonstrated to have high catalytic activity for aflatoxin B_1-8,9-epoxide (Hayes et al., 1991b).

All of these findings taken together suggest that, although the Ya and Yc subunits are categorized in the same class, they appear to be functionally distinct in that the Yc isoform has a unique substrate specificity. The Yc gene may be expressed in the absence of the Ya gene, in rat brain for example (Li et al., 1986).

8.12.2 Mu Class Enzymes

Mu enzymes are highly reactive toward epoxides which are compounds often produced during xenobiotic metabolism, or, as in the case of cholesterol epoxide, produced from endogenous substrates. The adrenal gland has high levels of GSTs, which might serve to protect it from injury caused by oxygen toxicity. It has been suggested that the adrenal gland is exposed to relatively high concentrations of reactive oxygen species as a result, among other things, of its high content of cytoplasmic P450. In the adrenal gland, GSTs may influence the accumulation and secretion of steroid hormones. Hypophysectomy increases μ expression, adrenocorticotropin suppresses it (Mankowitz et al., 1990).

For class μ isozymes, a clear polymorphism has been observed in humans; isozyme μ was found to be expressed in only 60% of the samples analyzed (Seidegard and Pero, 1985). In addition to the phenotype 1-0, which is characterized by an absence of near-neutral enzymes, three other phenotypes exist, with varying activity toward the model substrate trans-stilbene oxide (Van Omman et al., 1990). Using PCR-based methods to detect gene deletions, it has been found that the deleted μ phenotype is associated with a higher risk for adenocarcinoma of the stomach, colon, and bladder cancer (Bell et al., 1992). The null phenotype is more common in smokers with lung cancer compared to controls (Seidegard et al., 1986), suggesting that the expressed phenotype offers some protection against this disease. Unlike the above cancers, the absence of a class mu GST gene has not been associated with the susceptibility to developing breast cancer (Howie et al., 1989). Studies on the regulation of GST μ have not been reported.

8.12.3 Pi Class Enzymes

The relationship between drug resistance and the expression of GST π is not clear since chemotherapeutic drugs are not known substrates for this enzyme.

The pi class isozyme has been studied extensively in human tumors because of its potential usefulness as a marker of malignant transformation. GST π is the predominant isozyme in tumors derived from colon, kidney, breast, lung, uterus, ovary, stomach and skin (Shea et al., 1988) and is often elevated in tumors relative to the surrounding normal tissue (Moscow et al., 1989; Mekhail-Ishak et al., 1989).

It is possible that the increased expression of GST π isozyme found in cells with innate resistance to anti-cancer agents and induced in cells in response to carcinogens and anti-cancer drugs is part of the generalized coordinated enzymic response of cells to transforming events. Increased expression of this isozyme would serve as a marker of neoplastic transformation rather than a selective mechanism whereby cells have acquired resistance. Such a model has been proposed to account for the induction of GST Ya isozymes by a wide variety of compounds which contain or acquire, by metabolism, electrophilic centers (Friling et al., 1990). In addition to being inducers of the Ya isozyme, these compounds are also substrates. In an aqueous environment, the nitrogen mustards undergo spontaneous dechlorination, with formation of an aziridinium ion (Stout and Riley, 1985). This intermediate species contains two electron-deficient carbons, is a putative substrate and may be an inducer of α isozymes.

GST π is not inducible by phenobarbitol or 3-MC. Expression of the π gene occurs upon neoplastic or hyperplastic transformation in rat liver (Sugioka et al., 1985). Human hepatocellular carcinomas, however, unlike those of the rat, are not generally associated with overexpression of the π isozyme (Sato et al., 1984).

Transfection experiments with genes carrying rat (Sakai et al., 1988) and human (Dixon et al., 1989) pi class promoters demonstrate considerable differences in the

transcriptional control of the rat and human genes - differences that may underlie the expression pattern of pi class GSTs in rat and human hepatocarcinomas.

The GST π gene has sequence motifs associated with "housekeeping" genes such as GC-rich regions around their promoters, and GC boxes matching the consensus sequence for their binding site of the transcription factor SP-1. In addition, it has a phorbol ester responsive element (TRE) (Cowell et al., 1988). It has been shown that c-Ha-*ras* and phorbol esters, both of which can activate the polyoma virus enhancer, act through an enhancer element closely related in sequence to TRE (Imler et al., 1988). The 5' flanking region of the Ya subunit does not appear to contain either motifs associated with "housekeeping genes".

The π gene is *ras* responsive because it can be induced *de novo* in rat liver epithelial cells following transformation with an N-*ras* gene (Power et al., 1987). The possibility that *ras* acts through TREs of cellular genes like π is relevant to the tumor-specific induction of GST π expression, as amplified or activated *ras* genes are frequently found in tumors.

9. AUTOLOGOUS BONE MARROW TRANSPLANTATION

Despite the availability of more than 15 active agents and multiple clinical trials, no curative therapy is available for the treatment of metastatic breast cancer. However, in the treatment of both metastatic disease and adjuvant breast cancer, a steep dose-response effect has been demonstrated.

It has been suggested that improved response rates and both disease-free and overall survival may be correlated with increased dose of drug delivery to women receiving treatment for breast cancer in the adjuvant and metastatic settings (Hryniuk and Bush, 1984; Hryniuk and Levine, 1986). Frequent responses are obtained in resistant breast cancer but dose escalation is limited by dose-related toxicities (Peters et al., 1986). Lack of progress in the development of effective new drugs has focused attention on dose

escalation. Positive correlations between dose and response have encouraged the use of high-dose chemotherapy. Doses are limited, however, by toxicity to normal tissues. In these circumstances, techniques such as autologous bone marrow transplantation (Au BMT) may be valuable in overcoming toxicity. The reported trials of high-dose therapy with autologous marrow support have indicated that most patient's tumors recur, however, at sites of prior bulk disease.

A single treatment with intensive combination alkylating agents with bone marrow support can produce more rapid and frequent complete responses than conventional chemotherapy when used as initial chemotherapy for metastatic breast cancer (Peters et al., 1988). Achieving a higher percentage of complete responses may be possible with further dose escalation.

Micrometastatic cancer can be cured by standard chemotherapy in the clinic. Macrometastatic disease may be less sensitive to chemotherapy because of high tumor burden with an increased likelihood of resistant cells, hypovascularity, poor perfusion of the tumor by chemotherapy, hypoxia, low growth fraction, and increased mutation rates in association with hypoxia and, therefore, a greater propensity for drug resistance.

Extensive experimental and clinical evidence (Skipper and Schmidt, 1962; Frei and Caneltos, 1980) supports the notion that higher doses of cytotoxins can kill a larger fraction of the tumor. Among the active cytostatic agents suitable for delivery in high dosages are a number of alkylating agents, notably melphalan, cisplatin, thiotepa and BCNU (Souhami et al., 1983; Lazarus et al., 1983). One of the most widely active cytostatic drugs when applied in normal dosage is doxorubicin, but dose escalation is limited because of mucositis and cardiotoxicity.

The alkylating agents have been the major chemotherapeutic agents employed in bone marrow transplantation, primarily because of their broad spectrum of anti-tumor activity and because myelosuppression is dose-limiting. Among the solid tumors seen in adults, breast cancer is one of the most responsive to chemotherapy. A steep dose-response curve

is exhibited by many of the agents used to treat this disease, making it an attractive target for high dose chemotherapy with AuBMT (Steel, 1977; Tormey, 1984). For MCF-7 cells, the dose-response curve has been analyzed over multiple logs of depletion of stem cells. The reduction in stem cell viability was linear on a semilog plot for alkylating agents. For non-alkylating agents, the corresponding plot was curvilinear, with a substantial loss in activity after the first two to three logs of cell reduction because of resistance (Frei and Caneltos, 1980). The optimal agent for a curative regimen should maintain fractional tumor cell kill through multiple logs (i.e., straight line on a semilog plot).

Myelosuppression is a common and serious complication of the treatment of cancer because most chemotherapeutic agents lack specificity for malignant cells. The use of autologous bone marrow transplantation has allowed the development of more intensive and effective therapy for a variety of neoplasms by overcoming myelotoxicity that would otherwise limit doses, morbidity and mortality are high during the nearly 3-week period required for marrow engraftment and hematopoietic reconstitution (Souhami et al., 1983).

Three- to ten-fold the standard dose of alkylating agent can be delivered in the presence of bone marrow transplantation (Frei and Caneltos, 1980). Because resistance to alkylating agents is at best low level, this degree of dose escalation with AuBMT might be capable of eradicating even the resistant tumor cell population.

The solid tumors suitable for these types of study are those that exhibit substantial response rates to standard doses of chemotherapeutic agents appropriate for dose escalation with AuBMT. Tumors that fulfill these criteria include small cell lung cancer, metastatic breast cancer, and advanced ovarian cancer.

If it is assumed that one dose of an alkylating agent can produce 1/2 log cell kill and BMT allows a 6-fold increase in dose of alkylating agents, the use of the two together would produce three logs of kill. With the combination of three active alkylating agents delivered at full transplant doses, assuming no cross-resistance, 9 log kill would occur. Patients with metastatic breast cancer have, at time of presentation, approximately 10^{11}

cells. The growth fraction for metastatic breast cancer is approximately 5-10%. This could eradicate both sensitive and resistant cells.

In vivo and *in vitro* studies have suggested that the combination of BSO and melphalan does not increase the myelotoxic effect of melphalan (Ozols et al., 1987b; Dorr et al., 1986; Russo et al., 1986). However, in an *in vivo* myelotoxicity study with mice, BSO pretreatment markedly inhibited the recovery of peripheral leukocytes at the melphalan 10% lethal dose (Kramer et al., 1987). Similar results were found in patients treated with BSO followed by administration of melphalan. These clinical studies demonstrated depletion of GSH in both normal and tumor tissue (O'Dwyer et al., 1992). In addition, a high dose of BSO appeared to increase non-hematopoietic toxicities (hepatotoxicity or cardiotoxicity) of melphalan (Soble and Dorr, 1987). These studies suggest that the potentiation of melphalan myelotoxicity may preclude successful clinical studies of this combination. In another study, the myelotoxic effect of melphalan were not severely enhanced by L-BSO. Modest potentiation of melphalan myelotoxicity was observed, although not significantly enough to preclude successful clinical studies of this combination (Du et al., 1990).

Increasing GST in bone marrow may be of therapeutic value in the treatment of patients with breast cancer. If GST leads to a dose modifying factor in cancer patients such that the effective dose of alkylating agents or cisplatin can be increased 3- to 4-fold, then it may represent an important new therapeutic modality. The relevance of dose increases of this magnitude or less have been previously demonstrated in phase II trials of high dose therapy with either cisplatin or the non-nephrotoxic cisplatin analog carboplatin in refractory ovarian cancer patients (Ozols et al., 1985). In these trials the dose of platinum drugs was doubled and administered to patients who had progressive disease or standard dose cisplatin regimens. A 2-fold increase in the dose of cisplatin or carboplatin produced objective response rates of 32 and 35%, respectively, and a prolongation of survival was observed in those patients responding to treatment. A more recent study (Dunphy and

Spitzer, 1992) demonstrated that there is a subgroup of stage IV breast cancer patients with poor prognoses (estrogen-receptor-negative) who achieve extended disease-free survival after high dose treatment, which may not be expected with standard-dose chemotherapy. Mortality rates in AuBMT trials have been significantly reduced with supportive care which includes administration of hematopoietic growth factors and antibiotics. These AuBMT studies support this approach as being an encouraging prospect for improved long-term disease-free survival.

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CHAPTER 2

IN VIVO AND *IN VITRO* MECHANISMS OF DRUG RESISTANCE IN A RAT MAMMARY CARCINOMA MODEL

PREFACE TO CHAPTER II

Exposure of tumor cells to cytotoxic chemotherapeutic agents results in the development of cellular resistance.

This chapter describes the biochemical and molecular phenotype associated with the development of chemotherapy resistance. Among these biochemical phenotypes are included the ubiquitous molecule, glutathione, and its corresponding conjugating enzyme glutathione-S-transferase. Elevations of these two cellular components were considered to be indicators of the development of resistance to some chemotherapy drugs, especially those whose mechanism of action is mediated by DNA alkylation.

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ABSTRACT

Many *in vitro* tumor models have been examined to help understand the precise mechanisms responsible for drug resistance. The importance of these results *in vivo* remains uncertain. MatB 13762 is a rat mammary adenocarcinoma cell line that can be grown both *in vitro* and as a solid tumor in Fisher 344 rats, thus permitting the examination of tumor cell drug resistance under both conditions. Two cell lines have been selected *in vitro* for resistance to adriamycin (Adr^R) and melphalan (Mln^R) respectively. Each subline has the following features; Adr^R : increased MDR-1 mRNA, a high level of cross-resistance to vincristine and atypical low level resistance to melphalan and BCNU, decreased cellular glutathione content and increased expression of Yc and Yp glutathione-S-transferase (GST) isozymes; Mln^R: low level drug resistance to melphalan and cross-resistance to BCNU, adriamycin and vincristine, increased cellular concentration of glutathione, elevated GST activity as well as greatly increased mRNA specific to the Yc and Yp GST subunits. Most of the biochemical and molecular features described above are present but significantly less prominent in tumors grown *in vivo* . This model provides the opportunity to examine the magnitude of expression and the clinical significance of *in vitro* resistance in an *in vivo* model.

INTRODUCTION

Most metastatic or unresectable tumors are incurable. The existence or emergence of drug-resistant cells limits the effectiveness of chemotherapy. Breast carcinoma is an example of a tumor that generally has an initial response to chemotherapy but the duration of response is limited and recurrence is inevitable. The emergence of this acquired resistance is not avoided by treating with combination chemotherapy which includes a number of drugs with different chemical structures and mechanisms of action. Cross-resistance of tumors to a number of different drugs is therefore a common clinical feature.

Many *in vitro* experimental models have been developed to better understand the mechanisms of drug resistance (Zwelling et al., 1981; Suzukake et al., 1983; Batist et al., 1986; Paston and Gottesman, 1987; Slovak et al., 1988). In general, these human and animal-derived drug resistant cell lines express a phenotype that is characteristic for resistance to a particular class of drugs. One common experimental model of drug resistance is termed multi-drug resistance (MDR). Selection of mammalian cells *in vitro* for resistance to any one of a limited group of lipophilic antineoplastic agents results in cross-resistance to other distinctly different members of the group. Alkylating agents are specifically not among these drugs. The MDR phenotype is associated with decreased intracellular drug accumulation resulting from energy dependent drug efflux (Riordan and Ling, 1985; Roninson et al., 1984; Tsuruo, 1988). A high molecular weight membrane-associated glycoprotein called P170, and encoded by the MDR-1 gene, forms this pump. Compounds such as verapamil inhibit P-glycoprotein function and result in increased drug accumulation and sensitivity to cytotoxicity. Enhanced MDR-1 expression may be due to amplification of the gene (seen most commonly in *in vitro* models) or increased transcription of a single copy gene (seen in clinical specimens) or a combination of both (Roninson et al., 1984; Riordan et al., 1985; Van der Bliek et al., 1986).

Studies have shown elevated MDR-1 expression in some clinical specimens of both solid and hematologic malignancies including ovarian ascites cells (Bell et al., 1985), acute non-lymphoblastic leukemia (Ma et al., 1987), colon cancer (Dalton et al., 1988) and breast cancer (Fuqua et al., 1987). However, there is not a consistent relation between the level of expression in these tissue specimens and clinical resistance to chemotherapy. While MDR-1 expression is a feature of some *de-novo* resistant tumors, (eg. colon cancer) it is also apparently absent from others (eg. lung carcinoma and melanoma), (Goldstein et al., 1989). In addition, MDR-1 expression is a common feature of a number of normal tissues with secretory or membrane transport functions (eg. colon mucosa, adrenal, blood-brain barrier), so it may play an important role in normal physiological function (Fojo et al., 1987). Finally, there appears to be a relation between cellular differentiation and MDR-1 expression, such that" tumor stem cells ", the target of anticancer therapy, have lower MDR-1 expression. These findings have important potential implications for the strategy of targeting P-glycoprotein as a part of cancer therapy. Other potentially important features of multidrug resistant cells have also been described, including: (i) enhanced cellular defense against xenobiotic toxicity resulting from glutathione-S-transferase (GST) (Batist et al., 1986; Ozols et al., 1987; Deffie et al., 1988), increased glutathione (Ozols et al., 1987) and increased glutathione peroxidase (Batist et al., 1986; Myers et al., 1977); (ii) decreased levels or altered structure of topoisomerase II (Ross et al., 1988); (iii) and increased drug metabolism to non-cytotoxic products (Vasanthakumar and Ahmed, 1986).

Drug resistance has also been described in a number of cell lines selected for resistance to alkylators and nitrogen mustards. Resistance to these agents has been associated with alterations in cellular glutathione (GSH) content and GST activity. Both human ovarian cancer cells as well as a rat brain tumor cell line selected for resistance to nitrogen mustards have increased GSH concentration (Green et al., 1984; Evans et al., 1987). Buthionine sulfoxamine (BSO), which depletes cellular GSH concentration, results in sensitization of tumors to melphalan *in vitro* and *in vivo* in both parental and resistant

tumor cells (Hamilton et al., 1985; Evans et al., 1987; Kramer et al., 1987; Ozols et al., 1987). Other proposed mechanisms of alkylator resistance include overexpression of specific GST isozymes, altered cellular drug accumulation and metabolism or enhanced removal of DNA crosslinks (Batist et al., 1989). Both rat mammary carcinoma cells and CHO cells selected for resistance to chlorambucil have significantly higher concentrations of an alpha class form of GST in association with slightly increased GSH concentration (Evans et al., 1987; Buller et al., 1987; Lewis et al., 1988). Moreover, recent reports of experiments in which full length cDNAs of the alpha GST gene family transfected into yeast (Puchalski and Fahl, 1990) and the pi form transfected into mammalian Cos cells and NIH-3T3 (Black et al., 1989; Nakagawa et al., 1990), provide direct evidence for GST's role in alkylator and adriamycin resistance, respectively. On the other hand, transfection of GST pi into a human breast cancer cell line did not confer resistance to either alkylators or adriamycin (Moscow et al., 1989; Fairchild et al., 1990). In addition, a recent study of tumor cells selected for alkylator resistance *in vivo* suggests that entirely different mechanisms are operative which are not even apparent *in vitro* (Teicher et al., 1990).

The precise mechanism(s) responsible for resistance to the nitrogen mustards and the magnitude of clinical expression and functional significance of the proposed mechanisms of MDR resistance *in vivo* are not certain. The studies reported here describe biochemical and molecular mechanisms associated with drug resistance in a rat mammary carcinoma cell line. MatB mammary tumors have many features consistent with the human disease. Some of these include responsiveness to natural product antineoplastics and alkylating agents, solid tumor vascularization and metastases to regional lymphatics. This model, therefore, provides a potentially useful preclinical system in which to study drug resistance and evaluate appropriate maneouvers to circumvent this.

MATERIALS AND METHODS

1. Cell Growth *in vitro* and *in vivo*

MatB 13672 is a cell line derived from a female Fischer rat mammary tumor. Cells grow both *in vitro* and *in vivo* . Wild type (WT) MatB cells are grown *in vitro* in alpha Minimal Essential Medium (Gibco) (supplemented with 1.3% sodium pyruvate, 2.6% glutamine 1.3% non essential amino acids) containing 10% fetal bovine serum and 100,000 units/liter gentamycin. Adriamycin-resistant (Adr^R) MatB cells were selected by exposing the cells to escalating drug concentrations beginning at 10^{-10} M adriamycin. The final resistant subline was established when cells were able to survive in 10^{-6} M adriamycin. Cells are maintained in medium containing this concentration of adriamycin. Similarly, a melphalan-resistant (Mln^R) MatB subline was selected in escalating doses beginning at 10^{-8} M melphalan. Cells surviving at 10^{-5} M melphalan are maintained at this concentration.

Both drug resistant sublines were passaged in drug free media for at least 2 weeks prior to use in studies of drug sensitivity, enzyme assay and nucleic acid analysis. The WT and resistant MatB sublines were grown as solid tumors in 10-12 week old Fischer 344 rats. After an injection of 5×10^5 cells subcutaneously a solid mass is palpable within two weeks. On post-mortem examination metastases to regional lymph nodes are present.

2. Drug Sensitivity Assays

Logarithmically growing cells were harvested by gently tapping the side of the flask (in fresh media) and plated in triplicate into 6-well plastic tissue culture dishes (Nunc) at a density of 400 cells/well in a total volume of 5 ml containing 0.35% agar, 20% fetal bovine serum, 25% 2 × alpha Minimal Essential Media (MEM) and 34% alpha MEM.

Varying amounts of drugs were added to the dishes. Melphalan was freshly prepared for each assay in acid-alcohol. The cells were incubated under 5% CO_2 at 37°C for 10-14 days without changing medium. Colonies containing more than 50 cells were counted. The

cellular drug resistance was expressed as the IC_{50} value which is the drug dose resulting in 50% of colonies surviving compared to the number in the untreated samples.

Drug resistance levels *in vivo* of Mln^R cells was determined from tumor growth delay experiments. Rats were transplanted with either 5×10^5 WT or Mln^R cells. When the tumors were palpable, three groups of three animals each were treated with a single i.v. injection of melphalan at either; 0.5, 1.5., or 3.5 mg/kg for WT tumor bearing animals or 3.5, 5.0, or 6.5 mg/kg for Mln^R tumor bearing animals. Tumor size (L × W) was measured every other day following drug treatment. Six animals per group bearing either WT or Adr^R tumors were treated with a single i.v. injection of 20 mg/kg adriamycin. Twenty-four hours after treatment, tumors were removed and disaggregated in 0.05% collagenase (Sigma Chemical Co.) in Hanks Balanced Salt Solution (Gibco). Viable cells were counted based on their ability to exclude the dye, trypan blue. Their ability to form colonies *in vitro* was examined in triplicate.

3. Materials and Reagents

Melphalan, vincristine and BCNU were purchased from Sigma Chemical Company. Adriamycin was purchased from Adria Laboratories. Guanidinium isothiocyanate and cesium chloride were from IBI. DNA size standards were from Boehringer Mannheim. Hybond-N membranes and deoxycytidine 5'[alpha 32p] triphosphate (3000 Ci/mmol) were from Amersham. SDS polyacrylamide gel reagents including secondary antibodies, molecular standards, and nitrocellulose membranes were from Bio Rad.

4. Tissue Fractions and Enzyme Assays

Tissues were harvested immediately after sacrifice of the animals and were dissected free of necrotic or hemorrhagic material. Tissue fractions were prepared at 4°C. The tissues were weighed, homogenized with a Polytron homogenizer in a 10% solution containing 0.25 M sucrose, 150 mM KCl and 50mM Tris, pH 7.4. The homogenate was spun at

10,000 x g for thirty minutes. The remaining supernatant was centrifuged at 100,000 × g for 1 hr and the supernatant (cytosol) was assayed for GST using CDNB according to the method of Habig et. al. (1974). Total GSH was assayed according to the technique of Ellman (1959) in tissue homogenized in 3% sulfosalicylic acid. Se-dependent GSH peroxidase activity was assayed using H_2O_2 and Se-independent GSH peroxidase activity was assayed using cumene hydroperoxide according to the method of Paglia and Valentine (1967). Protein concentrations in all fractions were determined by the method of Lowry. Students t-test was used to assess the degree of significance between the different tissue samples assayed for enzyme activity.

5. Western Blotting

Polyacrylamide gel electrophoresis was performed according to the method of Laemmli. A 4% stacking gel was layered over a 12% resolving gel. Fifteen ug of protein from each sample was allowed to stack under 100 milliamps of current, the remaining running time was under 80 milliamps. The protein trapped in the gel was transferred onto zeta-probe membranes by electrophoretic transfer for 3 hours at 50 volts. The membrane was then blocked in 5% skim milk/PBS for 1 hour at room temperature and subsequently reacted with the selected polyclonal antisera (anti pi; 1:250 dilution in PBS, anti alpha-mu; 1:500 dilution in PBS) overnight at room temperature. Following three washes with wash buffer (0.05 M phosphate, 0.5M NaCl, 0.1% Tween 20, pH, 8.0), a goat anti-rabbit horseradish peroxidase conjugated second antibody was added at a 1:2000 dilution in PBS. The blots were then incubated with diaminobenzidine and the sites of antibody binding were revealed by a brown precipitate. Glutathione-S-transferase is a multi-enzyme family encoded by three distinct gene families termed alpha (Ya and Yc) subunits, mu (Yb1, Yb2, Yb3) and pi (Yp). Dimers of subunits from each family form the functional enzyme. Rat liver cytosol was used as a control for Ya, Yb and Yc subunits and purified human GST π is the positive control for the Yp GST subunit.

6. Isolation of Nucleic Acids and Hybridization Studies

Approximately 1×10^8 cells were grown, harvested, and washed in PBS. The cells were then resuspended in 10 mls of PBS. To that volume, NaCl and SDS were added to achieve a final concentration of 0.5 M and 0.5% respectively. The mixture was put on ice for 10 minutes and the DNA containing aqueous phase was extracted three times with an equal volume of phenol equilibrated with TE (10mM Tris - HC1, pH 8.0, 1mM EDTA, pH 8.0). The remainder of the protocol was performed essentially as described (Maniatis et al., 1984). The DNA was verified to be of high molecular weight by agarose gel electrophoresis.

Tissue DNA was prepared by pulverizing two to three grams of frozen tumor (cleaned free of necrotic tissue and blood) in liquid nitrogen, to a fine powder. One per cent SDS in RSB buffer (10mM Tris, pH, 7.4, 10 mM NaCl, 25mM EDTA) released DNA from nuclei. Purified DNA was further prepared according to Maniatis, et. al. (1984). The final DNA pellet was dissolved in TE to a final concentration of 2-3 ug/ul.

Total RNA was extracted from cultured cells and pulverized tumor tissue by homogenizing in guanidinium isothiocyanate followed by centrifugation over a cesium chloride cushion. Twenty ug of total RNA was electrophoresed in 1% agarose/6% formaldehyde gels as described (Maniatis et al., 1984). Hybridization was in 1% BSA, 0.1 mM EDTA, 0.5 M NaHP04, 5% SDS, 50% formamide for 18 hours at 42°C. The blots were washed (15 min. each time) by addition of 1 x SSC (0.15M NaCl, 15mM sodium citrate), 0.1% SDS at room temperature 4 times, followed by 4 washes at 65°C. For subsequent hybridizations, membranes were boiled in 0.01 x SSC, 1% SDS 4 times for 20 minutes each to remove previously used probes.

DNA probes were labeled *in vitro* with ^{32}P-dCTP by nick translation essentially as described (Maniatis et al., 1984). The rat cDNA GST probes used in these studies were the following: Yp-SalI/Eco RI insert of plasmid pGP5 (kindly provided by Dr. M. Muramatsu), and Ya/Yc and Yc-PstI inserts of plasmids PGTB38 and PGTB42,

Dr. C. Pickett). Hybridizations were also performed with an Eco RI generated MDR-1 pCHP1 cDNA insert. The levels of gene expression were determined by densitometry of the autoradiograms.

RESULTS

1. Drug Sensitivity Studies

The drug sensitivities of the wild type and drug resistant sublines determined in clonogenic assays are shown in Figure 1. Each point represents the mean of at least three separate experiments. The standard error for each determination is less than 10%. The Adr^R cells are 200-fold more resistant to adriamycin than the WT cell line (Fig. 1A). The stability of resistance was determined by cloning Adr^R cells passaged in drug-free media for 7 months. After this period of time these cells displayed 166-fold resistance to adriamycin. The Adr^R cells are 230-fold resistant to vincristine, (Fig. 1B). In addition, Adr^R cells show 2-fold resistance to melphalan (Fig. 1C) and 2.4-fold resistance to BCNU (Fig. 1D). This is consistent with other reports of primary resistance to doxorubicin associated with cross-resistance to alkylating agents (24).

The survival curve for Mln^R cells demonstrates a 10-fold level of resistance to melphalan (Fig. 1C) and 17-fold resistance to BCNU (Fig. 1D). Mln^R cells appear to be marginally cross-resistant to members of other drug classes. Figure 1A reveals that Mln^R cells are 2.3-fold resistant to adriamycin and they are 2-fold resistant to vincristine (Fig. 1B). There is little change in the resistance to melphalan seen in the Mln^R cells over a period of 7 months (10-fold vs. 9).

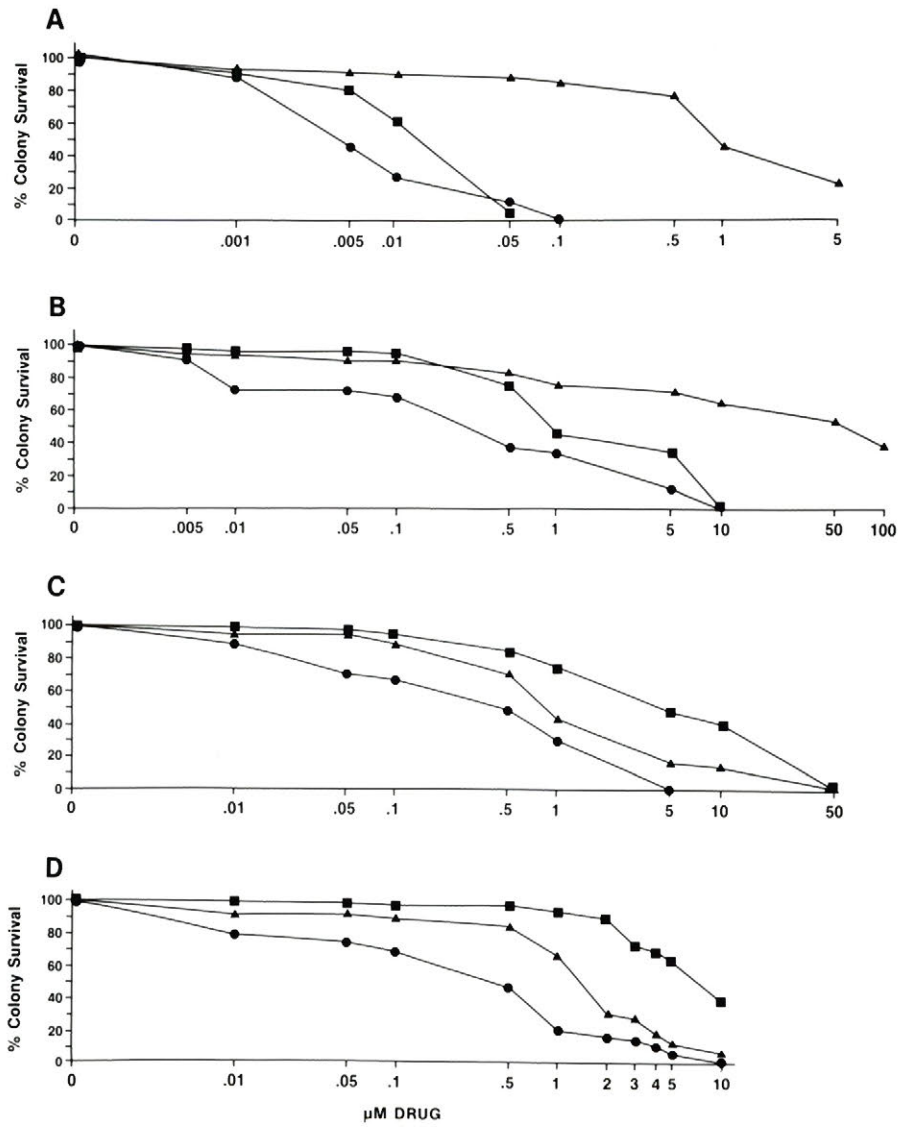

Figure 1. Survival patterns of WT and resistant sublines in A) adriamycin, B) vincristine, C) melphalan, D) BCNU. (●) WT (▲) Adr^R (■) Mln^R. Values are the means of three experiments, with the SE less than 10% of the reported values.

2. *In vivo* Tumors

Pathological examination of a MatB tumor indicates that this tumor is a poorly differentiated mammary adenocarcinoma. Hormone receptor assays for both estrogen and progesterone are negative, similar to high grade poorly differentiated human breast cancer malignancies. Preliminary studies of drug sensitivity of the sublines have been performed *in vivo* . Animals were injected with either the WT or the Mln^R cells (Fig. 2). After two weeks tumors were palpable. At this time, groups of at least three rats were treated with a single injection of melphalan administered i.v. via the tail vein. Tumor size was measured every other day. Using tumor growth delay it was possible to determine the fold resistance to melphalan of Mln^R cells *in vivo* . In WT tumor bearing rats 1.5 mg/kg melphalan resulted in prolonged tumor growth delay or complete disappearance of the tumor over three weeks. In Mln^R tumor bearing animals there was a tumor response in one third of animals treated with 3.5 mg/kg. At higher doses there was considerable toxicity but the one surviving animal treated with 6.0 mg/kg melphalan also had disappearance of the tumor. We conclude that *in vivo* resistance of Mln^R cells is 2-4-fold. Need data

Preliminary studies with i.v. adriamycin have demonstrated that the dose required to affect cell growth (even WT tumors) is greater than the maximal tolerated dose of 7.5 mg/kg. Excisional tumor assays were performed to overcome this problem. In these experiments animals bearing either WT or Adr^R tumors were treated with a single dose of adriamycin (20 mg/kg) administered via the tail vein. Twenty-four hours after treatment, tumor cells were removed from the rats and their ability to form colonies *in vitro* was examined. Resistance was measured as the surviving fraction relative to untreated controls. Tumor cells from drug treated WT tumors formed 7.6% colonies relative to untreated cells (25.5 ± 2.56 vs. 334 ± 12.9). Adr^R tumor cells treated with adriamycin had a 73% colony survival fraction relative to untreated cells (51 ± 6.8 vs. 70 ± 9.1). The Adr^R subline is approximately 10-fold resistant to adriamycin at 20 mg/kg.

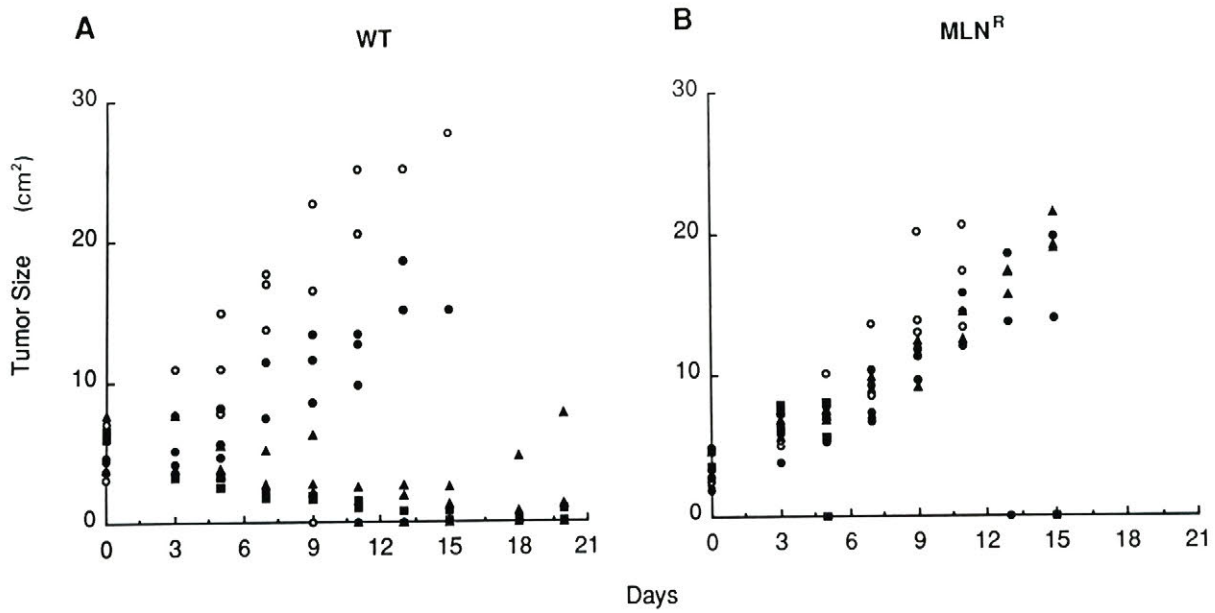

Figure 2. Turnover growth rate of tumors treated *in vivo*. WT tumor bearing animals received either no drug (O), 0.5 mg/kg (●), 1.0 mg/kg (▲) or 1.5 mg/kg melphalan (■). Mln^R tumor bearing animals received either no drug (O), 3.0 mg/kg (●), 5.0 mg/kg (▲) or 6.0 mg/kg melphalan (■). Each point represents one animal; at least three animals were used per drug dose.

3. Biochemical Characterization of WT and Resistant Sublines

The enzyme activities (mean ± SE) examined in cultured cells and tumor tissue derived from tumor bearing animals is shown in Table 1. There is no apparent difference in Se-dependent glutathione peroxidase activity between the MatB cell lines *in vitro* or in the tissue samples. Selenium-independent glutathione peroxidase activity is 3.7-fold increased in activity in Mln^R cells relative to WT cells *in vitro* and 1.7-fold increased in activity in a sample prepared from a Mln^R solid tumor. Mln^R cells *in vitro* have a 4.7-fold increase in GST activity, whereas activity measured in Mln^R tissue is increased 2.7-fold relative to WT tissue. For Adr^R cells grown both *in vitro* and *in vivo*, GST activity is increased 1.5-fold.

Glutathione was measured in cultured cells at plateau phase of growth, since this has previously been shown to correlate best with tumors *in vivo* (Lee et al., 1988; Lee et al., 1989). Adr^R MatB cells have a 67% reduction in total GSH concentration relative to WT cells while in Mln^R MatB cells the GSH concentration is almost double. In contrast to the Adr^R cells grown in tissue culture, those grown in an *in vivo* environment have a 2.2-fold increase in GSH concentration. Mln^R cells *in vivo* have a 2.8-fold increase in GSH concentration.

4. Western Blot Analysis

In both Adr^R and Mln^R cells *in vitro*, there is an increase in the amount of a protein whose immunoreactivity is consistent with the Ya subunit of GST (Fig 3). A larger protein consistent with the Yc subunit is present at extremely low levels in WT cells (not obvious from the photo reproduction), whereas the Yc subunit is increased in Adr^R and even more so in Mln^R cells.

WT tumor tissue, like cultured cells, express predominantly Ya protein. The Adr^R and Mln^R tissue samples have a slightly increased level of Ya protein, however. These samples also demonstrate the presence of the Yc subunit which is present in greater

TABLE 1. ENZYME ACTIVITIES IN WT AND RESISTANT SUBLINES *IN VITRO* AND *IN VIVO*

The results are expressed as the means ± SE of at least three separate determinations.

Enzyme	*In vitro*			*In vivo*		
	WT	Adr^R	Mln^R	WT	Adr^R	Mln^R
GSH peroxidase						
Hydrogen peroxide[a]	2.0 ± 0.8	2.1 ± 0.3	2.4 ± 0.3	4.2 ± 0.5	4.1 ± 0.5	5.6 ± 0.1
Cumene hydroperoxide[a]	6.2 ± 0.1	8.0 ± 0.1	23 ± 0.1[b]	2.0 ± 0.1	2.0 ± 0.1	3.4 ± 0.1[b]
Glutathione S-transferase[a]	7.9 ± 0.2	11.8 ± 0.5[c]	36.8 ± 1.2[b]	5.3 ± 0.3	5.3 ± 0.3	14.4 ± 1.0[b]
Glutathione[d]	48 ± 2.7	15.6 ± 1.9	88 ± 3.4	177 ± 2.8	392 ± 3.5	498 ± 12.7

a nmol/min/mg protein.
b Significantly different from WT value, $P < 0.001$.
c Significantly different from WT value, $P < 0.05$.
d nmol/mg protein.

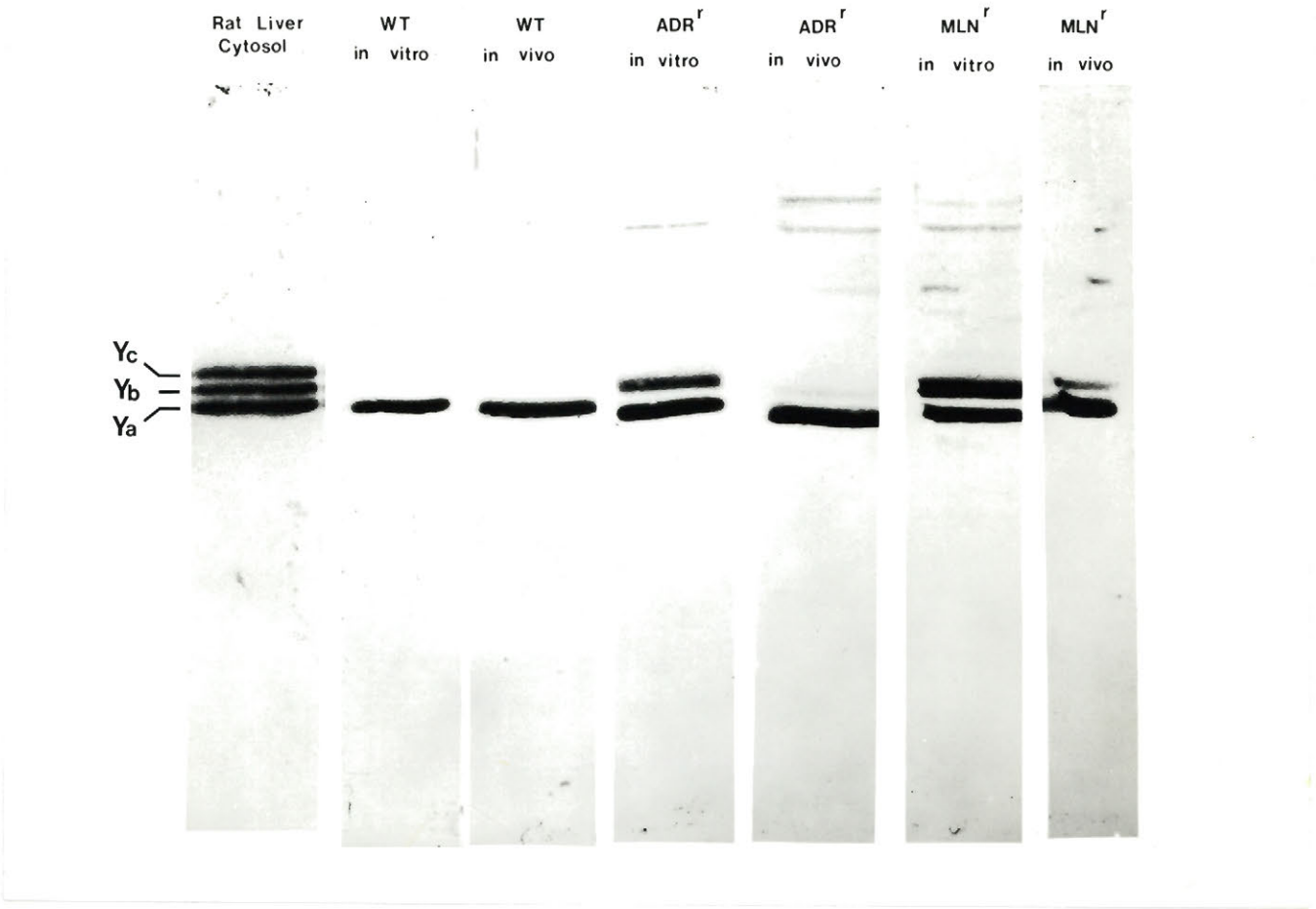

Figure 3. Western immunoblotting of cytosolic preparations of MatB cell lines (15 μg each). Lane 1 is a cytosolic preparation of normal rat liver containg Yc, Yb and Ya GST subunits. Protein was resolved on a 12% SDS-polyacrylamide gel, transblotted and reacted with polyclonal rabbit anti-GST alpha-mu antibody.

amounts in the Mln^R tumor sample. Thus, drug-sensitive MatB tumor cells, whether *in vitro* or *in vivo*, contain the Ya subunit as the principle glutathione transferase isozyme. However, drug resistant sublines express an altered GST pattern. This *in vitro* change is present, but to a lesser degree *in vivo* . None of the sublines demonstrated the presence of a band corresponding to the Yb subunit. Northern analysis, using a Yb specific cDNA probe revealed a weak signal that was equivalent in all sublines (data not shown). Thus, Yb protein levels may not be detectable using our antisera.

Reaction of the same samples with a polyclonal antibody directed against the Yp subunit is shown in Figure 4. In Adr^R cells *in vitro* , Yp levels are unchanged, whereas Mln^R demonstrate greatly increased amounts relative to WT cells. There is no significant change in the concentration of Yp protein levels in the resistant sublines *in vivo* versus WT tumor tissue.

5. Expression of MDR and GST mRNA

Radiolabeled glutathione-S-transferase cDNA probes were hybridized to total RNA prepared from cell lines *in vitro* and their corresponding solid tumors *in vivo* . Figure 5A illustrates the results obtained when an alpha gene family probe (Ya/Yc) is used. The amount of mRNA is greatest in the Mln^R cells *in vitro*. The signals *in vivo* reveal that the Mln^R cells express more Ya/Yc than Adr^R tissue. This data corresponds with protein quantitation results obtained in Figure 3. The same membrane was then stripped and rehybridized with an actin probe to control for variations in RNA loading. This figure (Fig. 5B) serves as the control for all northern analysis. When a Yc specific cDNA is used (Fig. 6) , a similar pattern is observed. Both resistant sublines *in vitro* have increased Yc mRNA ($Mln^R > Adr^R$). This is apparent *in vivo* as well, but to a lesser extent in both cell types. Densitometric quantification of the hybridizing bands demonstrates that GST-Yc is increased 14-fold *in vitro* versus 3-fold *in vivo* in Mln^R cells. All of these findings coincide with the data presented in Figure 4. In the Adr^R cells Yp expression is unchanged

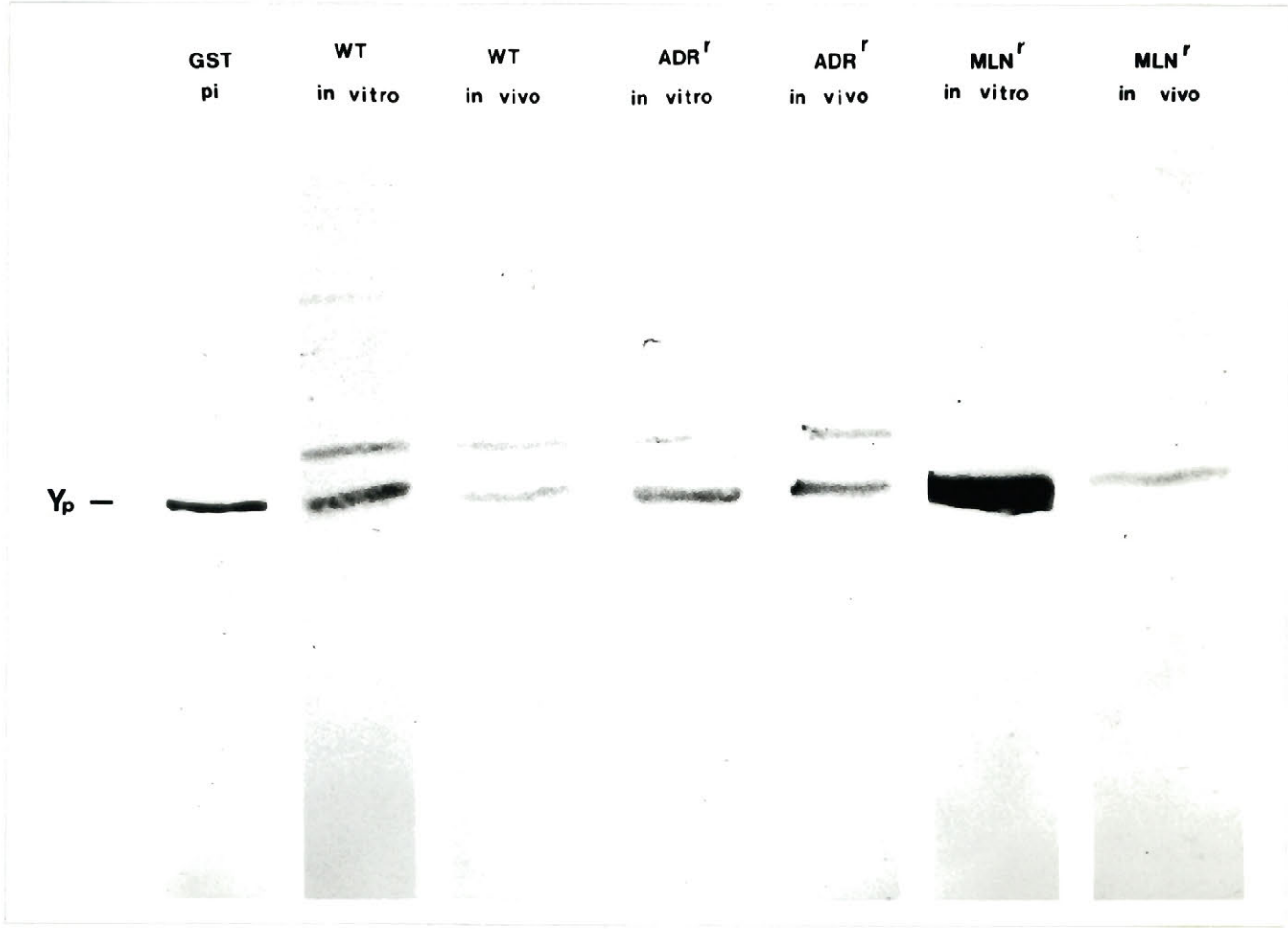

Figure 4. Western immunoblotting of cytosolic preparations of MatB cell lines (15 μg each). Lane 1 is purified human GST pi. Samples were reacted with a polyclonal rabbit anti-GST pi antibody.

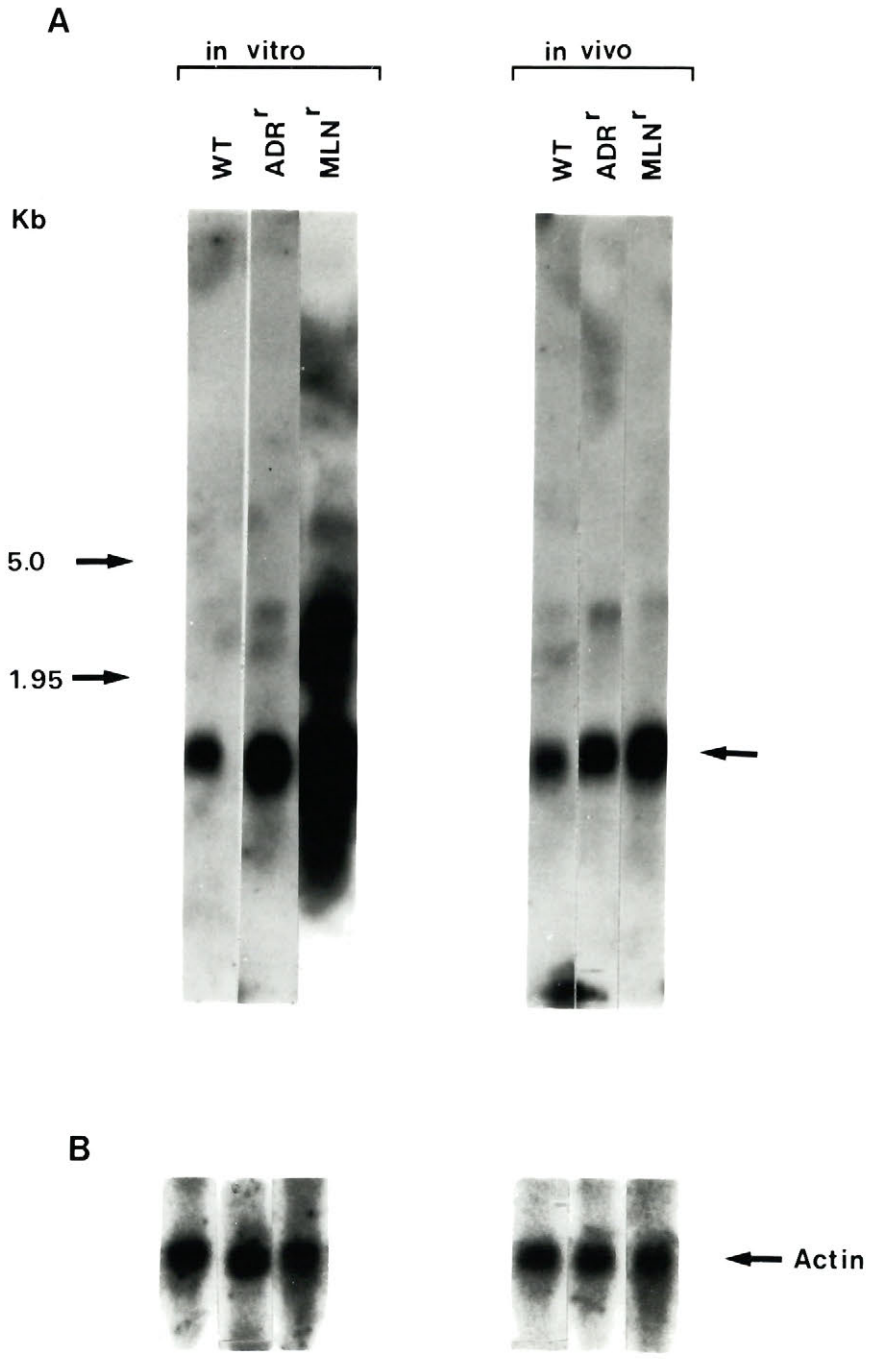

Figure 5. Northern blot analysis of: **A.** Ya/c expression. Denatured RNA (20 μg) was subjected to electrophoresis in a 0.8% agarose gel. Following transfer onto Hybond N membrane, hybridization was performed with the ^{32}P-labeled insert of probe pGTB38 prior to auto-radiography for 2 days. Arrows at left, positions of the 28S and 18S ribosomal bands; arrows at right, position of the hybridizing band, and **B.** Hybridization with actin to standardize the amount of RNA in each lane.

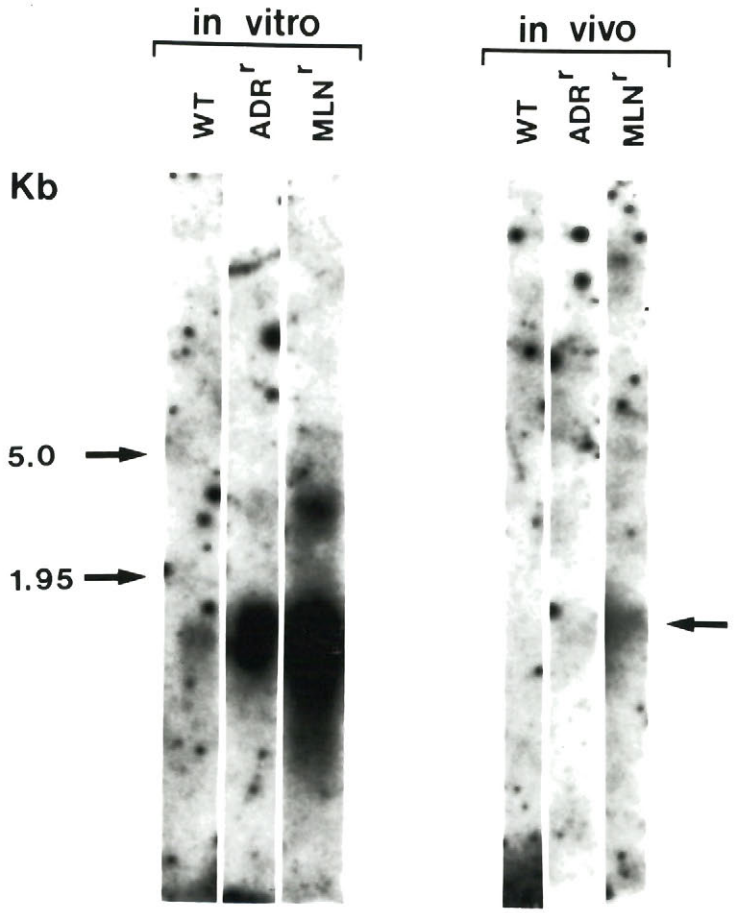

Figure 6. Northern blot analysis of Yc. Hybridization was performed with the ^{32}P-labeled insert of probe pGTB42. Arrow at right indicates position of hybridizing sequence.

from WT cells (Fig. 7). In Mln^R cells Yp expression is increased 10-fold consistent with the results of Western analysis (Fig.4). In both resistant sublines grown *in vivo* there is no change in Yp specific mRNA levels relative to WT tissue.

To assess expression of MDR-1, the steady state level of RNA containing related sequences was analyzed with the 32P-labeled complementary DNA subclone pCHP1, Figure 8. Adr^R cells grown *in vitro* express high levels of a 4.5 Kb mRNA that hybridizes to the probe. Examination of RNA prepared from Adr^R tissue also demonstrates the presence of the MDR-1 transcript, however, the level of this expression is 5-fold lower than that observed *in vitro* . MDR-1 RNA sequences are not present in any Mln^R cells.

6. Analysis of Gene Amplication in Wild Type and Resistant Sublines

To determine whether the adriamycin resistant subline has amplified MDR-1 gene, we analyzed DNA from cells grown both *in vitro* and *in vivo*. Figure 9 shows autoradiograms of a Southern blot analysis of DNA digested with HindIII and probed with a 680-base-pair insert from the pCHP1 plasmid containing a segment of the hamster MDR-1 gene. There is no evidence of rearrangement of this gene and a gene amplification (2-fold) is seen in Adr^R cells grown both *in vitro* and *in vivo* (Fig. 9A). The actin control for this blot is shown in Figure 9B.

The increased expression of glutathione-S-transferases, particularly in the Mln^R cells, is not associated with amplification of these genes (data not shown). Southern analysis of the resistant and drug-sensitive parental cell line did not reveal any gene rearrangement (data not shown).

7. *In vitro/in vivo* Differences in Gene Expression

It is not possible to attribute the *in vivo/in vitro* differences to a dilutional effect since the amount of stromal infiltration seen on light microscopy is extremely low (less than 5%).

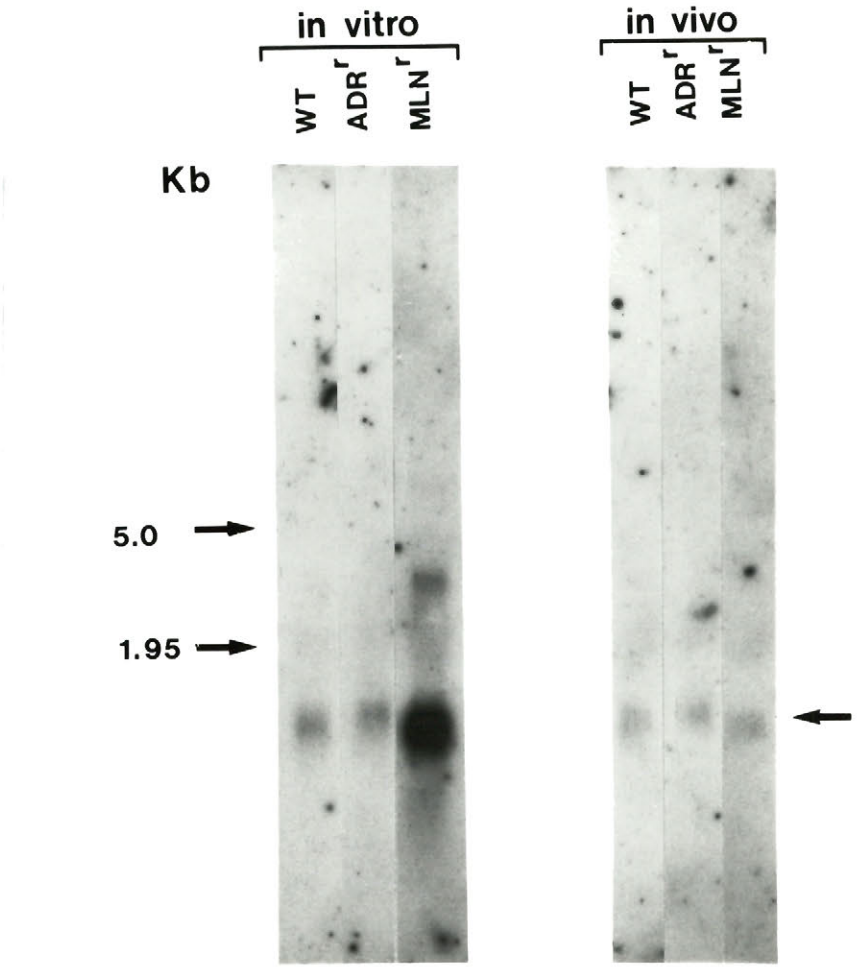

Figure 7. Northern blot analysis of RNA from sensitive and resistant cells using ^{32}P-labeled pGP5 DNA. Arrow at right indicates position of hybridizing sequence.

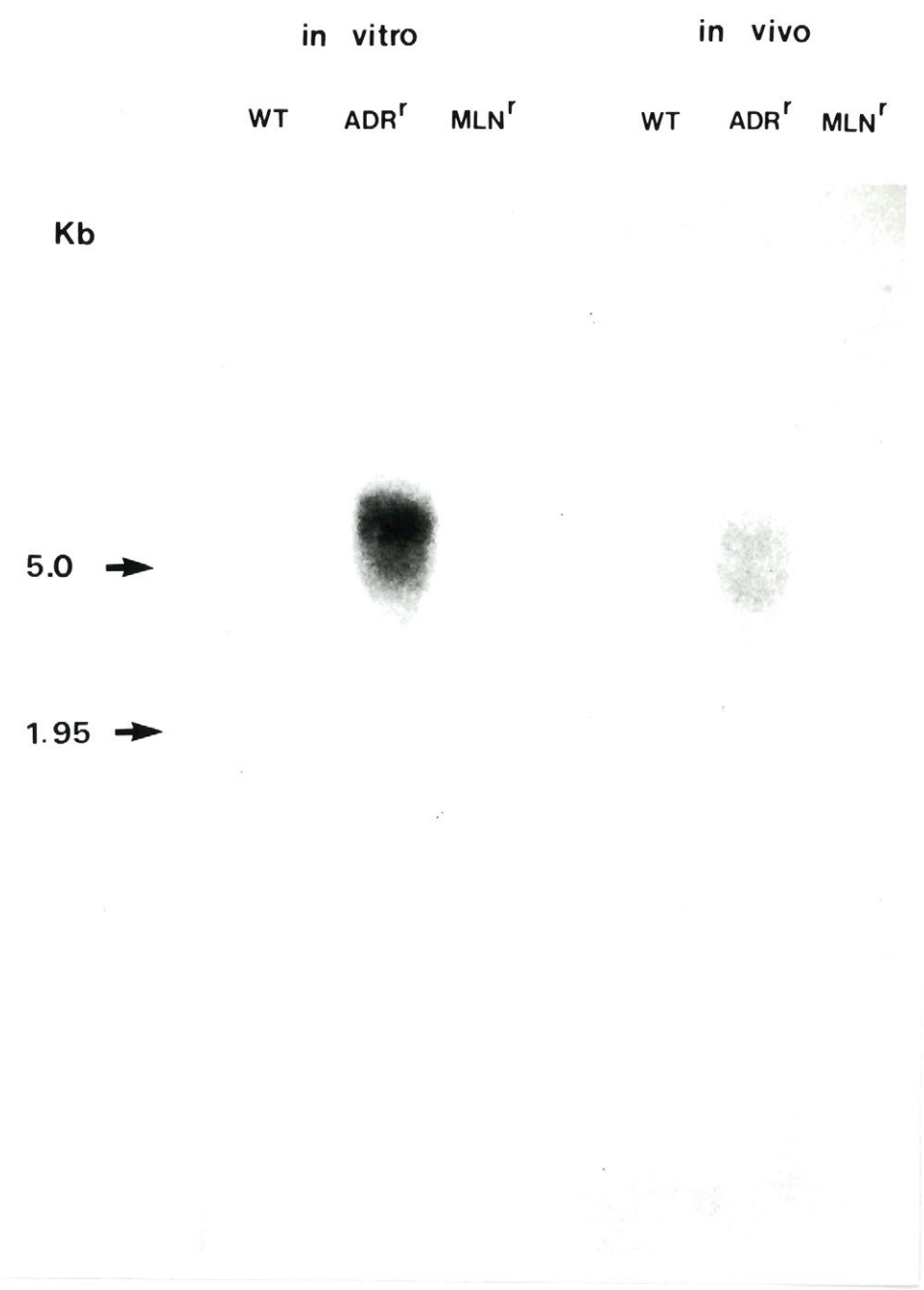

Figure 8. Northern blot analysis of P-glycoprotein expression following hybridization with the 680-base pair insert of plasmid pCHP1.

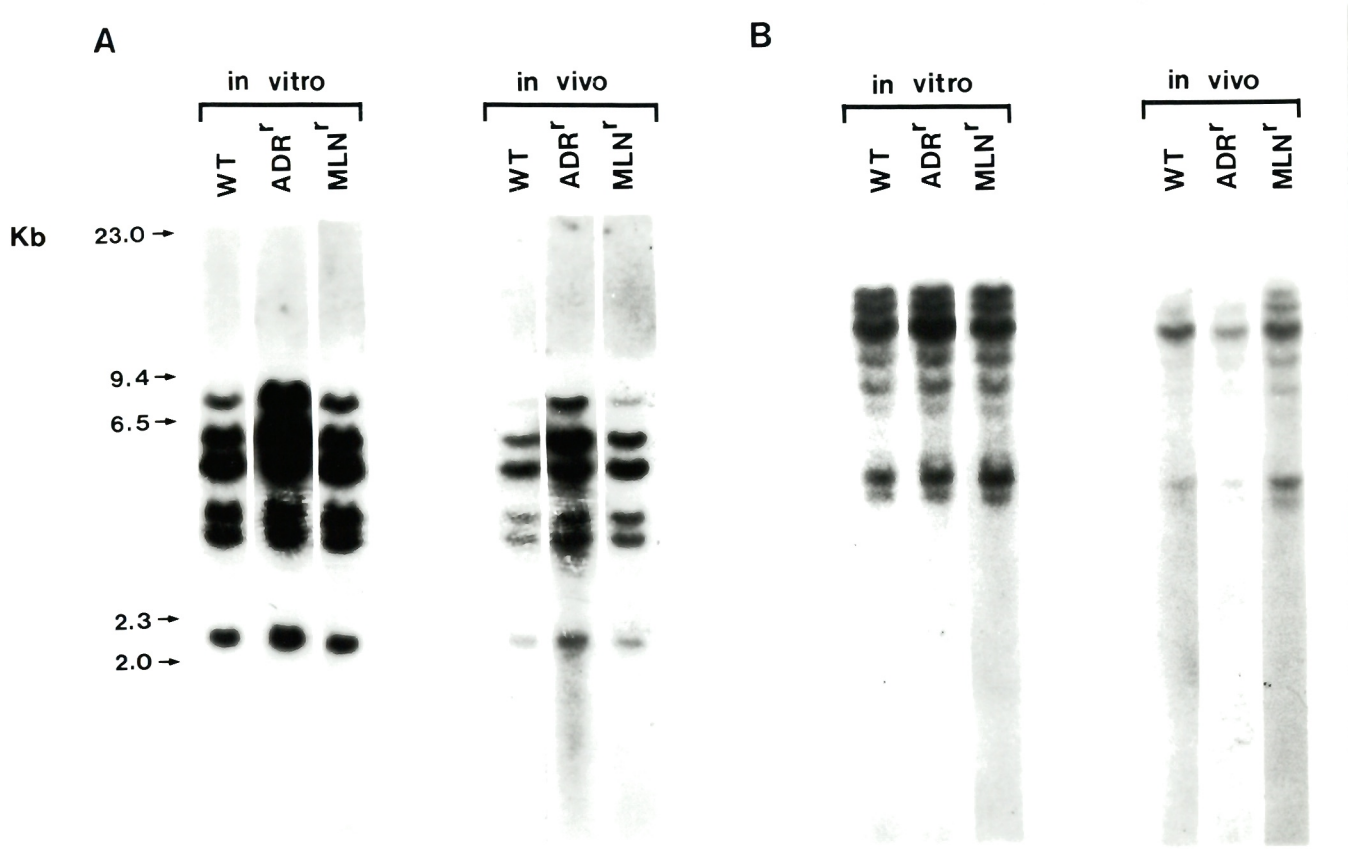

Figure 9. Analysis by Southern hybridization of sensitive and resistant sublines cultured *in vitro* and grown *in vivo*.

A. DNA was digested with Hind III and probed with the pCHP1 MDR-1 cDNA. Twenty µg of DNA from cultured cells and 10 µg from tissue samples were used.

B. Hybridization with actin to standardize the amount of DNA in each lane.

Endothelial cell content, determined by immunofluorescent staining for Factor VIII, is even smaller (data not shown). In addition, the ratio of the amount of a specific DNA sequence (eg. MDR-1) found in tumor relative to actin-specific DNA sequences present in both tumor and non-tumor cells is constant *in vitro* and *in vivo* . This indicates that there is not a significantly greater amount of non-tumor DNA present *in vivo* than *in vitro* . Therefore, there appears to be some effect on expression of the genes studied here by the micro-environment (*in vitro* and *in vivo*). Since we have previously shown that the proliferative state of some cells affects expression of GST (Batist et al., 1989), RNA hybridizations were done in cells grown and harvested in both confluent stationary growth and in the proliferative logarithimic stage. No consistent effect to explain the *in vivo/in vitro* differences was seen.

DISCUSSION

We have developed an animal model of drug resistant breast carcinoma to study the phenotype of resistant cultured cells in an *in vivo* environment. The MatB rat tumor is particularly well suited for this type of investigation because unlike so many other existing ascitic tumor models, it grows in rats as a solid mass with a vascular supply and metastasizes to regional lymphatics. This model allows for a direct comparison of *in vitro* and *in vivo* resistance and provides the opportunity to adapt *in vitro* manipulations of circumventing drug resistance to an *in vivo* environment.

The biochemical and molecular features of this model *in vitro* are similar to other models of natural product and alkylator resistance, which suggests that MatB may be useful as a preclinical model to study *in vitro* drug resistance. For the classic alkylating agents, prolonged *in vitro* selection pressure can produce only low levels of resistance compared with the parent line. However, extensive selection pressure using natural product antineoplastics can generate low level resistance within a short period of time and with prolonged exposure extremely high levels (up to 2000-fold in some cases) of resistance that

may not exist in a clinical setting. The degrees of resistance that we have achieved for the Adr^R and Mln^R MatB sublines are comparable to those previously described for other cell lines resistant to similar products. These results lend support to the value of MatB as a useful model.

The *in vitro* studies of biochemical and molecular parameters were performed with confluent cell cultures which have been shown to more closely match *in vivo* preparations (Lee et al., 1988; Lee et al., 1989). P-glycoprotein expression in Adr^R MatB cells is 5-fold less *in vivo* relative to *in vitro* grown cells. The proportion of cells that are both malignant and resistant apparently has not changed *in vivo* since the MDR gene remains amplified to the same extent *in vivo* and *in vitro* (2-fold), in the Adr^R cells. The pattern of both MDR and GST overexpression in Adr^R cells is consistent in culture and in tissue , however, the magnitude of expression in *in vitro* conditioned cells is higher. The decreased expression *in vivo* is common to most other tumors which express low or barely detectable amounts of MDR-1 RNA. It has been previously reported that just detectable MDR expression is associated with 6-fold resistance to adriamycin (Fairchild et al., 1987). A recent study showed that *in vitro* selected Adr^R cells accumulated 4-5 fold more drug when transferred to an *in vivo* environment which also suggests a lowered MDR-1 expression (Yin et al., 1989). Other studies have shown that decreased drug accumulation was not the primary mechanism of adriamycin resistance in ovarian cancer cells from clinically refractory patients, despite observing such a mechanism in cells with *in vitro*-induced adriamycin resistance (Louie et al., 1986).

A number of studies have reported elevated GSH concentration and GST activity associated with alkylator resistance (Ross et al., 1978; Hamilton et al., 1985; Zijlstra et al., 1986; Buller et al., 1987; Robson et al., 1987). There is some evidence that GST catalyzes the conjugation of GSH to drug or to drug-DNA adducts in the case of alkylators (Hansson et al., 1988). There is *in vitro* evidence that GST-μ is involved directly in the denitrosation reaction of nitrosoureas in a rat glioma cell line (Smith et al., 1989). Studies

have also shown that melphalan is a substrate for GST-catalyzed conjugation with glutathione (Dulik et al., 1986). Class α-GSTs have more consistently been demonstrated to be associated with cellular resistance to a number of chemotherapeutic agents. Although Ya and Yc encoding genes are both members of the alpha family, with approximately 70% homology in the protein coding region, it has been suggested that they are members of independent gene families based on their differential drug inducibility and organ distribution (Li et al., 1986).

Walker 256 rat breast carcinoma cells resistant to bifunctional nitrogen mustards show overexpression of glutathione transferase Yc subunit (Buller et al., 1987; Manoharan et al., 1987). Nitrogen mustard-resistant CHO cells demonstrate increased expression of both Yc and Yp subunits (Robson et al., 1987). As demonstrated in this study, growth of Mln^R MatB cells *in vivo* is accompanied by a small increase in Ya/Yc and Yp expression. The increase in GST activity in these cells (2.7-fold) is similar to the increase previously observed in human ovarian cancer cells resistant to cisplatin and chlorambucil (2.1-fold) (Wolf et al., 1987). In fact, the small degree of resistance to alkylating drugs observed clinically is of the same magnitude as that which has been demonstrated experimentally in Cos cells transfected with Ya and Yp GST isozymes (Puchalski and Fahl, 1990). The low level of GST overexpression observed in Mln^R MatB tumors is consistent with GST transfection data and suggests that the protective role of GST in *in vivo* drug-resistance represents an avenue worthy of further investigation.

In MatB cells GST expression is affected differently than GSH concentration. GST expression *in vivo* is reduced relative to cultured cells while a 2-fold increase in GSH content seen in cultured cells is maintained *in vivo*, suggesting that conditions for GSH biosynthesis are more favorable *in vivo* than *in vitro*. The role of glutathione in drug metabolism in an *in vivo* environment has been examined in resistant tumor models. Studies with murine L1210 leukemia cells (Suzukake et al., 1983), and human ovarian cancer cell lines established from patients exhibiting clinical signs of drug resistance (Green

et al., 1984) indicate that resistance is related to conversion of L-PAM to its non-cytotoxic derivative 4-(bis(2-hydroxyethyl)amino)L-phenylaline, which is associated with a 2-3 -fold increase in GSH in the resistant tumor cells.

There is strong evidence that GSH peroxidase plays a role in resistance to adriamycin and its increase in activity in the Mln^R cells may be functioning in the cross-resistance to adriamycin observed here. Glutathione transferases Ya, Yc and Yp all demonstrate selenium-independent GSH peroxidase activity. Increased expression of these subunits may explain the enhanced detoxification of adriamycin-mediated cellular effects. Glutathione peroxidase is thought to detoxify peroxides resulting from oxygen radicals generated by quinone containing compounds like adriamycin. The mechanism of resistance in the Mln^R cells to vincristine is not known but this has previously been described in other models (Horton et al., 1987; Rosenberg et al., 1989). The phenotype of reciprocal cross-resistance observed in MatB cells selected *in vitro* in either adriamycin or melphalan is consistent with the common clinical finding of broad cross-resistance of tumors in patients who were previously treated with any class of drugs.

Although *in vitro* models provide mechanistic explanations of drug resistance, the magnitude of these alterations may not necessarily be a true indication of resistance levels *in vivo* . MatB provides the opportunity to examine this problem in a more clinically relevant system. In addition to cellular factors which we have shown to be involved in drug resistance, there clearly are other factors observed only *in vivo* that may also contribute to mechanisms of clinical drug resistance (Teicher et al., 1990).

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CHAPTER 3

GLUTATHIONE-S-TRANSFERASE EXPRESSION IN MAMMARY TUMORS AND BONE MARROW CELLS

PREFACE TO CHAPTER III

Dose escalation of chemotherapy treatment in patients is limited by toxicity to normal tissues.

For alkylating agents such as melphalan, the dose limiting toxicity occurs in the bone marrow. This observation has led to the development of bone marrow transplantation in conjunction with high dose chemotherapy for solid tumors for which there is a linear and steep dose response curve for alkylators.

This chapter describes the relationship between mouse, rat and human bone marrow with respect to their glutathione and glutathione-S-transferase (GST) activity and subunit composition. These studies were performed in order to determine whether the bone marrow would be a suitable target tissue for gene transfer studies with glutathione-S-transferases. The purpose of introducing GSTs into these cells is to protect them from the cytotoxic effects of alkylating agents.

ABSTRACT

The glutathione-S-transferase (GST) isozyme encoding genes have been classified into multigene families (alpha, mu, pi) based on nucleotide sequence homology. These isozymes appear to play an important role in cellular defense against toxic chemicals including chemotherapeutic drugs. Both tissue and species-specific expression of the various GST forms have been observed. In this study, we have examined GST expression in rodent and human malignant mammary tissue and normal bone marrow. The latter is commonly the dose-limiting organ for systemic chemotherapy. Both mouse and rat mammary tumor tissue expressed alpha class GST; in addition, the rat tissue expressed the pi class subunit. We have also examined GST isozyme composition in human and rat bone marrow and have found expression of both alpha and pi class GST in these tissues. GST enzyme activity measurements revealed that human marrow has a 6-fold higher level of activity relative to rat bone marrow. The information obtained in this study will be instrumental in planning therapeutic strategies to modulate GST activity to overcome drug-resistance, or to confer drug protection to dose-limiting organs such as the bone marrow by retroviral gene transfer of GST isozymes.

INTRODUCTION

Changes in cellular detoxifying enzymes induced by previous drug exposure and resulting in resistance to further drug administration constitute a major limitation of current anticancer treatments. Several of these drugs are metabolized to electrophilic products. GST enzymes, which are formed by dimerization of homotypic or heterotypic subunits, play important roles in the cellular detoxication of electrophilic compounds. These compounds, and other potentially toxic molecules, may react with glutathione (GSH) directly or may be conjugated in a reaction catalyzed by glutathione-S-transferases (GST). Direct evidence for GST's involvement in chemotherapy disposition includes studies where GST mu has been shown to inactivate BCNU (1,3 bis(2-chloroethyl)-1-nitrosourea) and quench DNA crosslink precursors of BCNU (Smith et al., 1989) and where class alpha GST has been implicated in the conjugation of GSH to melphalan (Bolton et al., 1991). Numerous reports have provided additional evidence for a relation between resistance to alkylating agents and elevated GSH and GST. There are many examples in which the development of resistance to alkylating agents is associated with increases in cellular GSH content (Suzukake et al., 1982; Ahmed et al., 1987; Evans et al., 1987; Schecter et al., 1991) or increased expression of various GST forms, most commonly the Yc form (Buller et al., 1987; Robson et al., 1987; Lewis et al., 1988; Schecter et al., 1991). In one example of *in vivo* induction of drug-resistance apparently related to GST, administration of low doses of cyclophosphamide to mice protected them against the subsequent administration of a normally lethal dose of the same drug (Millar et al., 1975). More specifically, the bone marrow tissue was protected, and a 2-fold elevation in granulocyte GST activity was observed in these cells. Additional data in support of GST's role in resistance comes from the use of known inhibitors of GST activity to overcome resistance to nitrogen mustard *in vitro* (Tew et al., 1988). The same effect has been shown more recently *in vivo* in mice carrying human tumor xenografts (Clapper et al., 1990). Finally, recent transfection studies have described small but seemingly significant increases in

resistance to alkylating agents following stable transfection with cDNAs encoding GST alpha into mammalian cells (Puchalski and Fahl, 1990). Expression of GST class alpha into these cells conferred resistance to nitrogen mustard and cisplatin. Moreover, reversion of GST expression correlated with the loss of the drug-resistance phenotype in these cells. These findings, taken together with the recent evidence for the role of GST class alpha in conjugating melphalan (Bolton et al., 1991) and chlorambucil to GSH (Ciaccio et al., 1990) which in the latter case has been shown to be inhibited by ethacrynic acid (Ciaccio et al., 1991), lend strong support for a protective role of GST against the cytotoxic effects of alkylating agents. Mechanisms whereby elevations in cellular GST levels might contribute to tumor cell resistance include enhanced inactivation of electrophilic agents (Dulik et al., 1986), quenching of chloroethylated-DNA monoadducts (Eastman and Richon, 1986), and scavenging of reactive organic peroxides (Mimnaugh et al., 1989). In order to understand the potential risks to bone marrow cells of using GST inhibitors as well as the feasability of protecting these cells by transferring GST genes, we have examined the expression of GSH and GST isozymes in rodent and human mammary tumors and normal bone marrow.

MATERIALS AND METHODS

1. Cell Lines and Tumors

MatB 13762 is a cell line derived from a carcinogen-induced female Fischer 344 rat mammary tumor. These cells can be grown both *in vitro* and *in vivo*. After an injection of 5×10^5 cells subcutaneously (s.c.) into mammary fat pads, a palpable mass develops within two weeks. Tumors were processed once they reached a size of 1.5-2.0 cm^3. C4 mouse mammary tumors are obtained by injecting 5×10^5 cells into mammary fat pads of BALB/c mice. A pool of 3-6 fat pads (approximately 0.5cm^3 each) provided one sample. All tumor tissues were isolated free of host mammary tissue prior to preparation of tissue extracts.

2. Bone Marrow Collection

Fischer 344 rat bone marrow cells were obtained by flushing femurs with Iscoves Modified Dulbecco's Medium (IMDM,Gibco) supplemented with 12.5% fetal bovine serum (Gibco). Aliquots of human bone marrow were obtained from bone marrow transplantation donors after informed consent with the approval of our Institutional Ethics Committee. Light density (d< 1.077) mononuclear bone marrow cells were isolated by centrifugation over a Ficoll Paque (Pharmacia) density gradient and then washed twice in IMDM. Cytosolic preparations were prepared by lysing the cells in distilled water.

3. Biochemical Analysis

Approximately 5×10^6 mononuclear bone marrow cells were assayed for GST expression following lysis by vortexing at 4°C in distilled water. Total GST enzyme activity was assayed using 2-chloro-1,3-dinitrobenzene (CDNB) as a substrate (Habig et al., 1974). Protein levels were determined according to the method of Lowry with bovine serum albumin as a standard. The concentration of GSH was determined following deproteinization in 3% sulfosalicylic acid according to the method of Ellman (1959).

4. Western Blot Analysis

The GST isoenzyme composition of the various samples was determined using Western immunoblotting. Cytosolic proteins (25 μg) were separated by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membranes by electroblotting. Non-specific binding to the membrane was blocked by incubation in 5% BSA in PBS for 1 hour at room temperature. Blots were then incubated overnight in 0.5% BSA containing either a 1:500 dilution of a rabbit polyclonal antibody directed against the rat cationic Yc, Yb and Ya subunits of GST generated by immunization with purified rat liver GST subunits Yc, Yb and Ya, or a 1:250 dilution of a rabbit polyclonal antibody directed against the human π subunit. Controls were run in parallel to

determine the position of migration of each isoform. After three washes in 0.05 M phosphate, 0.5 M NaCl, and 0.1% tween 20 (pH 8.0), the blots were incubated for three hours at room temperature in 0.5% BSA containing a 1:2000 dilution of horseradish-peroxidase-conjugated goat anti-rabbit IgG. The blots were then developed in PBS containing 0.52 mg/ml diaminobenzedine and 0.04 % H_2O_2.

RESULTS

1. Mammary Tissue Analysis

Figure 1A shows the results of Western immunoblotting to characterize GST isoenzymic subunits of various tissues. Lanes 1-3 were reacted with an antibody directed against cationic GST subunits. A cytosol preparation from normal rat hepatocytes was run in lane 1. Three subunits are present that correspond to GST subunit Yc (MW = 29.5 Kd), Yb (MW = 28.5 Kd) and Ya (MW = 27.5 Kd). A mouse C4-derived neoplastic mammary tissue extract was loaded in lane 2, and lane 3 was loaded with an extract from rat MatB-derived mammary tumor. Both mammary tumors show only a single band corresponding to the Ya subunit. Neither of the rodent tumors were found to express the Yb subunit with our antibody. In Figure 1B the same samples were reacted with a polyclonal antiserum prepared against the anionic GST Yp subunit. Lane 1 serves as a control and was loaded with a preparation of purified human GST π. The Yp subunit is seen only in the rat tumor (lane 3), and not in the sample from mouse (lane 2). We have previously shown that increased levels of expression of the Yc subunit are observed when a rat mammary tumor cell line is selected for melphalan resistance (Mln^R) (Schecter et al., 1991).

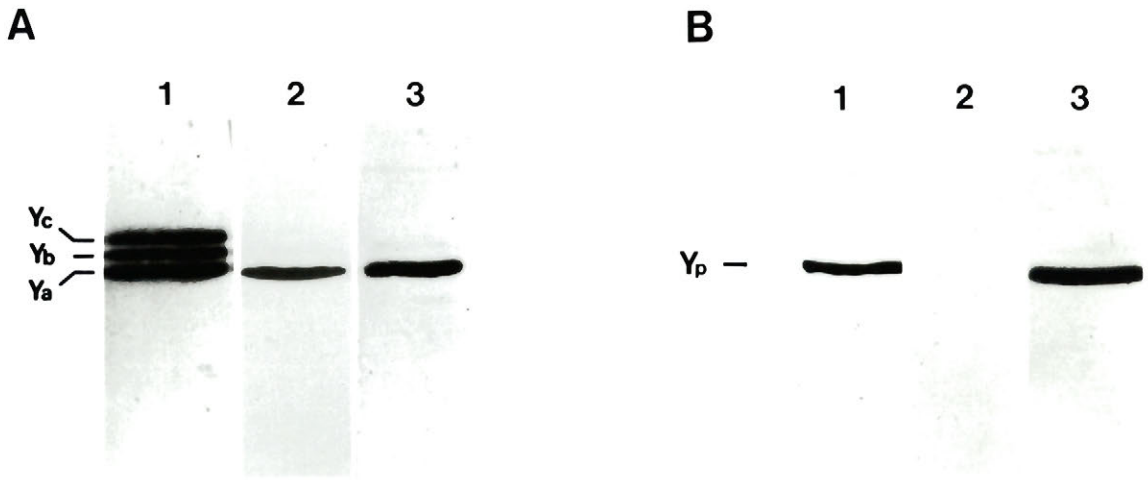

Figure 1. Immunoblotting of GST isozymes in mouse and rat mammary tumors. Cytosolic preparations (20 μg of total protein) were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes for immunoblotting.

A. Lanes 1-3 were reacted with a polyclonal antibody against cationic GST subunits. Purified rat liver GST subunits Yc, Yb and Ya served as markers in lane 1. Lanes 2 and 3 are from mouse and rat mammary tumor tissue, respectively.

B. Lanes 1-3 were reacted with a polyclonal antibody against anionic GST subunits. Lane 1 was loaded with purified human GST pi. Lanes 2 and 3 are the same as in A.

2. Biochemical Characterization of Bone Marrow

The biochemical profile of the bone marrow samples is presented in Table 1. Total cellular GSH content was higher in human bone marrow cells relative to rat marrow, however, the difference in GSH concentration between rat and human did not reach statistical significance. GST activity using CDNB as substrate showed that human bone marrow has a considerably higher level of transferase activity relative to rat marrow.

3. Western Immunoblotting of Bone Marrow Cells

The isoenzymic subunit composition of bone marrow cells was also examined by Western immunoblotting. Figure 2 shows a Western blot reacted with the same anti-cationic GST antibody used in Figure 1A. As shown, both human and rat bone marrow cells express similar levels of an immunoreactive form corresponding to the Ya subunit, but no detectable levels of Yc or Yb forms. In Figure 3, the same samples were reacted with an anti-Yp antisera. Both human and rat marrow demonstrate similar levels of expression of an immunoreactive form corresponding to Yp.

Table 2 summarizes GST subunit expression detected by Western immunoblotting in mammary tumors and bone marrow cells from mouse, rat and human samples. Of note, none of these tissues expresses readily detectable levels of the Yc subunit which is most often associated with alkylator resistance.

DISCUSSION

The present study was undertaken to determine the level of GST activity and isoenzyme composition of rat and human bone marrow cells and mammary tumors. One long-term goal of these studies is to determine whether the expression of GST isozymes might serve a protective role in bone marrow cells against the myelosuppressive effects of chemotherapeutic drugs, particularly the alkylating agents. The GST enzymes have been

Table 1. Biochemical characterization of normal rat and human bone marrow

	GSH (nmol/1 $\times 10^6$ cells)	GST (nmol/min/mg protein)
Rat Bone Marrow	6.1 ± 0.21	1.70 ± 0.30
Human Bone Marrow 1	8.0 ± 0.72	7.74 ± 0.47
Human Bone Marrow 2	12.0 ± 1.10	7.97 ± 0.26

Abbreviations used are: GSH, glutathione; GST, glutathione-S-transferase.

The results are expressed as the means ± SE of three separate determinations.

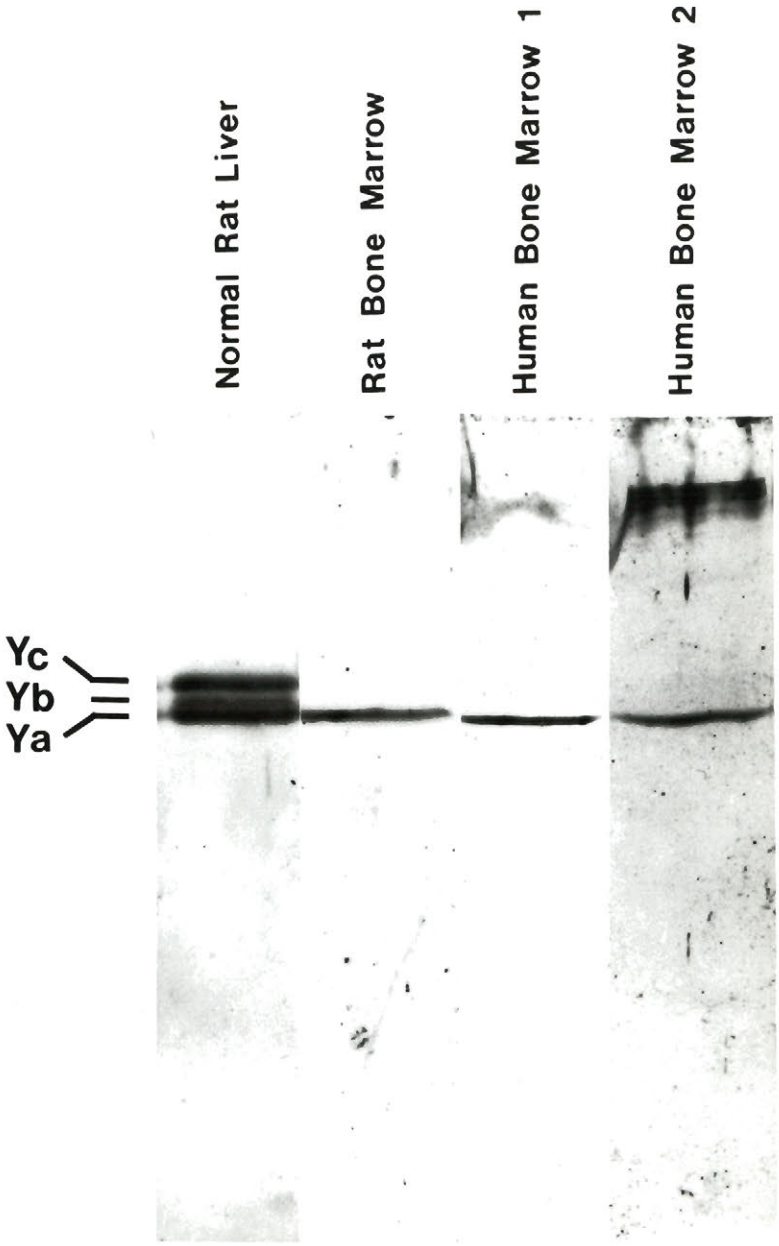

Figure 2. Immunoblotting of GST isozymes in rat and human bone marrow cells. Cytosolic preparations (20 μg of total protein) were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes for immunoblotting. Samples were reacted with the anti-cationic GST antibody.

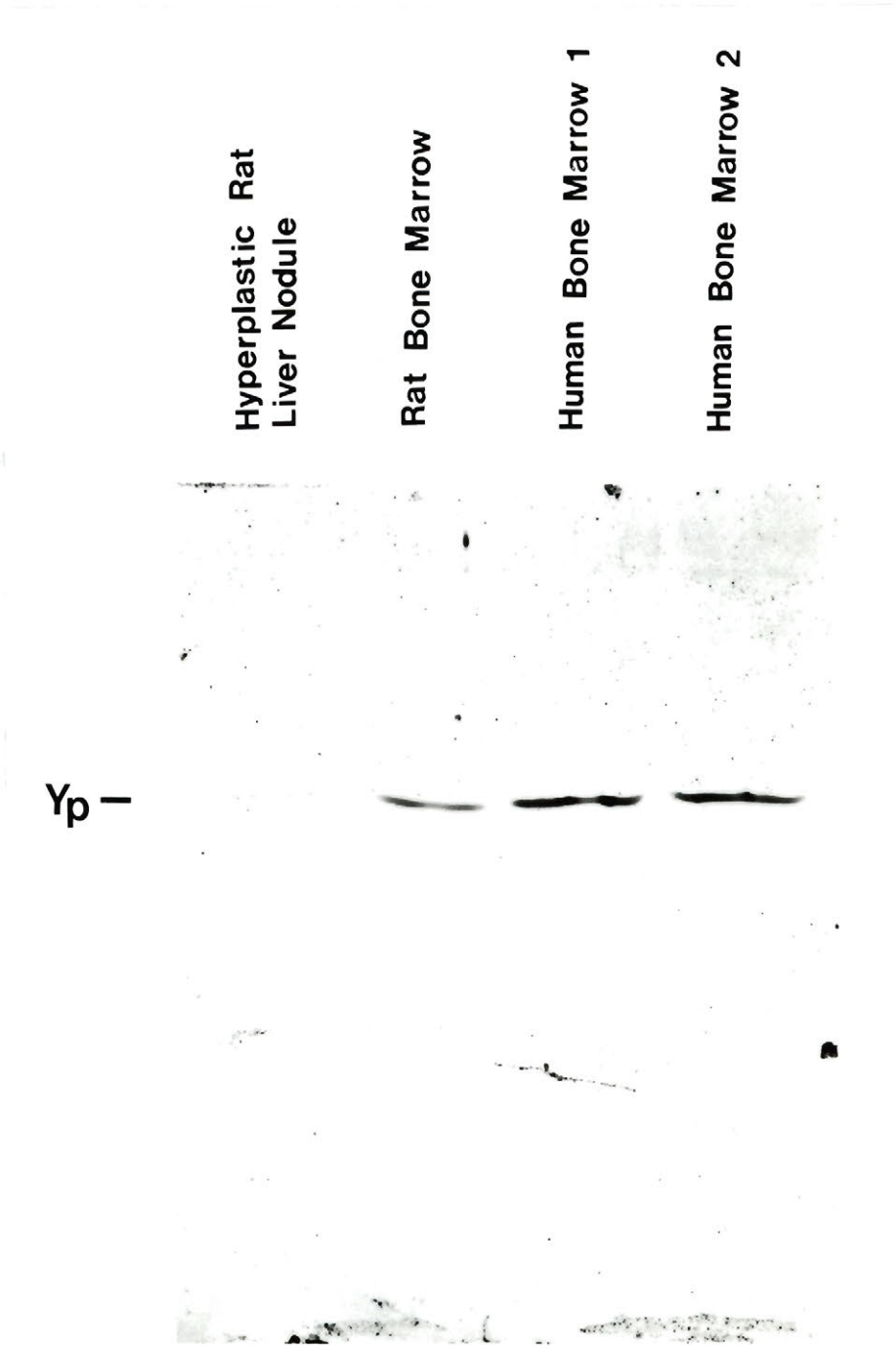

Figure 3. Immunoblotting of GST isozymes in rat and human bone marrow cells. Cytosolic preparations (20 μg of total protein) were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes for immunoblotting. Samples were reacted with the anti-anionic GST antibody.

Table 2. GST subunits detected by Western immunoblotting

	Species		
Tissue	Mouse	Rat	Human
Mammary Tumor	Y_a	Y_p, Y_a	Y_p, Y_b(50%)[a]
BM	Y_a, Y_b[b]	Y_p, Y_a	Y_p, Y_a

a Lewis et al., 1988.
b Isola and Gordon, 1986.

implicated in the resistance of cells to a variety of chemicals including anti-cancer drugs (Suzukake et al., 1982; Ahmed et al., 1987; Buller et al., 1987; Evans et al., 1987; Robson et al., 1987; Lewis et al., 1988; Bolton et al, 1991; Schecter et al., 1991). The alpha class GST subunits (Ya,Yc) have most often been associated with alkylator resistance. Although the dimeric forms YaYa and YcYc are both classified into the alpha family on the basis of their amino acid sequence homology and immunological cross-reactivities, differences in substrate specificities, drug inducibility and organ distribution have been observed between these two isozymes (Wolf et al., 1987; Mannervik et Danielson, 1989; Shea et al., 1990). For example, YaYa preferentially catalyzes conjugation and isomerization reactions whereas YcYc more efficiently catalyzes the peroxidation reaction . The multiplicity of alpha class subunits has been reported in rat. Two Ya forms (Pickett et al., 1984; Lai et al., 1984) in addition to the Yc form (Telakowski-Hopkins et al., 1985) have been defined by cDNA sequencing. It is estimated that there are at least two genes for Yc and 5 genes for Ya (Chow et al., 1988). All of these findings taken together suggest that Ya and Yc subunits may be under separate expression control mechanisms and may actually be located on independent genetic loci.

In this report we demonstrate inter-species variation in GST expression in both a tumoral and normal tissue that are targets of chemotherapy. Rat and mouse mammary carcinomas express the Ya subunit, however, expression of the Yp form is found only in rat mammary tumor tissue. All untreated human breast cancers examined by Shea et al. (1990) expressed GST pi isozyme protein and none expressed alpha class GST protein. Approximately 50% of the samples expressed mu protein (which corresponds to the rat Yb subunit). In those studies, substantial differences existed among the primary breast carcinomas in both the amount of GST activity and isozyme composition. Although breast tumors generally demonstrate a good initial response to chemotherapy the development of tumor cell resistance eventually limits this treatment modality.

We have previously reported that following the development of *in vitro* resistance to melphalan, MatB cells transplanted *in vivo* expressed a three-fold increase in Yc mRNA and a corresponding increase in Yc gene product (Schecter et al., 1991). There was, however, no change in Ya or Yp expression in these cells. Tumors derived from melphalan-resistant (Mln^R) MatB cells were also determined to be 2- to 4-fold resistant to the drug *in vivo*. The association of increased Yc expression and alkylator resistance is quite firm (Ahmed et al., 1987; Buller et al., 1987; Robson et al., 1987; Schecter et al., 1991). We have recently shown that transfection of rat liver Yc cDNA into drug-sensitive MatB cells confers low level resistance to melphalan (Schecter et al., 1992).

Using a polyclonal antiserum prepared against the cationic Ya, Yb and Yc subunits of rat GST, we show here that both rat and human mononuclear bone marrow cells express subunits corresponding to the Ya form. These cells also express the anionic Yp form of GST. Species specific differences in affinity of GST for CDNB may explain differences in enzyme activity levels despite equal band intensities on Western immunoblots. Wolf et al. have previously shown that mouse bone marrow cells expressed both mu and alpha class GST subunits (Wolf et al., 1987). An important feature of the GST profile in rat and human bone marrow cells that we have shown is the absence of the Yc subunit. Although the sensitivity of bone marrow cells to drug may be related to lower total GST activity compared to tumor tissue, the evidence suggests a particular importance for alpha class GSTs in bifunctional alkylator detoxification (Ciaccio et al., 1990; Bolton et al., 1991).

Because one of the major toxicities of chemotherapy is myelosuppression, the concept of transferring genetic material into bone marrow cells to confer drug-resistance and protect these cells from chemotherapy has become an important issue. A number of chemotherapeutic agents show a steep and linear dose-response curve in their ability to kill various tumor cell types, which suggests a potential benefit from dose-intensification of these drugs. This is particularly true of alkylating agents which maintain a dose-related effect through multiple logs of tumor cell kill (Tormey et al., 1984; Peters, 1985).

Autologous bone marrow transplantation (AuBMT) has provided a means to intensify cancer therapy by "salvaging" the patient from normally lethal doses of myelotoxic drugs. Because breast cancer initially tends to respond well to chemotherapy, it is a good candidate tumor for dose-intensification and several groups of patients suffering from advanced breast cancer have received high-dose alkylating drugs followed by AuBMT. Although many patients responded with a complete disappearance of clinically detectable disease, indicating sensitivity of the tumors to dose-intensification, the duration of response to treatment has, in most studies, been short (Frei et al., 1989; Dunphy and Spitzer, 1990). If the bone marrow cells to be transplanted could be protected from further high-dose chemotherapy, additional cycles could be delivered. This, in theory, would result in a higher tumor cell-kill and might therefore improve treatment results. Several lines of evidence support the feasibility of this approach. Transgenic animals expressing a mouse dihydrofolate reductase (DHFR) gene became tolerant to methotrexate (Isola and Gorden, 1986). Likewise, transgenic mice expressing the mouse MDR-1 gene in their bone marrow were shown to be resistant to leukopenia induced by drugs involved in the MDR phenotype, but not by other myelotoxic agents (Galski et al., 1989). Other experiments have shown the possibilty to confer both *in vitro* (Corey et al., 1990; McLachlin et al., 1990) and *in vivo* (Corey et al., 1990) drug-resistance to hematopoietic cells by retroviral-mediated gene transfer into somatic cells. With regard to the use of GST isoenzymes to protect the hematopoietic system from the ablative effect of chemotherapy, the absence of the Yc isoform in both human and rodent bone marrow cells appears to be a very relevant observation since this form appears to be most often associated with alkylating drug-resistance. This observation is in contrast with the expression of P-glycoprotein by rodent bone marrow cells (Aihara et al., 1991) and human hematopoietic stem cells (Chaudhary and Roninson, 1991). Therefore, the transfer of a GST Yc expression vector into the hematopoietic system could potentially augment the patient tolerance to alkylating agents

and result in an increased therapeutic index for those drugs. We are now exploring the feasibility of this approach in an experimental model.

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CHAPTER 4

EXPRESSION OF A RAT GLUTATHIONE-S-TRANSFERASE cDNA IN RAT MAMMARY CARCINOMA CELLS: IMPACT UPON ALKYLATOR-INDUCED TOXICITY

PREFACE TO CHAPTER IV

Glutathione-S-transferase activity is found to be elevated in a number of cell lines including a MatB subline, selected for resistance *in vitro* to alkylators, as well as in human solid tumors following treatment with alkylating agents. The GST α class isozymes in particular have been shown to conjugate glutathione to agents such as chlorambucil and melphalan.

The Yc subunit, which is thought to be responsible for resistance to alkylating agents, was investigated. Yc cDNA was transfected into drug sensitive mammary tumor cells. Individual clones overexpressing this subunit were studied for their responsiveness to a panel of alkylating drugs. The results of these experiments are reported in this chapter.

ABSTRACT

The role of glutathione-S-transferase (GST) in alkylator drug resistance has been studied in MatB rat mammary carcinoma cells. A series of GST transfectant cell lines was established by using an expression vector containing the cDNA for the rat GST-Yc gene under regulation of the SV40 early region promoter and the antibiotic resistance plasmid pSV2neo. Transfectant cell lines expressing up to 4-fold higher total GST activity than in the parental wild type cell line were identified. Southern blot analysis confirmed a DNA fragment corresponding in size to the transfected GST Yc cDNA. Wild type MatB cells contain very low levels of Yc protein, whereas the Yc^+ clones showed greatly increased amounts of the Yc subunit. The effect of increased GST Yc activity on the sensitivity of the transfected clones to various cytotoxic agents was assessed using the MTT cell-survival assay. The clones expressing recombinant GST Yc were more resistant to melphalan (6-12-fold), mechlorethamine (10-16-fold) and chlorambucil (7-30-fold). In late passage populations of the GST Yc^+ clones that had been grown over a period of 14 months under continuous selection in G418, GST activity was decreased and it was paralleled by a decrease in Yc protein. These late passage clones with diminished GST Yc content also demonstrate a partial reversion toward the wild type phenotype as determined by cytotoxicity assays using melphalan, mustargen and chlorambucil. Interstrand DNA crosslinks induced by mechlorethamine were significantly lower at 0 hr, 2 hr and 20 hr post-treatment in one of the GST Yc^+ clones when compared to wild type MatB cells. These studies indicate that GST Yc overexpression can confer resistance to alkylating agents and that this correlates with inhibition of DNA crosslink formation.

INTRODUCTION

In spite of the advances that have been made in the treatment of malignancies, the development of tumor cell resistance to cytotoxic drugs is a clinical phenomenon that presents a major obstacle to effective therapy. A variety of factors determine the response of tumor cells to chemotherapy. *In vitro* studies have demonstrated a number of mechanisms that are able to protect against the cytotoxic effects of anticancer agents. Elucidation of resistance mechanisms has relied primarily on comparative studies of sensitive and selected drug resistant cell lines.

The GSTs are a family of enzymes with a range of functions and substrate specificities. These enzymes can confer resistance to anticancer drugs through a variety of mechanisms: through conjugation of electrophilic compounds by covalent addition to the thiol-containing peptide glutathione (Mannervik and Danielson, 1988); by noncovalent and covalent binding of hydrophobic compounds (Ketterer, 1986); and by detoxification of lipid and DNA hydroperoxides through an intrinsic peroxidase activity (Jakoby, 1978). A number of factors have strongly supported a direct role of GST forms in chemotherapy resistance, particularly for alkylating agents. Several alkylator resistant sublines have been shown to overexpress GST alpha (Ya, Yc) activity (Buller et al., 1987; Evans et al., 1987; Robson et al., 1987; Lewis et al., 1988; Schecter et al., 1991). GST inhibitors are capable of overcoming resistance in these cell lines (Tew et al., 1988; Yang et al., 1991). The Ya and Yc forms have been assigned to the same gene family, however, on the basis of their differential drug inducibility and organ distribution (Wolf et al., 1987a; Mannervik and Danielson, 1988) it appears likely that the expression of these two proteins is under seperate control mechanisms. It has also been reported that certain members of the GST alpha family can be selectively induced by alkylating agents (Clapper et al., 1991). Resistant cell lines that revert to a more sensitive phenotype lose their elevated GST activity (Xue et al., 1988; Hansson et al., 1991). GST inhibitors are capable of overcoming

resistance in these cell lines (Xue et al., 1988; Yang et al., 1992).There has been a limited examination of clinical specimens, but in CLL cells (Schisselbauer et al., 1990) and in specimens from ovarian and neuroblastoma cancer patients whose tumors were clinically resistant to chemotherapy there is elevated GST activity (Wolf et al., 1987; Kuroda et al., 1991). GSTs have been shown to catalyze the conjugation of glutathione to chlorambucil (Ciaccio et al., 1990), cyclophosphamide (Yuan et al., 1990), 1,3-bis(chloroethyl)-1-nitrosourea (Smith et al., 1989) and melphalan (Bolton et al., 1991).

The critical event leading to cell killing by alkylating agents is thought to be drug-DNA interaction (Chasseaud, 1979; Tew and Clapper, 1988). GSH may act nonenzymatically (Bolton et al., 1990; Yuan et al., 1990) or through GST-catalyzed reactions (Tew and Clapper, 1988; Xue et al., 1988) to compete with DNA for drug binding. GSH has been found to quench DNA monoadducts in alkylated DNA and inhibit DNA crosslink formation (Ali-Osman, 1989). It has also been suggested that both GSH and GST modulate other cellular processes such as DNA repair (Xue et al., 1988; Gi-Ming et al., 1989; Ketterer and Myer, 1989). In addition to numerous observations of alkylator resistance associated with overexpression of the alpha-subclass of GST, involvement of an alpha-subclass GST in the resistance phenotype was also implicated through enzyme inhibition studies. Pretreatment of chlorambucil resistant tumor cells with the competitive GST inhibitor ethacrynic acid resulted in enhanced sensitivity to the drug in both rat and human tumor cells (Tew et al., 1988). Similarly, in chlorambucil- resistant mouse fibroblasts, inhibition of GST activity by ethacrynic acid or indomethacin significantly enhanced toxicity to chlorambucil (Yang et al., 1992). The same effect has been shown *in vivo* in mice carrying human tumor xenografts (Clapper et al., 1990).

The introduction of isolated GST genes into lines of cultured cells has provided the opportunity to study the expression of GST and its involvement in drug resistance most directly. Transfection of alpha-class GST into yeast cells conferred 3-16-fold resistance to chlorambucil (Black et al., 1990). Low level resistance to melphalan, chlorambucil and

cisplatin was observed following transfection of the rat Ya GST cDNA into monkey Cos cells (Puchalski and Fahl, 1990). In the same study reversion of transient expression in Ya^+ Cos cell clones to a Ya^- phenotype was associated with total loss of drug resistance. Transfection of the human alpha class GST into NIH 3T3 cells conferred a 6-fold level of resistance to 4-OH-cyclophosphamide (Lewis et al., 1992). However, transfection into human MCF-7 breast cancer cells of either the human alpha GST (Leyland-Jones et al., 1991) or the rat Yc GST (Bailey et al., 1992) failed to induce resistance to either melphalan, chlorambucil or cisplatin.

We have previously reported a Mln^R MatB cell line which displays several mechanisms of drug resistance. Mln^R cells have increased cellular glutathione (GSH) levels, elevated GST activity *in vitro* and *in vivo* where the Yc subunit is specifically elevated (Schecter et al., 1991). This cell line is also cross-resistant to chlorambucil and mechlorethamine. Mln^R cells have also been shown to accumulate significantly less DNA-DNA crosslinks following exposure to melphalan and chlorambucil relative to the sensitive wild type cell line (Alaoui-Jamali et al., 1992).

To examine the importance of GST in alkylator resistance we have constructed an expression vector containing the full length coding sequence of the rat Yc subunit. Wild type MatB cells which normally express very low levels of Yc were transfected with the Yc expression vector. A number of cell lines shown to be expressing the recombinant Yc were then analyzed to determine their sensitiviy to a group of alkylating chemotherapeutic agents.

MATERIALS AND METHODS

1. Materials

Melphalan, chlorambucil, cisplatin and 2-chloro-1,3-dinitrobenzene (CDNB) were obtained from Sigma Chemical Co (St. Louis, MO.). Adriamycin was from Adria Laboratories. Nitrogen Mustard was from Merck Sharp and Dohme (Kirkland, Quebec). Reagents for biochemical analysis of GST and GSH levels were from Boehringer-

Mannheim (Laval, Quebec). Molecular biology reagents were obtained from BRL (Burlington, Ontario), and radioisotopes were from ICN (St.Laurent, Quebec). Blotting membranes were from Amersham (Oakville, Ontario). Immunoblotting reagents were from BioRad (Mississauga, Ontario). Tissue culture reagents were from Gibco (Burlington, Ontario).

2. Construction of Expression Vectors

The rat alpha class Yc expression vector was constructed by subcloning the pGTB42 Yc cDNA (kindly provided by Dr. Cecil Pickett) into the pSM-1 expression vector. The cDNA insert is 888 bp long and contains 22 nucleotides of the 5'-noncoding region and a 734 bp open reading frame. Expression of the Yc cDNA is driven by the SV40 early-region promoter. The SV40 late polyadenylation sequence from pSV2gpt is located 3' to the cDNA insertion site. The resulting plasmid (called pSVYc) was transformed into Escherichia coli HB101 and isolated by previously described techniques (Maniatis et al., 1984).

3. Transfection of MatB Cells

Rat MatB cells were grown in minimal essential medium (supplemented with 1.3% sodium pyruvate, 1.3% non essential amino acids and 2.6% glutamine) containing 100,000 units/L gentamycin and 10% fetal bovine serum. MatB cells (5×10^5/3-cm plate) were cotransfected with the pSVYc expression vector (18 μg) and pSV2neo (2 μg) using the calcium-phosphate precipitation technique according to the manufacturer's directions (Pharmacia). Transfected cells were selected for resistance to G418 (5.2 units/ml).

4. Biochemical Analysis

One hundred clones surviving G418 selection were assayed for GST activity following cell lysis by hypotonic shock at 4°C. Total GST enzyme activity was assayed

using CDNB as substrate according to previously described methods (Habig et al., 1981). GST peroxidase activity was assayed using cumene hydroperoxide as the substrate (Paglia and Valentine, 1967). Cellular GSH content was assayed according to previously described techniques (Ellman, 1959). Total cellular protein levels were determined by Lowry protein assays.

5. Immunoblotting Analysis

Cytosolic protein (75 μg) was subjected to electrophoresis on a 12% SDS-polyacrylamide gel and transferred overnight to nitrocellulose by electroblotting. The blots were blocked with 5% BSA/PBS and incubated overnight in 0.5% BSA containing a 1:500 dilution of polyclonal rabbit antisera directed against rat liver Yc, Yb and Ya GST subunits. After 3 washes (0.05 M phosphate, 0.5M NaCl and 0.1% Tween 20, pH 8.0) the blots were incubated for 3 hr at room temperature in 0.5% BSA containing 1:3000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG. Following 3 washes, the blots were developed in PBS containing 0.52mg diaminobenzidine/ml and 0.04% H_2O_2.

6. Nucleic Acid Analysis

Transfected cells were harvested and lysed in guanidinium isothiocyanate and RNA was isolated by CsCl gradient centrifugation (Maniatis et al., 1984). Total cellular RNA was denatured in 10 mM NaOH and applied directly onto nitrocellulose membrane according to manufacturers suggestions (BioRad). High molecular weight DNA was isolated according to standard techniques (Maniatis et al., 1984). For Southern analysis, genomic DNA (10 μg) was digested with Xho I and Bam HI and electrophoresed on a 0.8% agarose gel, depurinated, transferred to Hybond N membrane and hybridized to ^{32}P-labeled pSVYc by the random-priming method. Hybridization was carried out in 50% formamide, 0.05 Na_2HPO_4, 1% BSA, 0.1 mM EDTA, 5% SDS at 42°C for 18hr. The

membrane was washed 3 times in 2 × SSC, 0.1% SDS at room temperature and 4 times in 0.1 × SSC, 0.1% SDS at 65°C.

7. Nuclear Run-on

These procedures were performed with modifications of a previously described protocol (Linial et al., 1985). Each 100 µl reaction consisted of 21 µl of nuclei from WT and Mln^R cells, 36 µl of reaction buffer (reaction buffer is 0.3 M $(NH_4)SO_4$, 100 mM Tris HCl, pH 7.9, 0.4 mM $MgCl_2$, 4 mM $MnCl_2$, 50 mM NaCl, 0.4 mM EDTA, 0.1 mM PMSF, 1.2 µM DTT, 1 mM GTP, ATP, and CTP triphosphate mixture, 10 mM creatine phosphate, 20 U/ml of RNasin, 150 µM (alpha-^{32}P UTP, >600 Ci/mM) in 29% glycerol. The reaction was carried out for 30 min at 26-28 °C, was stopped with 100 µg tRNA and 10x DNase. Further steps included proteinase K treatment, filtration through a G-50 Sephadex column, TCA precipitation and sodium hydroxide treatment. The RNA was then resuspended in a solution of 20 mM HEPES, and 5 mM EDTA and 1 N NaOH. 1 M HEPES, pH 5.5 and 3M sodium acetate were then added, the solution was mixed, and the precipitate was washed in 70% ethanol. The pellet was finally resuspended in hybridization mixture, and aliquots were counted (newly transcribed nuclear RNA 8.53 × 10^6 c.p.m.). Nitrocellulose filters containing varying quantities of the GST-Yc cDNA (from pGTB42) were hybridized in this solution for 48hr at 42°C. The membranes were then washed as described and placed on film.

8. Cytotoxicity Assays

Cells were plated out in 100µl of medium at a concentration of 2-10 × 10^3 cells per flat-bottomed well in 96-well microtiter plates. Plates were incubated for 24 hr at 37°C in an atmosphere of 5% CO_2. One hundred µl of media containing drug dissolved in appropriate solvent were added to triplicate wells and incubated for a further 72 hr. One hundred microliters of medium were then removed from the wells and 25 µl of MTT were

then added to each well and incubated for 4 hr. The formazen crystals were dissolved in 100µl of isopropanol/1NHCl (24:1). The absorbance was recorded in an ELISA plate reader (BioRad) at a wavelength of 570nm. The IC_{50} values were determined for chlorambucil (CLB), mechlorethamine (HN2), melphalan (MLN), and cisplatin (CDDP). The fold resistance for Mln^R cells was determined by dividing the MLNr IC_{50} for each drug by the IC_{50} value obtained for WT cells. The fold resistance of the transfected cell lines was calculated by dividing the IC_{50} values obtained for neo-transfected cells.

9. DNA-DNA Crosslink Studies

DNA interstrand crosslinks were quantified by the alkaline elution assay. Exponentially growing cells were labeled with either 0.05 µCi/ml ^{14}C-thymidine or 0.5 µCi/ml ^{3}H-thymidine at a final concentration of 10^{-6}M for two cell doublings. The labeled cells were washed twice with cold PBS and the radioactivity was chased by an additional 16hr incubation in medium containing 10^{-5} M cold thymidine. The ^{14}C-labeled cells were treated in serum-free medium for 30 min, and treatment was then stopped by immediate chilling of cells on ice. Aliquots of cells were used immediately (0 time) or further incubated in drug-free medium for the accumulation and removal of DNA crosslinks. Control or drug-treated ^{14}C-labeled cells (0.5×10^6) were then mixed with ^{3}H-labeled cells (0.5×10^6) and irradiated with 6Gy using ^{60}Co gamma rays at a dose rate of approximately 1 Gy/min, at 4°C. The combined cell suspension was immediately prepared for alkaline-elution as described previously (Alaoui-Jamali et al., 1989). Briefly, 1×10^6 combined ^{3}H and ^{14}C labeled cells were layered on a polyvinyl chloride filter, washed with cold PBS and lysed with 5ml lysis solution (2% SDS, 0.025 M EDTA,) with or without 0.5mg/ml proteinase K (pH 9.7) for 45 min. Filters were then washed with 2×2 ml 0.02 M EDTA, pH 10.3 and the DNA was eluted with 30ml tetrapropylammonium hydroxide-EDTA buffer (pH 12.1) containing 0.5%SDS solution, at a flow rate of approximately 0.035ml/min, in the dark at room temperature. The ^{14}C and ^{3}H activities on filters and

fractions were analyzed by liquid scintillation counting and DNA-crosslink frequency, expressed as DNA-DNA crosslink index, was estimated as: $(1\text{-Ro})^{1/2}/(1\text{-R})^{1/2} - 1$, where Ro and R are the final fractions of ^{14}C and ^{3}H DNA retained on the filter, respectively.

10. Transport Studies

Exponentially growing cells (2×10^6 cells/ ml) were incubated with chloroethyl-^{14}C melphalan for different periods of time at 37°C. At the end of each incubation time, 400 µl of the incubation mixture were layered onto 1 ml of vestilube F-50 silicone oil in microfuge tubes and centrifuged at 12,000 ×g for 1 min at room temperature. The radioactivity in the medium and the cell pellet was determined as previously described (Panasci et al., 1988). Nonspecific absorption of labeled drug was determined by layering 200 µl of untreated cells onto 200 µl of medium containing labeled melphalan, at 4°C followed by immediate centrifugation as described above.

RESULTS

1. Expression of GST in Mln^R MatB Cells

Selection of WT cells in 10 µM melphalan (Mln^R) results in overexpression of GST-Yc subunit mRNA. To determine whether enhanced transcription of the GST Yc gene is responsible for the observed increase in Yc mRNA, nuclear run-on experiments were performed (Fig. 1). Drug- sensitive (WT) cells showed very low levels of initiated transcription complexes of the Yc gene whereas Mln^R cells demonstrated a much higher rate of transcription (Fig. 1A).

We have also isolated a Mln^R variant subline (Mln^{R+}) which has been grown in 10 µM melphalan for over a period of 18 months. These cells are shown to have markedly higher levels of Yc mRNA relative to Mln^R cells which were isolated far earlier in the

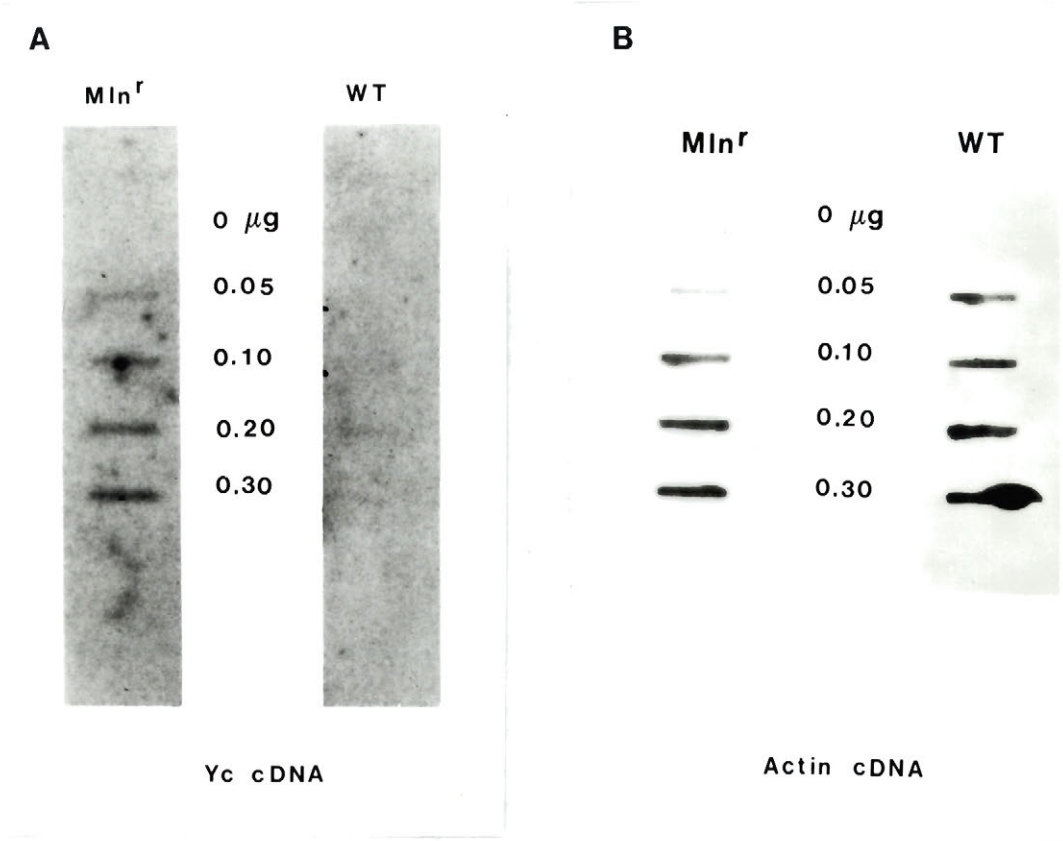

Figure 1. Nuclear run on analysis of initiated GST Yc transcription complexes in nuclei from WT and Mln^R cells. Nitrocellulose filters containing increasing amounts of cDNA (0.05, 0.10, 0.20 and 0.30 μg) were hybridized with ^{32}P-UTP-labeled newly transcribed RNA from WT and Mln^R cells.

A. Hybridization with GST-Yc cDNA.

B. Hybridization with actin cDNA.

protocol which selected for resistance to melphalan (Fig. 2A). Mln^R cells growing in 10 μM melphalan are 96-fold resistant to this drug based on MTT assays (Table 2), while Mln^{R+} cells are more resistant (183-fold) to melphalan.

2. Expression of Yc cDNA.

The pSVYc expression vector was co-transfected with pSV2neo into rat MatB mammary carcinoma cells and selected in the presence of the neomycin analog antibiotic G418 (5.2 units/ml). A total of 100 clones were isolated. Two clones designated R49 and M49-8 were found to have elevated GST activity. Integration of the recombinant GST Yc expression gene in the R49 and M49-8 clones was confirmed by Southern blot analysis (Fig. 3). A single band of approximately 700bp was detected in the GST-transfected, R49 and M49-8 cell lines but not in the WT or control pSV2neo-transfected MatB cells. This band corresponds to the expected restriction fragment size of the Yc cDNA in the pSVYc expression vector. The several other hybridizing fragments in all cell lines examined represent DNA sequences corresponding to the endogenous Yc gene.

Whole cell extracts were prepared from the various cell lines to quantitate the level of the Yc cDNA gene product. Drug sensitive cells (Fig. 4 lane 2) express very low levels of Yc protein. Selection in 10 μM melphalan resulted in a sharp increase in the level of this subunit (Mln^R lane 4). Transfection of the Yc cDNA yielded 2 clones, R49 and M49-8 with significantly elevated levels of Yc protein. We observed variable stability of expression of the transfected Yc gene. Clone R49 (early vs. late) demonstrated a significant decline in Yc protein expression over 18 months (despite the continued presence of G418 in the growth media) to a level just above that seen in the neo cells, whereas clone M49-8 does not show as appreciable a loss in expression over time (early vs. late).

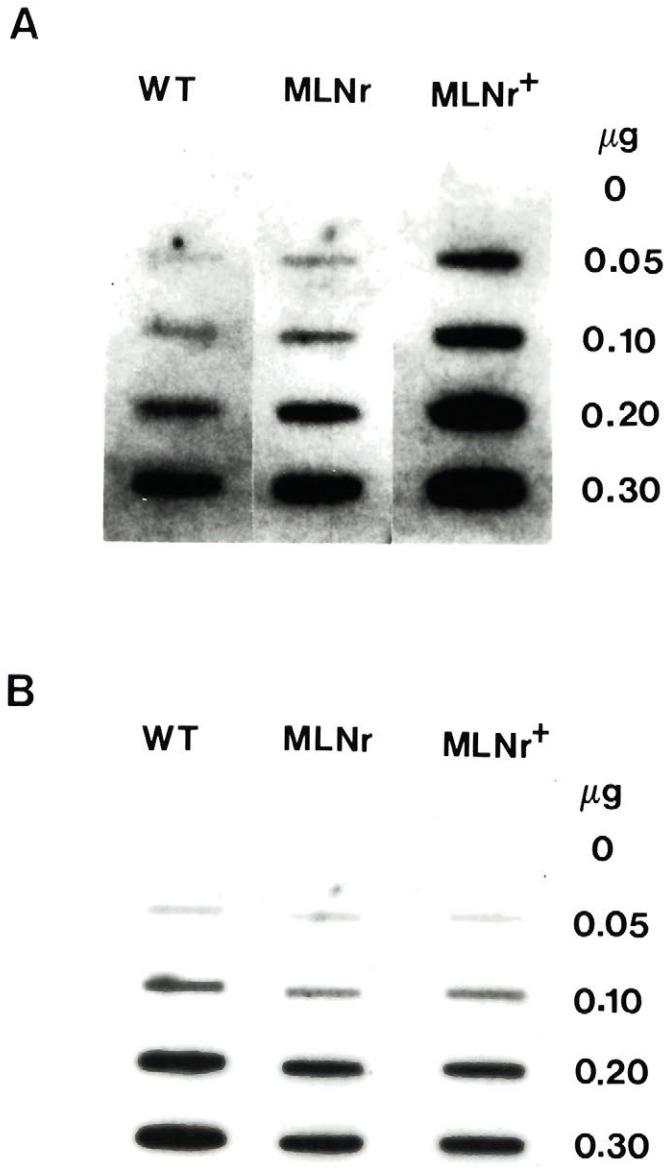

Figure 2. RNA slot blot analysis of total RNA isolated from WT, Mln^R and Mln^{R+} cells. Total RNA (0.05, 0.10, 0.20 and 0.30 μg) were blotted onto nitrocellulose membranes.

A. Samples were hybridized with Yc cDNA.

B. Samples were hybridized with actin cDNA.

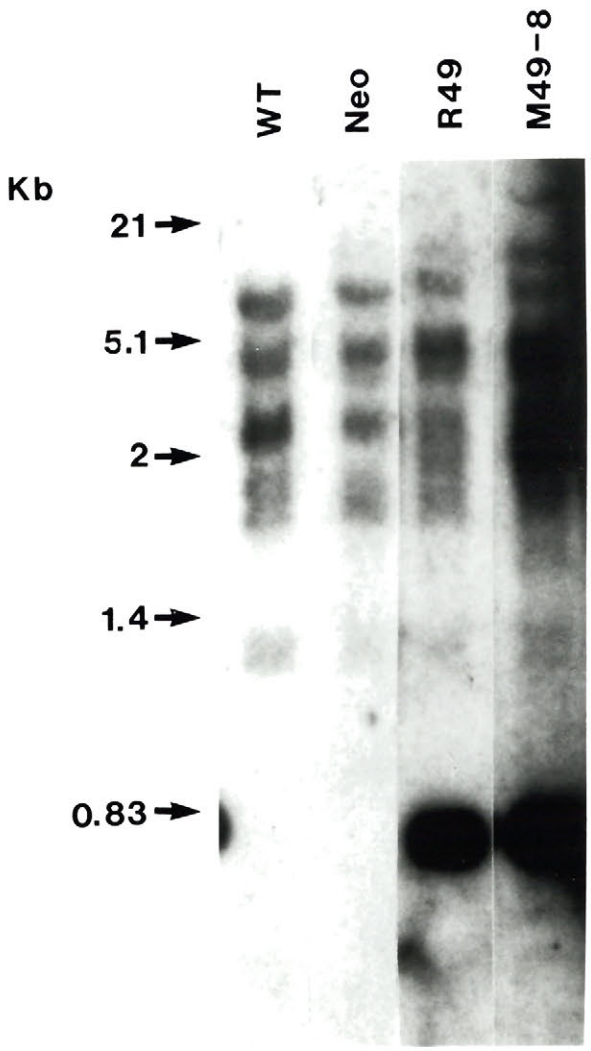

Figure 3. Analysis by Southern hybridization of WT, WT-neo and GST-Yc-expressing clones (R49, M49-8) of MatB cells. Ten µg of genomic DNA were digested with BamHI and XhoI, electrophoresed, blotted, and probed with a GST Yc cDNA.

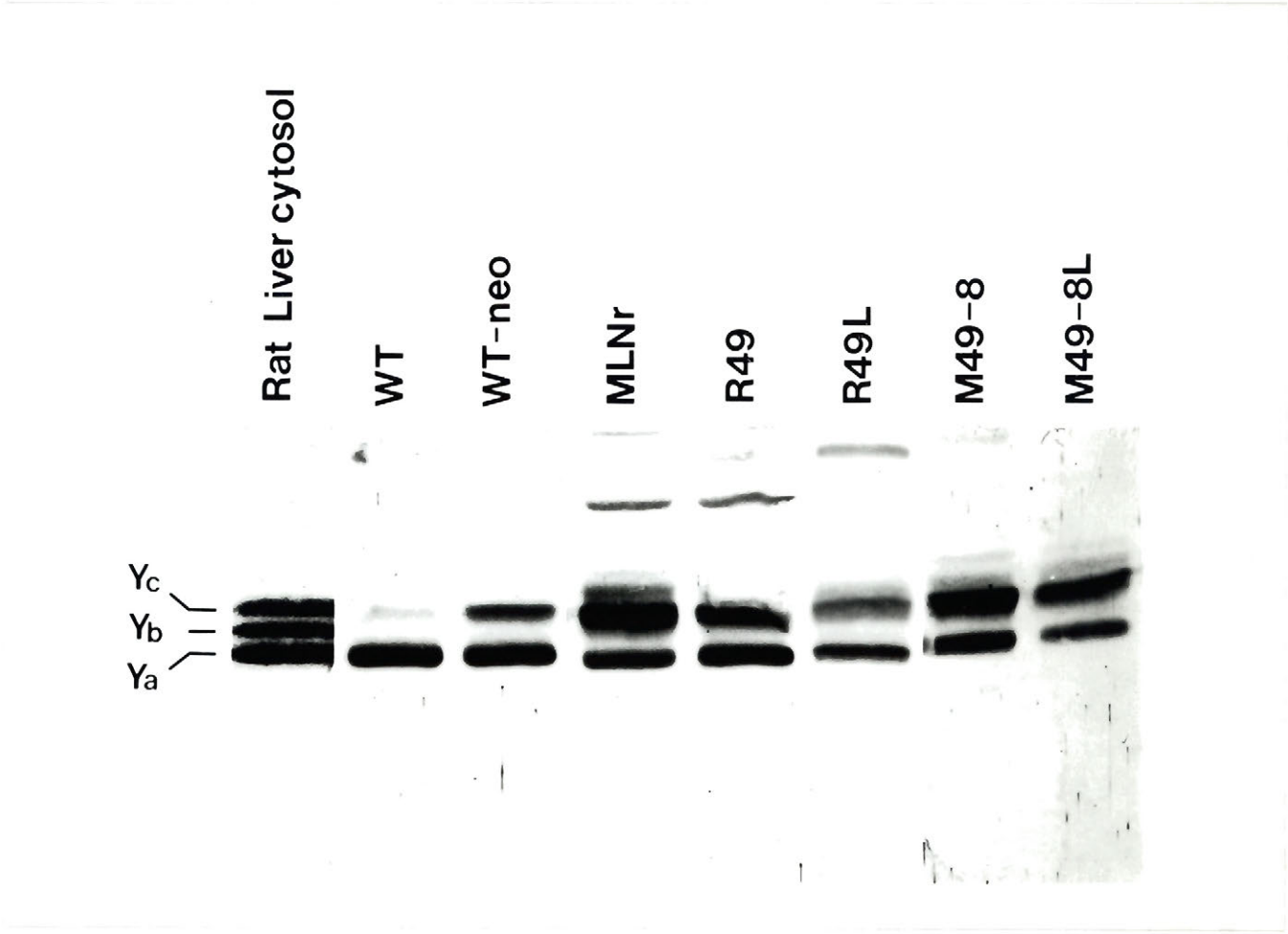

Figure 4. Quantitation of GST isozymes by immunoblotting in control (WT, WT-neo) and GST-Yc-expressing lines (Mln^R) and clones of MatB cells. Cytosolic proteins (75 μg of total protein) were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes for immunoblotting. Samples were reacted with a polyclonal antibody against cationic GST subunits. Normal rat liver cytosol containing GST subunits Yc, Yb and Ya served as markers in lane 1.

3. Biochemical Analysis of GST and Cytotoxicity.

Enzyme assays were carried out to determine GST activity levels in the isolated clones. Total GST enzyme activity was studied using the universal substrate CDNB. A range of activity levels was observed for the various clones isolated. Table 1 summarizes the data for clones R49 and M49-8 that consistently expressed higher GST enzyme activity (approximately 4-fold); Mln^R cells are included for comparison. In late passage cells, the GST activity levels correspond with Western analysis of the Yc subunit. Selenium-independent organic peroxidase activity has been shown to be associated with dimerized Yc/Yc enzyme (Jakoby, 1978). Cumene hydroperoxide was used to measure enzyme activity associated with Yc expression. GSH-peroxidase activity correlates well with the increase in total GST activity which presumably results from expression of the transfected pSVYc plasmid. Although GST has been shown to enhance the conjugation of drugs such as chlorambucil and melphalan with GSH, spontaneous conjugation of these drugs with GSH may be a mechanism of resistance in cell lines with elevations of this thiol. Consequently, intracellular GSH measurements were taken on the MatB transfectants. Clones R49 and M49-8 have cellular GSH levels which are comparable to neo-transfected cells.

Cytotoxicity assays were carried out using an MTT assay on MatB clones expressing recombinant GST Yc to assess their sensitivity to alkylating agents versus control cells (Table 2). Cells expressing increased GST Yc, either encoded by the endogenous gene (Mln^R) or by the expression gene (R49, M49-8), showed significant resistance to the alkylating drugs examined here. Importantly, the degree of resistance was significantly reduced in revertant cell populations (R49L, M49-8L), which had reduced levels of the recombinant GST Yc protein (Table 2).

For all of the drugs examined, the IC_{50} values were not significantly different between WT cells and WT cells transfected with pSV2neo (Table 2). These results indicate that transfection of the marker plasmid pSV2neo does not contribute to the drug sensitivity

Table 1. Intracellular levels of glutathione (GSH), glutathione-S-transferase (GST) and glutathione-peroxidase activity in MatB cells

Total GST activity using CDNB as substrate, glutathione-peroxidase activity using cumene hydroperoxide as substrate and total GSH content was measured in control cells (WT, WT-neo) and in a cell line (Mln^R) or clones of cells which were shown to express GST Yc. Each assay was performed in triplicate as three separate experiments.

Cell Line	GST activity nmol/min/mg protein	GSH peroxidase activity nmol/min/mg protein	GSH nmol/10^6 cells
WT	7.8 ± 1.0	4.7 ± 0.9	0.96 ± 0.05
Mln^R	37.0 ± 1.1	18.2 ± 1.2	2.12 ± 0.98
WT-neo	10.3 ± 1.2	5.7 ± 1.0	0.96 ± 0.05
R49	28.6 ± 1.1	9.5 ± 0.1	1.16 ± 0.33
R49L	13.6 ± 0.5	7.3 ± 0.2	1.16 ± 0.28
M49-8	30.1 ± 0.9	12.2 ± 0.9	0.89 ± 0.06
M49-8L	20.7 ± 1.0	9.5 ± 0.8	1.28 ± 0.28

Table 2. Relative resistance to alkylating chemotherapy drugs in MatB cells which were expressing GST Yc.

Sensitivity to each drug, as reflected by IC_{50} values, was determined using the MTT assay as described in Materials and Methods. Each toxicity study was performed at least seven times in triplicate. To establish fold resistance values (shown in parentheses) the IC_{50} value for Mln^R cells was divided by the IC_{50} value of WT cells, and the IC_{50} values of transfectants were divided by the IC_{50} value of the control cell line WT-neo. IC_{50} values are expressed in μM.

	IC_{50}						
Drug	WT	Mln^R	WT-neo	R49	R49L	M49-8	M49-8L
chlorambucil	0.73 ± 0.08	48.5 ± 5.6[a](68)	0.79 ± 0.09	6.5 ± 2.0[b](8.2)	3.7 ± 1.1[c](4.7)	24.3 ± 7.7[b](30.8)	18.2 ± 5.6[b](23)
mechlorethamine	0.04 ± 0.01	3.5 ± 0.47[a](87)	0.06 ± 0.01	0.6 ± 18[b](10)	0.08 ± 0.03(1.3)	0.96 ± 0.21[b](16)	0.34 ± 0.11[b](5.6)
melphalan	0.26 ± 0.03	24.9 ± 2.2[a](96)	0.29 ± 0.02	1.9 ± 0.36[c](6.6)	0.49 ± 1.0 (1.7)	3.7 ± 1.1[c](12.8)	0.52 ± 0.18 (1.8)
cisplatin	0.26 ± 0.04	40.0 ± 9.1[b](155)	0.25 ± 0.04	0.3 ± 0.04(1.2)	ND	1.1 ± 3.2[b](4.4)	ND

a $p < 0.001$
b $p < 0.01$
c $p < 0.05$

even though this results in overproduction of the Yc subunit (Fig. 4). The level of resistance for the Yc-expressing clones ranged from 11.7 to 30.8-fold for chlorambucil, 10 to 16-fold for mustargen, 6.6 to 12.8-fold for melphalan and 1.2 to 4.4-fold for cisplatin. The M49-8 clone, which expresses the highest GST activity (Table 1), was consistently more resistant to each of the drugs tested. The sensitivity of clones R49 and M49-8 were tested after an extended length of time in tissue culture (18 months). Clone R49L, which demonstrates a significant reduction in Yc expression on immunoblot (Fig. 4) with a coordinate loss of GST activity (Table 1), also becomes significantly more sensitive to the alkylating drugs tested. In clone M49-8L, where the loss of Yc expression and activity is not as dramatic, the reduction in the fold resistance is likewise smaller than that seen with the R49/R49L pair. These paired early and late passage samplings of GST Yc^+ clones, which demonstrate a change from high GST Yc activity to low GST Yc activity and a concomitant change to greater drug sensitivity, argue against the likelihood that clonal variation explains the drug resistance results in these MatB clones expressing recombinant GST Yc.

Because melphalan is actively transported into cells by two separate amino acid transport systems, the sodium-dependent ASC-like (alanine-serine-cysteine) system and the sodium-independent L system (leucine- preferring) (Begleiter et al., 1979), drug transport studies were carried out to determine whether alterations in the membrane of Yc-expressing clones could account for their resistance to melphalan. The uptake of ^{14}C melphalan by MatB cells is shown in Fig. 5. These data show that between the two GST Yc-expressing clones and the control cells there is no significant difference in melphalan accumulation which might account for the observed differences in cytotoxicity of melphalan.

4. DNA-DNA Crosslink Studies

The cytotoxicity and antitumor activity of bifunctional nitrogen mustards is related to their ability to undergo bifunctional addition reactions with DNA producing interstrand and

intrastrand crosslinks as well as DNA-protein crosslinks (Ewig and Kohn, 1977; Kohn et al., 1981). DNA crosslinking, especially of the interstrand type, has been shown to correlate with cytotoxicity to nitrogen mustard derivatives (Ewig and Kohn, 1977; Kohn et al., 1981). The alkaline-elution technique allows for the measurement of both interstrand crosslinks and DNA-protein crosslinks. DNA crosslink formation was estimated based on diminished X-ray sensitivity of cellular DNA. Single strand DNA breaks induced by irradiation produces enhanced elution. In cells that have been treated with a crosslinking agent the effect of X-irradiation is reduced and elution is retarded. Since DNA is a target for alkylating drugs, we quantitated the presence of DNA crosslinks induced by mechlorethamine in the most resistant GST Yc^+ clone to see if they would be reduced because of GST Yc-catalyzed conjugation of mechlorethamine. Control WT-neo cells and clone M49-8 cells were exposed to 30μM mechlorethamine for 30 min and DNA crosslinking was measured at various times following this treatment. Figure 6A illustrates the results of this experiment. Immediately following treatment, the accumulated interstrand crosslinks in M49-8 cells were significantly less than in control cells. At 2 and 20 hr post-treatment, DNA crosslinks measured were uniformly less in M49-8 than in WT-neo cells. When proteinase K was omitted from the lysing solution (see Materials and Methods), the amount of DNA-protein crosslinks was similar between control WT-neo cells and clone M49-8 cells (data not shown).

Because extended passaging of M49-8 cells resulted in a partial revertant line with a decreased Yc level and an increased sensitivity to alkylating agents (M49-8L), alkaline elution analysis was carried out to determine whether the loss of drug resistance in M49-8L cells was associated with a diminished ability to prevent mechlorethamine-DNA crosslinks (Fig. 6B). In M49-8L cells where GST activity is significantly decreased from the early passage M49-8 cells, there is no longer a significant difference in the level of crosslinks between the M49-8L and WT-neo control cells.

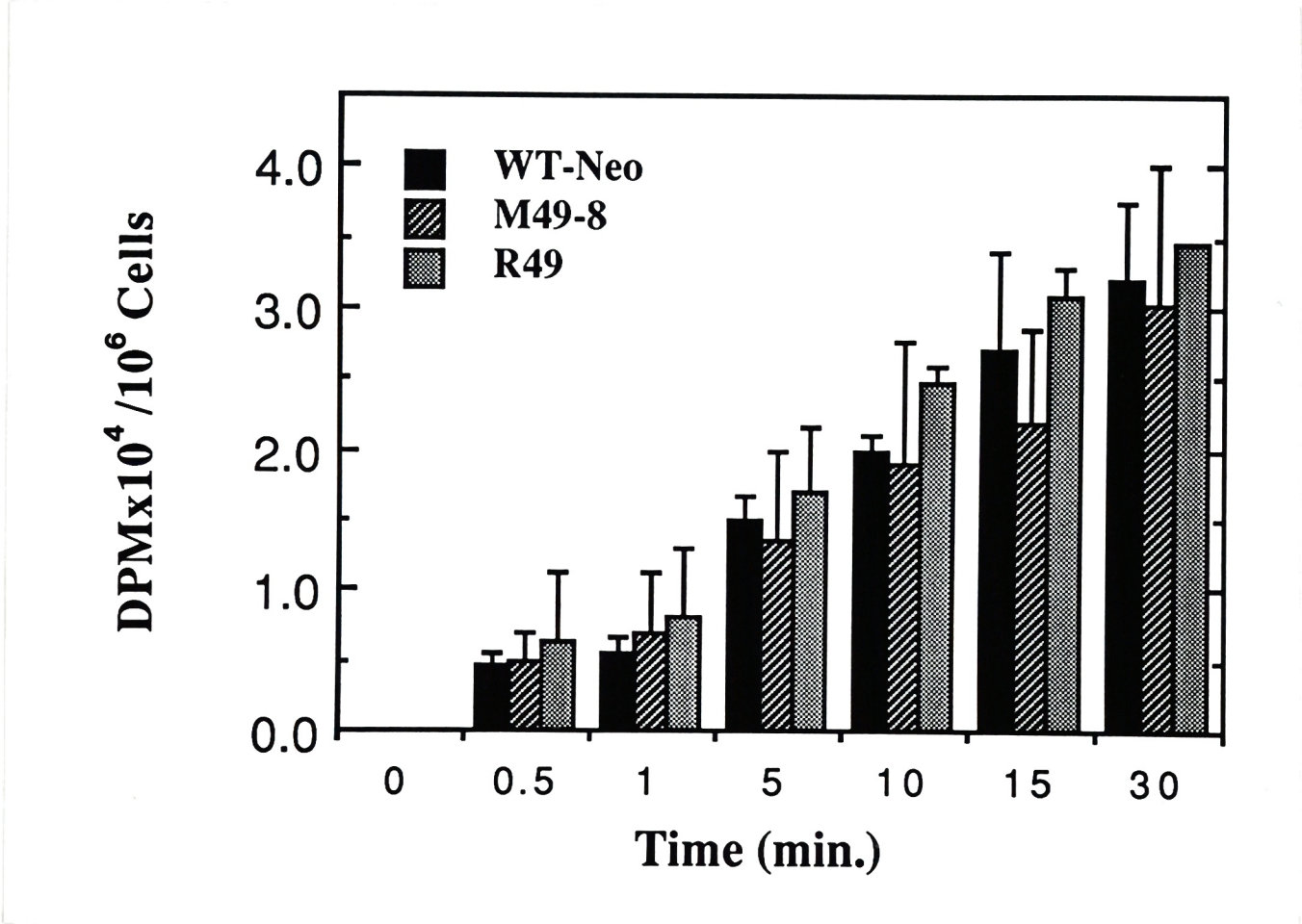

Figure 5. Melphalan accumulation in WT-neo cells and GST-Yc-expressing clones of MatB cells. Exponentially growing cells (2×10^6) were incubated with ^{14}C-MLN for different time intervals at 37°C. Each point corresponds to the mean value ± SE generated from at least three experiments.

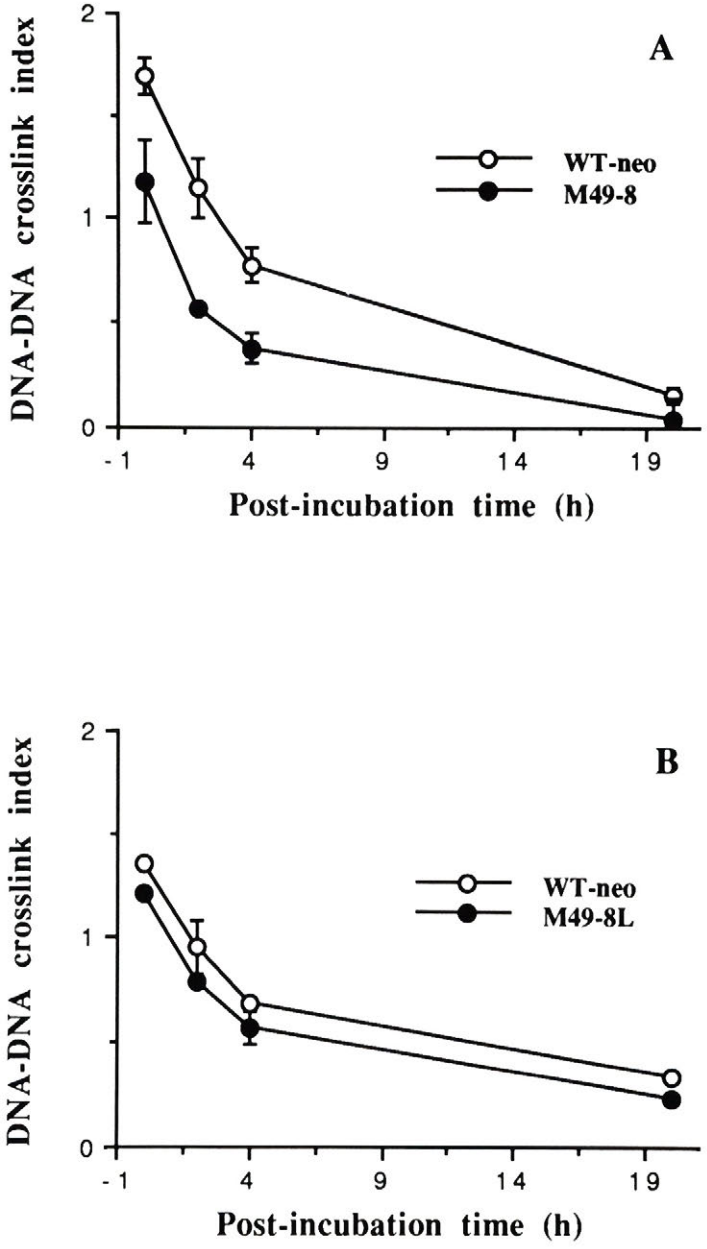

Figure 6. DNA crosslinks induced by mechlorethamine in WT-neo and GST-Yc-expressing clones of MatB cells. Exponentially growing cells (1×10^6) were labeled with ^{14}C-thymidine and treated with 30 μM mechlorethamine for 30 min in serum-free medium. Cells were then either used immediately for alkaline elution or further incubated in drug-free medium for the removal of crosslinks over the time indicated. Each point represents mean value ± SE calculated from three independent experiments. **A.** Analysis of WT-neo vs M49-8 cells. **B.** Analysis of WT-neo vs M49-8L cells.

DISCUSSION

To determine the role of GST-catalyzed detoxification in diminishing DNA crosslink formation by bifunctional nitrogen mustards, we previously used a competitive inhibitor of GSTs to treat Mln^R MatB cells (Alaoui-Jamali et al., 1992). Ethacrynic acid exposure results in an increase in DNA crosslink formation in cells treated with nitrogen mustard; this suggested that GST was playing a role in inhibiting the formation of these crosslinks rather than contributing to repair of damage. In this study we examined this directly by transfecting a GST-Yc cDNA into drug sensitive cells. We have demonstrated that stable expression of a rat Yc cDNA yields functional enzyme in MatB mammary carcinoma cells, and that these Yc-expressing cells are resistant to a variety of bifunctional alkylating agents by virtue of decreased DNA crossslink formation. Resistance to the alkylating drugs correlated with the amount of Yc protein present in the cells, both between clones as well as within a single clone. Clone M49-8, which expresses the highest level of resistance to these agents, demonstrates a higher level of GST activity than does clone R49. Partial reversion of Yc expression in both clones, although to different degrees, is mirrored by increases in the respective sensitivities of the cells to all the drugs tested. These two clones demonstrated variability in retention of the transfected gene, perhaps related to the site of integration of the expression cassette within the genome and in relation to the neomycin resistance gene. The number of integrated Yc cDNAs in the transfected cells ranges between 40-50 copies. The technique used for gene transfer was designed to encourage stable transfection of the Yc cDNA. Cells that replicate episomal DNA without selection will usually lose the episome over a 4-week period (Puchalski and Fahl, 1990). We have not observed such a phenomenon; it is therefore likely that the cDNA transferred has become incorporated into the host cell genome.

Although there is a relationship in our experiments between the concentration of the Yc subunit and the level of sensitivity to alkylating agents, for Yc to confer resistance to alkylating agents, a threshold overexpression of Yc was necessary. This was observed in

an earlier published report (Schecter et al., 1992) where Yc transfectants with lower levels of Yc than those achieved here were not resistant. A novel observation here is that selection of WT MatB cells transfected with pSV2neo alone in G418 results in overexpression of the endogenous Yc gene, however, to a level below that observed in Yc transfectants that are resistant to alkylating drugs. This observation also supports the hypothesis that a minimum level of Yc protein in tumor cells is necessary to confer resistance. Selection of the parental WT MatB cell line in G418 results in equivalent overexpression of the endogenous Yc gene as that observed in pSV2neo-transfected/G418-selected cells. It therefore appears that G418 or one of its metabolites induces expression of the endogenous Yc gene.

There is significant published evidence which correlates bifunctional alkylator cytotoxicity to the formation of DNA-DNA interstrand crosslinks (Ross et al., 1978; Kohn et al., 1981). Using an alkaline elution assay, we have demonstrated that in Yc-overexpressing clone M49-8, the initial crosslinks which accumulated following mechlorethamine treatment are significantly less than that seen in WT-neo control cells. Furthermore, in late passage cells of the M49-8 clone which partially revert to a more sensitive phenotype, DNA crosslink formation approaches the pattern observed in WT-neo control cells.

GST Yc conferred resistance is highly specific for alkylating agents. Similar studies using the same target cells and the identical vector containing a Ya cDNA instead, showed no resistance to alkylating drugs despite equivalent increases in GST activity (data not shown). Furthermore, the Yc^+ transfectants in the present study were not significantly resistant to adriamycin or radiation, but rather only to the alkylating drugs shown in Table 2. This confirms and extends a previous study demonstrating that the Yc peroxidase activity has very specific and defined substrates that result from oxygen-radical reactions. T47D cells transfected with Yc cDNA were resistant to cumene hydroperoxide and singlet oxygen but not to free-radical generating adriamycin (Lavoie et al., 1992). According to

our data H_2O_2 produced by ionizing radiation is not reduced by peroxidase mediated GST-Yc.

Transfection of a Ya cDNA into COS and 10T1/2 cells (Puchalski and Fahl, 1990) conferred resistance to chlorambucil and melphalan (1.3 to 2.9-fold). These cells do not express highly constitutive levels of the Ya subunit. Similar studies using the identical Ya expressing vector in MatB cells failed to produce any resistance to alkylating agents. Transfection of the Ya gene into MatB cells did not results in a marked elevation in Ya subunit protein. The discrepancy in results between the different cell lines may be explained by this observation and may additionally indicate a difference between the Ya and Yc subunits in terms of their conjugating affinity towards alkylating agents.

Given the fact that GSTs are enzymes requiring co-substrates and sufficient enzyme properly localized, it is not surprising that some cells may not be effective transfection targets. The unusually low basal GST activity in MCF-7 cells may explain the consistently negative results. The lack of resistance to nitrogen mustards in alpha/Yc transfected MCF-7 cells may be due to the target cell examined in combination with the gene transferred. These cells may require accessory proteins which act in conjunction with Yc to exert their protective role.

The results reported here corroborate the findings of earlier reports which hypothesized an association between nitrogen mustard resistance *in vitro* and *in vivo* to the increased expression of an alpha class GST. We have shown that the Yc GST is specifically overexpressed in cells chronically exposed to melphalan. If enough of this protein is present in transfected cells, the cells will be protected from the cytotoxic effects of drugs in this class. This represents important additional evidence that despite the high degree of sequence homology between the Ya and Yc subunits (75%) both the regulation and substrate specificity are significantly different. The regulatory region of the Ya gene has been studied in some detail (Rushmore et al., 1991; Nguyen and Pickett, 1992). Regulation of the Yc gene has not been determined.

Modulating the catalytic efficiency of GST Yc by identifying specific inhibitors or protecting sensitive tissues such as the bone marrow by introducing a recombinant Yc

We are currently isolating and analyzing the specific Yc that is overexpressed in Mln^R MatB cells. This will be compared to the rat liver Yc used in these experiments in order to determine whether there is tissue and inducer -specific forms of this GST.

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5.1 General Discussion

A significant limitation of the effectiveness of antineoplastic chemotherapy is the appearance of drug resistance. Elucidation of the cellular mechanisms responsible for this resistance could be of benefit to cancer patients.

As more effort has been focused on understanding the cellular, molecular and physiological mechanisms underlying drug resistance, it has become clear that a number of different factors are involved in the development of drug resistance. Studies at the cellular level have demonstrated that overexpression of membrane gylcoproteins involved in drug efflux, elevated levels of redox active molecules, and elevated activities of enzymes involved in detoxification can provide significant cellular resistance to chemotherapy drugs. The extent to which these biochemical adaptations contribute to drug resistance *in vivo* is at present not clear.

This thesis has examined the biochemical changes associated with the development of chemotherapy resistance. A novel experimental model has been developed for studying resistance in tumor cell lines in both *in vitro* and *in vivo* conditions. MatB 13762 is a mammary carcinoma cell line established in Fischer female rats. The tumor cells grow *in vitro* with good cloning efficiency, and when injected subcutaneously they grow as solid masses that develop a vascular supply and metastasize to regional lymph nodes. The *in vivo* characteristics of the tumor are similar to clinical breast cancer. The tumors grown both *in vitro* and *in vivo* are estrogen-receptor negative, and histologically appear to be poorly differentiated adenocarcinoma.

To further understand drug resistant phenotypes, two different cell lines were established following exposure to antineoplastic drugs. In one case, drug sensitive cells were exposed to an anthracycline antibiotic (Adr^R) and in the other, they were treated with a commonly used alkylating agent (Mln^R). One of the important features of this experimental model is that animals bearing either the WT or the resistant tumor cell lines

can be treated intravenously to determine the *in vivo* resistance of the cells to any particular drug and the effect of a biochemical modulator on sensitivity to the drug.

The Adr^R cells display classic overexpression of the multidrug resistance gene (MDR). They also demonstrate enhanced glutathione-S-transferase activity. Following implantation of these cells into syngeneic rats, the GST activity returns to WT levels. The predominant feature, however, remains the overexpression of the MDR gene although it is significantly diminished in comparison to that observed *in vitro*.

The loss of expression of these two markers *in vivo* cannot be simply explained by a dilutional effect *in vivo*, as determined by histological and immunocytochemical (Factor VIII) examination of tumor tissue, or a loss of resistance since Adr^R maintained in drug free culture for over 12 months do not revert to a more sensitive phenotype. These data suggest that the host environment affects the expression of tumor cell genes, including some related with resistance.

Cells selected for resistance to melphalan display a significant increase in GST activity *in vitro*. These cells also have elevated levels of the corresponding conjugating molecule, GSH. We have determined that continuous exposure of MatB cells to this drug results in overexpression of the Yc gene and that this is by transcriptional activation. The MatB melphalan-resistant cells selected *in vitro* for resistance to melphalan when grown *in vivo* have a 2.7 fold increase in GST activity relative to the WT tumor grown *in vivo*. Sensitivity to intravenous melphalan in *in vivo* grown tumors is approximately one quarter (i.e., four-fold resistant) that of WT cells. These levels of modulation biochemically and biologically are small; however, they are consistent with the findings both *in vitro* and *in vivo* of alkylator resistance. Tumor tissue from a patient with ovarian cancer that was clinically resistant was compared with specimens from the same patient obtained at the start of therapy. These cells demonstrated a 2.1-fold increase in GST level and a 3-fold level of resistance to cisplatin and to chlorambucil, both of the latter measured *in vitro* from the cells harvested from the patient. Similarly in patients with CLL, it has been found that

patients resistant to nitrogen mustards have approximately a two-fold increase in GST activity. A consistent observation *in vitro* and *in vivo* in the Mln^R MatB cells is the overproduction of the Yc GST subunit.

Gluthathione and GSH-related enzymes (GSTs) are known to function in the cellular detoxification of potentially harmful xenobiotics and oxygen-related toxic species. Due to the nature of the role of GSTs they might be expected to be altered in some way in cells which become resistant to antitumor drugs, in order to enhance the efficiency of the cells' detoxification of such agents. A number of factors have strongly supported a direct role of GST forms in chemotherapy resistance. The data are strongest with regard to alkylating agents. Many alkylator-resistant sublines have been shown to overexpress GST alpha class activity. Alkyl chlorides and aziridinium ions, spontaneously forming reactive intermediates of alkylating agents, react with GSH to form conjugates and these reactions can be catalyzed by GSTs. Resistant cell lines that revert to a more sensitive phenotype lose their elevated GST activity.

The importance of GSTs in altering cellular response to certain chemotherapy drugs has been demonstrated by virtue of agents that can inhibit enzyme activity. Inhibition of GST function in a variety of cell types has been shown to enhance the cytotoxicity of chemotherapy drugs. These findings, in conjunction with elevations in GST activity in resistant tumor cells, provide strong evidence that these detoxifying enzymes play a major role in cellular resistance to anticancer drugs.

As stated above, overexpression of alpha class GST is most likely to be associated with alkylating agents, and in particular, nitrogen mustards. The finding of increased Yc activity in alkylator resistant sublines, together with observations by Colvin and colleagues that α class GSTs conjugate GSH with melphalan, led to studying the role of Yc directly in drug resistance using the MatB model. A full-length cDNA complementary to the GST Yc subunit was transfected into WT cells with a baseline drug sensitivity to chemotherapy

drugs. Individual clones expressing varying levels of Yc protein could tolerate growth at higher drug concentrations.

These clones were shown to overexpress the Yc subunit. The overall GST activity observed in transfected Yc clones paralleled those found in Mln^R MatB cells. Using a colorimetric cytotoxicity assay, it was determined that there exists a relationship between the concentration of the Yc subunit and the level of sensitivity or resistance to alkylating agents. A significant amount of Yc protein must be expressed to confer a relatively small level of resistance. It is possible, therefore, that the GST Yc overexpressed in alkylator selected MatB mammary cells is more effective than the rat liver Yc at conferring resistance.

To determine the mechanism of resistance to nitrogen mustard in the transfected cells, the interaction of the drug with DNA was examined. For nitrogen mustard-type drugs, there is a significant amount of evidence correlating cytotoxicity to the formation of DNA-DNA interstrand crosslinks. Using alkaline elution, the kinetics of DNA crosslinks were studied.

The overexpression of Yc in transfected clones was associated with decreased DNA crosslink formation by nitrogen mustard. In clones that revert toward the WT level of Yc expression, there is an increased sensitivity to alkylating agents with a corresponding increase in DNA crosslinks. The demonstration that the Yc form of GST can confer resistance specifically and selectively to the bifunctional alkylating agents is important from a chemotherapy perspective because of the frequent use of these compounds in the clinic. These drugs may also be seen as representative of a number of environmental xenobiotics which alkylate DNA and form interstrand crosslinks.

These studies confirm a direct role of GST, particularly Yc, in protecting tumor cells from the cytotoxic effects of alkylators, namely inhibiting the formation of drug-induced DNA crosslinks, and suggest that glutathione-S-transferase is a cause rather than a consequence of resistance.

In the clinic, some patients may benefit from strategies to overcome resistance. It is reasonable to assume that the chances of responding to, for example, high dose alkylator administration depend on both the level of resistance in the individual tumor and the specific mechanism(s) of resistance involved. The potential mechanisms of resistance available to malignant cells may depend on their tissue of origin and on their stage and degree of differentiation, i.e., on the specific repertoire of genes expressed. Thus, mechanisms of resistance activated in response to drug exposure are likely to depend on many factors, the combination of which may be unique for each individual tumor.

Therapeutic strategies to enhance chemotherapy effectiveness have generally attempted to overcome or block the mechanism of resistance. An alternative approach would be to use a known mechanism to confer resistance to accessible normal tissue.

The principal limitation of cytotoxic agents is that they lack specificity. Because hematotoxicities constitute the dose-limiting toxicities for most drugs, the hematopoietic system would be the principal target in this type of strategy.

Clinical trials using high dose chemotherapy followed by autologous bone marrow transplantation "rescue" suggest a benefit to dose-intensification, but this strategy is limited to one or two courses of high-dose treatment. Protecting the hematopoietic system would permit repeated cycles of high-dose chemotherapy.

Alkylating agents have a dose-related effect through multiple logs of tumor cell kill and, therefore, are very appropriately used in dose-intensification regimens; they are also active against a wide range of tumor cell types. In the studies presented in Chapter III it was demonstrated that the Yc isoform is not constitutively expressed by rat or human bone marrow cells. Bone marrow cells are, therefore, an ideal target tissue for studying the protective effect of the Yc subunit in dose-intensification regimens.

All the known classes of GST have the potential to become overexpressed in drug resistant cell lines. The finding that different selective agents can result in the overexpression of different GST subunits in the same line (in Mln^R MatB cells where both

π and α class subunits become overexpressed, for example) indicates that the GST subunit which is actually changed may depend on a range of factors such as the type of selective agent, how the selection is carried out, and the nature of the target cell. The capacity to induce different GST subunits, dependent on the selective agent, suggests that GSTs have evolved as part of an adaptive response to environmental stress. Transfection of a GST gene into drug sensitive MatB tumor cells has provided evidence that these enzymes are part of a protection mechanism against toxic stimuli.

5.2 CLAIMS TO ORIGINAL RESEARCH

The following novel findings and observations have been demonstrated in this thesis:

1. Two new drug resistant mammary carcinoma cell lines have been established, Adr^R and Mln^R MatB. These cells have the capacity to be grown in tissue culture and in syngeneic animals permitting a multitude of *in vivo* experiments with regard to minimizing the risk of the development of resistance and devising new strategies for overcoming resistance (Chapter II).

2. An apparent relationship between the selecting agent and the mechanism of resistance has been identified using these different tumor cell lines *in vitro* and *in vivo* (Chapter II). The predominant feature in the alkylator-resistant subline is increased GST activity. In the adriamycin-resistant subline, resistance appears to be mediated by expression of p-glycoprotein.

3. Most previous work on drug resistant cell lines selected *in vitro* have identified biochemical changes which have been thought to also exist *in vivo*. This theory is unsupported in these studies, as demonstrated by the loss of GST-Yp expression in Mln^R cells once implanted *in vivo* (Chapter II). Thus, not all *in vitro* observations may apply to the *in vivo* situation, suggesting an important role of the tumor cell microenvironment in determining gene expression.

4. Overexpression of the GST-Yc subunit *in vitro* (via increased transcription, Chapter IV) and *in vivo* is especially related to the acquisition of alkylator resistance. Cells selected for adriamycin resistance do not diplay these features (Chapter II).

5. Transfection of a GST-Yc expression vector into drug-sensitive WT MatB cells and isolation of positive clones with increased Yc protein levels (Chapter IV),

demonstrated a causal relationship between drug resistance and the expression of a specific protein.

6. Yc transfected clones resistant to alkylating agents, as determined by cytotoxicity assays, demonstrated decreased DNA crosslink formation. Transfectants which reverted toward the WT Yc phenotype and were more drug sensitive showed enhanced DNA crosslink formation (Chapter IV). This finding supports a direct role of GST in diminishing the cytotoxic potential of alkylating drugs.

7. The GST subunit profile of bone marrow cells has been characterized in rat and humans. Similar to rat and human mammary tumors, bone marrow cells from these species either lack or express amounts of Yc protein too low to visualize using immunoblotting (Chapter III). Hematopoietic cells may, therefore, be an appropriate target tissue for GST-Yc gene transfer studies.

5.3 SUGGESTIONS FOR FURTHER WORK

To enhance the therapeutic efficacy of antineoplastic drugs in the treatment of mammary cancer, further molecular characterization of the various components of these resistant sublines may be of potential clinical utility.

1. Calcium channel blockers have proven successful in reversing the multidrug resistant phenotype *in vitro*. However, because of host toxicity, the use of these agents in the clinic is prohibited. The ability of Adr^R MatB cells to grow *in vivo* in syngeneic animals provides the opportunity to study novel modulators of the MDR phenotype which may be of clinical benefit.

2. Preliminary studies of the Yc transfected MatB cells *in vivo* has indicated that expression of this protein confers resistance to melphalan. A significant tumor growth delay was observed in rats bearing Yc transfected tumors compared to neo-control tumor bearing animals. Further *in vivo* studies would permit an evaluation of Yc protein content and the associated resistance pattern.

3. The ability of the Yc form of GST to confer alkylator resistance together with the absence of the Yc isoform in human and rodent bone marrow is an observation with potential importance. Transferring a Yc expression vector, using retroviral techniques, into the hematopoietic system may augment the patient tolerance to alkylating drugs. This novel approach can be tested experimentally using the MatB model.

4. From the transfection studies performed here, the involvement of GST Yc in alkylator resistance has become clearer. However, it is apparent that a significant amount of protein must be expressed to confer a relatively small level of resistance. This is in comparison to the Yc protein content of alkylator-selected MatB cells and their considerably higher level of conferred resistance. This finding suggests that the Yc

gene product in resistant tumors may be different (greater reactivity or affinity for melphalan) than the Yc overexpressed in transfected cells using a rat liver cDNA. Cloning the Yc cDNA from the Mln^R cell line would provide valuable information regarding the efficiency of the Yc protein. Studying the regulation of the Yc gene would provide insight into the mechanism responsible for transcriptional activation, as has been demonstrated in this work.

5. In order to more accurately reflect the events which result in expression of drug resistance, a longitudinal study of cell lines throughout serial drug selection should reveal more information about the sequence of events which may be involved in the induction or selection for MDR and GST and, thereby, provide information about possible prevention or modulation of these events in the clinic during therapy. Additionally, tumor biopsies could be obtained from patients with various disease stages and studied to determine the relevance of protein alterations to clinical drug resistance.