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**MUTATION INDUCTION BY
REACTIVE NITROGEN SPECIES AND NEUTROPHILS
IN AN IN VITRO/IN VIVO MURINE TUMOUR MODEL**

Helen Privora

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in
partial fulfillment of the requirements for the degree of Master of Science

University of Ottawa
Ottawa, Ontario, Canada

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ABSTRACT

Our laboratory has been interested in factors in the tumour microenvironment that may contribute to mutagenesis, and thus lead to tumour progression. The well documented relationship between chronic inflammatory states and cancer implicates inflammatory cells, such as macrophages and neutrophils, and their products as having a role in carcinogenesis. Using the MN-11 system, an *in vitro/in vivo* murine tumour model developed in our laboratory, the mutagenicity of neutrophils and one of their products, reactive nitrogen species, was studied. To further investigate the mechanism of the observed mutagenicity of nitric oxide donors on MN-11 cells, cellular glutathione levels of cells treated with glyceryl trinitrate (GTN) and sodium nitroprusside (SNP) were manipulated. Contrary to expectations based on radiation-induced free radicals, depletion of glutathione to less than 2% of controls by buthionine sulfoximine (BSO), resulted in a dramatic reduction in GTN- or SNP-induced mutations. Paradoxically, overproduction of glutathione levels by cells following removal of BSO, or treatment with agents that act to increase glutathione levels (N-acetylcysteine and L-2-oxo-4-thiazolidine carboxylic acid) also resulted in a dramatic reduction in GTN- and SNP-induced mutations, to near control levels. These results suggest a dual role for glutathione, as both protector against NO donor-induced mutations, and as a participant in causing them. Similar trends were seen in the corresponding *in vivo* experiments. Treatment with other NO donors (3-morpholinopyrrolidine (SIN-1), S-nitrosoglutathione (GSNO), and diethylenetriamine (DETA-NO)) did not cause mutations in MN-11 cells.

Reactive nitrogen species, such as nitric oxide, are part of the microbicidal armament of neutrophils. Neutrophils have been implicated as a possible link between chronic inflammation and cancer, and are the most abundant inflammatory cell type observed in MN-11 tumours. Thus, the role that neutrophils have in causing mutations was investigated. Intratumoural injection of interleukin-8 was used to attract larger numbers of neutrophils to the tumours. Over 70 tumours were analyzed for mutation frequency, number of neutrophils, and percent necrosis. Both the number of infiltrating neutrophils and the percent tumour necrosis were highly statistically significant predictors of mutation frequency and were highly correlated with each other. These results provide supporting evidence for the role of inflammatory cells in carcinogenesis.

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LIST OF ABBREVIATIONS USED

ANCOVA	analysis of covariance
ANOVA	analysis of variance
BCNU	1,3-bis(2-chloroethyl)-nitrosourea
BSO	buthionine sulfoximine
c.c.	cubic centimetre
C57BL/6	C57 black6 syngeneic mice
CDTA	<i>trans</i> -1,2-diaminocyclohexane- <i>N,N,N',N'</i> -tetraacetic acid
CHO	Chinese Hamster Ovary cells
CNOS	constitutive nitric oxide synthase
DAB	diaminobenzidine
DETA/NO	diethylenetriamine
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FISH	fluorescence <i>in situ</i> hybridization
FCS	fetal calf serum
fMLP	N-formyl-methionylleucylphenylalanine
FMN	flavin mononucleotide
G418	Geneticin
GSH	reduced glutathione
GSNO	S-nitrosoglutathione
GTN	glyceryl trinitrate (also known as nitroglycerin)
H&E	hematoxylin and eosin
HAT	hypoxanthine, aminopterin, thymidine
hprt	hypoxanthine - guanine phosphoribosyltransferase
hr	hour(s)
HT	hypoxanthine, thymidine
IL-8	interleukin-8
INOS	inducible nitric oxide synthase
i.p.	intraperitoneal
LANA's	modified Zamboni's fixative
log	logarithm
LTB ₄	leukotriene B ₄
MF	mutation frequency
min	minutes
MN-11	mouse fibrosarcoma cell line
MNU	N-methyl-N-nitrosourea
NAC	N-acetylcysteine
neo	neomycin
NOS	nitric oxide synthase
OCT	optimal cutting compound

OPT	o-phthaldialdehyde
OTZ	L-2-oxo-4-thiazolidine-carboxylic acid
PAF	platelet activating factor
PB	phosphate buffer
PBS	phosphate buffered saline
PCA	perchloric acid
PGE2	prostaglandin E2
PMNs	polymorphonuclear neutrophils
RT	room temperature
SEM	standard error of the mean
SIN-1	3-morpholinopyrrolidine
SNAP	S-nitroso-N-acetyl-D,L-penicillamine
SNP	sodium nitroprusside
SOD	superoxide dismutase
SPER/NO	spermine bis (NO [•]) adduct
TBS	tris-buffered saline
6-TG	6-thioguanine
Tris	tris(hydroxymethyl)aminomethane

1. GENERAL INTRODUCTION

1.1 Objective

Our laboratory has been interested in identifying factors in the microenvironment of tumours that may contribute to mutagenesis and lead to tumour progression. Through the development of a transplantable murine tumour cell line (MN-11), it was previously found that cells grown as a subcutaneous tumour had a 3.4 fold higher mutation frequency than MN-11 cells cultured for the equivalent period of time (Wilkinson *et al.*, 1995). Products of infiltrating inflammatory cells (such as reactive oxygen and nitrogen species - ROS and RNS) were considered as potential contributors to this increased mutation frequency. Treatment of MN-11 cells in culture and mice with MN-11 tumours with various nitric oxide (NO) donors caused an increase in mutation frequency (Sandhu and Birnboim, 1997). The objective of the first part of my thesis was to explore and identify the pathway(s) through which NO donors cause mutations in the MN-11 system. The most abundant infiltrating inflammatory cell observed in MN-11 tumours is the neutrophil, known to produce both ROS and RNS. My second objective was to establish a link between number of infiltrating neutrophils and mutation frequency (MF) by attracting neutrophils into the tumour and scoring MF. These experiments may help provide an understanding of the mechanism of mutation induction believed to lead to tumour progression, as well as the association between inflammatory conditions and cancer.

1.2 Background

1.2.1 Carcinogenesis

Carcinogenesis is considered to involve a sequential accumulation of multiple genetic alterations. The development of cancer may involve the successive alteration of normal cells to premalignant foci to localized tumours to invasive tumours to metastatic lesions (Loeb, 1991). One of the most important changes that occur when normal cells become malignant is that they escape from the signals which control cell proliferation and apoptosis (Ilyas and Tomlinson, 1996). In order for normal cells to become malignant, a minimum number of necessary mutations must be acquired. These mutations probably occur randomly, but a nonrandom selection of mutations takes place that allows the cells to overcome growth regulations, and thus become cancerous (Ilyas and Tomlinson, 1996). The classic model of cancer development is considered to involve initiation, promotion, and progression steps. Initiation of cancer involves the induction of an irreversible change in the DNA of a cell. Promotion is thought to result in the formation of benign tumours, possibly through a reversible process of gene activation and cellular changes over a long latency period. Progression is the accumulation of more genetic hits causing the conversion of benign to increasingly malignant tumours. Progression involves alterations of DNA and usually results in more rapid growth, invasiveness, metastasis, and increased genomic instability of tumours (Weitzman and Gordon, 1990; Archer, 1992). Genomic changes associated with cancer include gene amplification, point mutations, chromosomal translocations, deletions, insertions, changes in DNA

methylation, and aneuploidy (Cheng and Diaz, 1991; Coleman and Tsongalis, 1995). Stable variants arise that have increased malignant potential, leading to resistance to chemotherapy and host defences, a faster growth rate, and more effective invasiveness (Perkarek *et al.*, 1995). Human cancers that have been reported to contain multiple chromosomal abnormalities include breast carcinoma (Paz-y-Mino *et al.*, 1997; Schmutzler *et al.*, 1996), colonic adenocarcinoma (Ruschoff *et al.*, 1997; Vogelstein *et al.*, 1988), malignant melanoma (Matsuta *et al.*, 1997; Dracopoli *et al.*, 1989), lung carcinoma (Balsara *et al.*, 1997; Sachse *et al.*, 1994), and numerous others (see Mitelman, 1991).

Central to carcinogenesis is the induction of a chemical change in a cell's DNA that eventually becomes a stable mutation. This damage to the DNA can result either from a "spontaneous" modification of the DNA molecule, or it can be the result of a physical or chemical insult (Coleman and Tsongalis, 1995). Spontaneous DNA alterations may include hydrolysis, oxidation, or methylation, and errors incurred during DNA replication (Lindhahl, 1993). Physical agents, such as ionizing radiation and ultraviolet light, and chemical agents, such as N-nitroso compounds, polycyclic aromatic hydrocarbons, and crosslinking agents, can lead to a number of genetic lesions, including single and double strand breaks, intrastrand and interstrand crosslinks, the formation of bulky adducts, and the formation of nucleotide base modifications (Coleman and Tsongalis, 1995).

1.2.2 Genomic Instability

It was originally postulated that at least two mutations are required to produce a cancer phenotype in the case of certain inherited cancers (Knudson, 1977). However, where it has been examined, most common cancers seem to have acquired a large number of genetic hits (Loeb, 1991; Fearon and Vogelstein, 1990). In order to explain the cause of these multiple genetic hits, some workers have proposed the existence of a “mutator phenotype” leading to the “genomic instability” seen in some malignancies. A mutator phenotype would be expected to predispose a cell to the accumulation of mutations at a greater rate than normal, as an early step in tumour progression (Nowell, 1976; reviewed by: Coleman and Tsongalis, 1995; Tlsty *et al.*, 1995; Cheng and Loeb, 1993). Certain human genetic diseases, such as Fanconi’s anemia, Bloom’s syndrome, ataxia telangiectasia, and xeroderma pigmentosum, are associated with a predisposition to cancer (Digweed, 1993; Eyfjord *et al.*, 1995; Coleman and Tsongalis, 1995). Numerous defects in cellular processes that would generate an increase in mutation rate have been studied and include: defects in DNA repair processes; inactivation of enzyme systems involved in detoxification of chemical and physical carcinogens; improper regulation of recombination and mutation events during the development of the heterogeneity required in lymphocytes; alteration in cell cycle control (Tlsty *et al.*, 1995). Recently, a relatively common form of DNA repair deficiency has been identified. In hereditary non-polyposis colorectal cancer (HNPCC) syndrome, a mutation in DNA repair genes (such as the hMSH2 gene) results in an increase in mutation rate and the development of a mutator phenotype, termed replication error phenotype (RER) (Radman and Wagner, 1993). A

germline mutation in one of the genes responsible for DNA mismatch repair results in an increase in replication errors throughout the entire genome, most easily detected at microsatellite sequences (Ilyas and Tomlinson, 1996). Individuals with this syndrome develop colorectal and /or other epithelial tumours (Peltomaki, 1995). Additionally, the RER phenotype has been observed in numerous other tumour types, including breast carcinoma (Yee *et al.*, 1994), gastric cancer (Ruyu *et al.*, 1994), myelogenous leukemia (Wada *et al.*, 1994), bladder carcinoma (Gonzalez-Zulueta *et al.*, 1993), and numerous others (see Coleman and Tsongalis, 1995), suggesting this to be a relatively common feature of human cancers.

Much has been learned about the contributions of factors within tumour cells to carcinogenesis. For example, the *p53* tumour suppressor gene has been of particular interest. It is probably the single most common locus with mutations in human cancers (Greenblat *et al.*, 1994), and alterations in this gene have been reported in a wide variety of human cancers including colon, lung, esophagus, breast, liver, brain, reticuloendothelial tissues, and hemopoietic tissues (Hartwell and Kastan, 1994; Harris, 1994). The *p53* gene product plays a role in cell cycle regulation at the G1 - S checkpoint, participating in the cellular response to DNA damage by eliciting cell cycle arrest (Kastan *et al.*, 1991). Elimination of normal *p53* function could lead to uncontrolled cell cycle progression, as well as the propagation of damaged DNA to generate stable DNA mutations and contribute to genomic instability (Hartwell and Kastan, 1994; Coleman and Tsongalis, 1995).

1.2.3 Inflammation and Genomic Instability

In contrast to intrinsic conditions (factors within tumour cells) affecting carcinogenesis (such as *p53* mutations), some researchers have become interested in studying extrinsic factors (in the tumour environment) capable of leading to genomic instability and tumour progression. It has long been recognized that chronic infection and inflammation are associated with a large number of human cancers (Ohshima and Bartsch, 1994). Examples include: the association of hepatitis B virus and hepatic cirrhosis with hepatocellular carcinoma; the association of ulcerative colitis, Crohn's disease, and chronic inflammatory bowel disease with cancers of the colon; the association of liver fluke with cholangiocarcinoma of the biliary tract; the association of *Helicobacter pylori* infection and atrophic gastritis with cancers of the stomach; the association of tuberculosis and particles (asbestos, silica dust) with cancers of the lung; the association of urinary infections, long-term catheterization, and chronic cystitis with cancers of the bladder; and numerous others (Tamir and Tannenbaum, 1996; reviewed by: Rosin *et al.*, 1994; Ohshima and Bartsch, 1994). Interest in the role of inflammation in carcinogenesis led to findings that stimulated inflammatory cells are capable of causing genotoxic events, such as DNA strand breaks (Birnboim, 1982), mutations (Weitzman and Stossel, 1981), sister chromatid exchanges (Weitberg, 1989), and malignant transformation (Weitzman *et al.*, 1985). One area that has received attention is the role of reactive oxygen and nitrogen species (ROS and RNS) generated by inflammatory cells in causing cytotoxicity and mutagenesis (Weitberg *et al.*, 1983; Tamir and Tannenbaum, 1996). Interest in further studying the role of exogenous factors present in the tumour

environment in contributing to genomic instability led to the development of the MN-11 tumour model system by our laboratory.

1.2.4 Development and Characteristics of the MN-11 System

The MN-11 system was developed as a means of studying exogenous factors presumed to be present in the tumour microenvironment that may cause genotoxicity, and therefore, contribute to tumour progression. Briefly, the development of the MN-11 system was as such: A tissue culture line was established from a mouse tumour (MC1A) originally isolated from a male C57BL/6 mouse that had been treated with methylcholanthrene. The tumour was known to be infiltrated with inflammatory cells and was transplantable in syngeneic animals (Kadhim and Rees, 1984; Kadhim *et al.*, 1987). This line was genetically altered to allow for sensitive detection of mutational events at the *hprt* (hypoxanthine-guanine-phosphoribosyltransferase) locus. Multiple *hprt* genes of MC1A-C1 cells were inactivated by treatment with N-methyl-N-nitrosourea (MNU), and *hprt*⁻ clones were selected by their ability to grow in 6-thioguanine (6-TG). Eighteen of these clones were tested for tumorigenicity in C57BL/6 female mice, of which MC-TGR17 was chosen. Finally, one of what was believed to be two or more inactive copies of the *hprt* gene spontaneously reactivated and *hprt*⁺ clones were selected in hypoxanthine/aminopterin/thymidine (HAT) medium. The selected clones were screened to identify ones that exhibited the most stable expression (lowest spontaneous reversion to *hprt*⁻). MN-11 is a derivative of one such clone (MC-TGS17-51), into which

a *neo* gene in a retroviral vector was introduced, conferring G418 resistance, and allowing the easy distinction of tumour cells from host animal cells. For more details of MN-11 development, see Wilkinson *et al.* (1995).

The MN-11 cell-line was developed so that the loss or inactivation of the X-linked marker gene (*hprt*) could be detected with high sensitivity. This locus is widely used for the detection of both intra-locus and multi-locus mutagenic events in mammalian cells (Evans *et al.*, 1986; Vrieling *et al.*, 1985; Vrieling *et al.*, 1988; Koberle and Speit, 1991). Since MN-11 cells appear to contain more than one active X chromosome (fluorescence in situ hybridization (FISH) analysis has revealed that the line has three X chromosomes), the reported limitation of *hprt* as a marker gene for chromosomal mutations is minimized. The typical shortcoming of the uses of the *hprt* locus for studies of multi-locus events is that there is usually only one active X chromosome; accompanying loss of neighbouring essential genes will result in non-viable mutants, and therefore will lower the sensitivity for detecting induced mutations (Evans *et al.*, 1986). In the MN-11 system, the functions of the neighbouring essential genes are presumed to be provided by the other X chromosomes (which are presumed to be active, but contain inactive *hprt* genes). While there is no direct proof that MN-11 cells have a heterozygous *hprt* genotype (*hprt*^{+/−}) capable of detecting large scale mutational events, different lines of evidence provide support for this model: MN-11 cells have a high sensitivity at the *hprt* locus to mutation induction by ⁶⁰Co γ radiation (a known clastogen) that is similar to other known heterozygous loci (Evans *et al.*, 1986; Schwartz *et al.*, 1991). About half of the radiation

induced *hprt*⁻ mutants exhibit a 'slow-growing' phenotype (Sandhu, 1998), which has been ascribed to large scale deletions at the *tk* locus in mouse lymphoma cells (Zhang *et al.*, 1996; Davies *et al.*, 1993).

MN-11 cells grow in an anchorage-dependent manner as a monolayer, and have a doubling time of approximately 16 hours. Prior to the start of any experiment, any pre-existing *hprt*⁻ cells must be removed by culturing cells in the presence of HAT for 7 days. After the completion of an experiment, *hprt*⁻ mutants are scored by plating 1×10^5 cells in a 10 cm dish supplemented with 6-TG. Only cells with no active *hprt* gene are able to grow in this medium. Although the nature of the mutation scored in 6-TG has not been identified, previous work using FISH analysis suggests that it is not whole chromosome loss. As well, indirect evidence exists to suggest that *hprt* gene inactivation is not due to methylation (Sandhu, 1998). Currently, a collaborative study is underway to determine if chromosomal-level deletions are detectable in 6-TG^R mutants. The MN-11 cell line was developed as an *in vitro/in vivo* model system to allow the study of clastogenic and/or mutagenic events occurring *in situ*. As such, it allows for a comparison between the mutation frequency occurring in an animal with that of cells cultured for the equivalent period of time. Although a comparison of doubling times between cells as tumours and in culture is not known, the doubling time *in vitro* is a rapid 14 to 16 hours, and thus it is unlikely to be faster *in vivo*. To ensure that tumours form in nearly 100% of injected animals, 5×10^5 HAT-cleaned MN-11 cells are injected into the flank region of syngeneic C57BL/6 mice. By day 14 following tumour inoculation, solid, subcutaneous tumours of

approximately 1 cm in diameter are formed. The tumour has been described as a malignant fibrosarcoma; it may be locally invasive, but does not form secondary tumours. Histochemical studies of MN-11 tumours show infiltration predominantly by neutrophils, but also macrophages, mast cells, and lymphocytes are seen in fewer numbers. For a complete characterization of the MN-11 tumour, refer to Sandhu (1998).

Using this model, it was previously found that the mutation frequency in MN-11 cells grown as a subcutaneous tumour was 3.4 fold higher than in cells cultured for the equivalent period of time (Wilkinson *et al.*, 1995), suggesting that factors exist in the tumour environment that are genotoxic. Treatment of MN-11 cells in culture and mice with MN-11 tumours with nitric oxide donors caused a large increase in mutation frequency. It is plausible that the observed 3.4 fold increase in mutation frequency in tumours may be due, at least in part, to nitric oxide or related species, released by inflammatory cells such as neutrophils and macrophages infiltrating into the tumour.

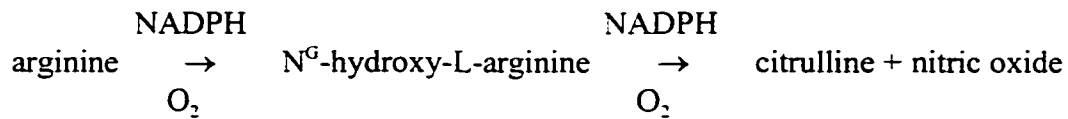
1.2.5 Nitric Oxide

Nitric oxide (NO) serves numerous major biological functions. Physiological roles of NO include controlling blood pressure (vasodilation) (Rees *et al.*, 1989), neurotransmission (Bredt and Snyder, 1989), antimicrobial and antitumour activities (Hibbs *et al.*, 1987), regulation of platelet function (Radomski *et al.*, 1990), bronchodilation, and penile erection (reviewed by: Moncada *et al.*, 1991; Gibaldi, 1993).

However, at elevated levels of NO, such as those found during infection and inflammation, harmful effects may occur. These include hypotension, septic shock, nitrosation, DNA deamination, oxidation, and neural damage (reviewed by Liu and Hotchkiss, 1995) (see below).

NO is a free radical, consisting of seven electrons from the nitrogen atom and eight electrons from the oxygen atom, thus leaving one unpaired electron (Liu and Hotchkiss, 1995). NO and its derivatives were recognized as a component of air pollution many years ago, resulting from fuel combustion processes from motor vehicles and power plants (Williams, 1996). Still, the major sources of nitrogen oxides (NO_x) are natural (bacteria, volcanic action, lightning). Indoor concentrations of NO_x may be considerably higher, the main sources being tobacco smoking, gas-fired appliances, and kerosene heaters (Victorin, 1994). The discovery that NO was generated by mammalian cells for specific biological functions opened a new field of study (reviewed by Moncada, 1992). Because of its unpaired electron, NO is highly reactive, and reacts rapidly with O₂. Its intracellular half-life in biological systems is estimated to be 4 - 50 seconds (Furchgott and Vanhoutte, 1995), and, because it is uncharged, NO can migrate freely through cell membranes similarly to O₂ (Laval and Wink, 1994), thus probably diffusing several cell diameters before being consumed by O₂ (Liu and Hotchkiss, 1995). S-nitrosothiols have been postulated to be carriers of NO, greatly increasing the span of action of NO to distant tissues (Stamler *et al.*, 1992).

NO is synthesized by the conversion of L-arginine to L-citrulline by NO synthase (NOS) using NADPH and oxygen as cosubstrates:



(Esumi and Tannenbaum, 1994).

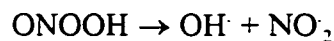
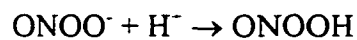
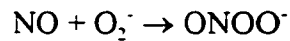
NOS exists as both constitutive and inducible forms, and is structurally related to the cytochrome *P*-450 family, consisting of a single polypeptide chain with L-arginine, heme, FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide) binding sites, and require tetrahydrobiopterin as a cofactor (Marletta, 1994). The constitutive form (cNOS) which is Ca²⁺/calmodulin dependent, is present in the endothelium of blood vessels and in brain neurons, and produces NO in small amounts (Bredt and Snyder, 1990; Palmer *et al.*, 1988). The released NO functions to activate soluble guanylate cyclase to regulate vascular tone (Palmer *et al.*, 1988), and as a neurotransmitter in the central nervous system (Bredt and Snyder, 1990). In contrast, inducible NO synthase (iNOS) in activated macrophages, neutrophils, and several other cell types, is Ca²⁺- and calmodulin-independent, and may release large amounts of NO (nanomoles of NO *versus* picomoles of NO by cNOS) continuously when substrate is available (Moncada *et al.*, 1990). The primary function of NO release by these cell types is as a defense mechanism against foreign organisms and tumour cells (Hibbs *et al.*, 1987). It is hypothesized that, as a result of this continual release of large amounts of NO when infection or

inflammation lasts months and years, neighbouring cells may become damaged, thus possibly linking NO to an increased risk of cancer.

As mentioned above, numerous reports have indicated that high levels of NO can cause deleterious effects, both cytotoxic and mutagenic. For example, Isomura *et al.* (1984) demonstrated that NO and NO₂ could induce mutations in rat lung cells. It was found that various NO releasing compounds and low concentrations of NO itself (with and without NO₂) were mutagenic in *Salmonella typhimurium* TA1535 using the Ames bacterial mutagenicity test. (Arroyo *et al.*, 1992; Wink *et al.*, 1991). NO gas was mutagenic in TK6 human lymphoblastoid cells (Nguyen *et al.*, 1992). IL-1 β -stimulated intracellularly-formed NO caused DNA damage in rat islets of Langerhans and HIT-T15 cells. This damage was prevented by the addition of nitromonomethyl arginine, a selective inhibitor of NO synthase (Delaney *et al.*, 1993).

Several potential mechanisms have been proposed to explain the observed genotoxicity and cytotoxicity of NO. Through diffusion, NO may react and interfere with transition metals, such as iron centers involved in iron storage or electron transfer reactions (Lancaster and Hibbs, 1990). NO may autoxidize to form N₂O₃, a powerful electrophilic nitrosating agent. This leads to the formation of carcinogenic *N*-nitroso compounds, capable of deaminating and crosslinking DNA (Tamir and Tannenbaum, 1996; Liu and Hotchkiss, 1995). Also, NO reacts rapidly with superoxide anion (O₂⁻) to form peroxynitrite (ONOO⁻), which is reactive and toxic, damaging DNA by attacking

deoxyribose and causing direct oxidation of purines and pyrimidines (Tamir and Tannenbaum, 1996) and inducing membrane lipid peroxidation (Radi *et al.*, 1991). As well, ONOO⁻ may decompose to form the highly reactive hydroxyl radical (OH[·]), as shown by the reactions below:



(Ohshima and Bartsch, 1994).

Interestingly, one of the most prevalent point mutations observed following treatment with NO gas and various NO donors is GC → AT transitions (Routledge *et al.*, 1993; Routledge *et al.*, 1994). GC → AT transitions are repeatedly seen in mutated *p53* from human colon tumours, brain tumours, lymphoma, leukemia, and small cell lung cancers (Hollstein *et al.*, 1991), possibly providing a link between NO and genomic instability.

1.2.6 Chronic NO Release by Phagocytes and Carcinogenesis

As stated above, the major endogenous source of large amounts of NO is activated phagocytes, namely macrophages and neutrophils. For some time it has been known that activated phagocytes can produce mutations in bacteria and mammalian cells, and that

reactive oxygen metabolites generated by these inflammatory cells are important for the production of these genetic lesions, and thus may play a role in carcinogenesis (Weitberg *et al.*, 1983; Weitzman *et al.*, 1985). Only more recently, with the discovery of NO as a product of immune system cells, has the involvement of NO in carcinogenesis been postulated too.

Although both macrophages and activated neutrophils are capable of producing large amounts of NO, neutrophils represent 50 to 60% of the total circulating leukocytes, constituting the first line of defence against “nonself” agents, and are the first cells recruited to sites of infection or injury (Smith, 1994). They are often seen infiltrating human tumours, but their effects on tumour progression and prognosis are unclear (Colombo *et al.*, 1996). As well, neutrophils are the predominant infiltrating inflammatory cell-type observed in MN-11 tumours (Sandhu, 1998). Neutrophils may be attracted to the site of infection or injury by chemotactic factors generated by infectious agents themselves or released by their contact with other components of the immune system. Once there, neutrophils release an armament of defenses including reactive oxygen and nitrogen species and various hydrolytic enzymes and antimicrobial polypeptides (Smith, 1994). Also, neutrophils produce and secrete other factors, such as cytokines, and thereby influence other cells of the immune system (Lloyd and Oppenheim, 1992). While neutrophils have generally been considered as the first line of defence against microbial infection, there is also some evidence of their role in lysing tumour cells *in vitro* and destroying tumours *in vivo* (Shaw and Roberts, 1992).

Neutrophils are essential to host defence; neutropenia or defects in neutrophil function are notable risk factors in developing potentially fatal bacterial and fungal infections (Malech and Gallin, 1987). However, neutrophils also have been implicated in the pathology of many chronic inflammatory conditions. Host tissue damage by neutrophils may occur due to several different mechanisms, including premature activation during migration, extracellular release of toxic products, or failure to terminate acute inflammatory responses (Smith, 1994). Conditions in which damage by neutrophils has been implicated include rheumatoid arthritis (Robinson *et al.*, 1992), lung tissue damage in chronic *Pseudomonas* infection (see Smith, 1994), and *Helicobacter pylori* infection (Talley *et al.*, 1991). *Helicobacter pylori* is a primary cause of chronic gastritis, development of peptic ulcers, and a risk factor for the development of gastric cancer (Talley *et al.*, 1991). Chronic inflammation has been implicated in the carcinogenic process (Huang *et al.*, 1995). Recent literature suggests that sustained production of NO by host neutrophils and macrophages infiltrating the gastric mucosa may cause DNA damage, and contribute to carcinogenesis (Mannick *et al.*, 1996), thus providing an example of a potential link between nitric oxide, neutrophils, and cancer.

2. MUTAGENICITY OF NO DONORS AND THE ROLE OF GLUTATHIONE IN NO DONOR-INDUCED MUTATIONS

2.1 Introduction

Glutathione is the ubiquitous tripeptide γ -glutamylcysteinylglycine, the most abundant thiol and low molecular weight peptide present in mammalian cells (Kosower and Kosower, 1978). It has a role in numerous cellular functions, including involvement in the conversion of ribonucleotides to deoxyribonucleotides, folding of newly synthesized proteins, maintenance of the thiols of proteins and the reduced forms of other compounds such as ascorbic acid and α -tocopherol, regulation of enzyme activity, and inter- and intra-cellular transport (Meister, 1992; Meister, 1983). It serves as a coenzyme for numerous enzymes, including formaldehyde dehydrogenase, maleylacetoacetate isomerase, glyoxalase, prostaglandin endoperoxidase isomerases, and dichlorodiphenyl-trichloroethane (DDT)-dehydrochlorinase and similar enzymes (Meister, 1988). Most relevant to my work is the role that glutathione has in providing cellular protection as an antioxidant. By acting as a sulfhydryl buffer in the cytoplasm, it helps to provide cells with a reducing milieu (Meister, 1992; Hwang *et al.*, 1992). Glutathione protects cells from oxidative damage, free radical damage, and other toxic compounds (both endogenous and exogenous) (Meister, 1992). Further, glutathione also participates in the detoxification of hydrogen peroxide and lipid peroxides by acting as a cofactor for glutathione peroxidases (Meister, 1983). Impaired glutathione status has been implicated in a number of diseases, including diabetes (Murakami *et al.*, 1989), Parkinson's disease

(Perry *et al.*, 1982), AIDS (Kalebic *et al.*, 1991), and cancer (Coles and Ketterer, 1990; reviewed in Richie, 1992)

Glutathione is synthesized in two steps. In the first (rate-limiting) step, γ -glutamylcysteine is synthesized enzymatically from glutamate and cysteine by γ -glutamylcysteine synthetase. Next, glutathione synthetase catalyzes the synthesis of glutathione from γ -glutamylcysteine and glycine. By negatively regulating the rate of formation of γ -glutamylcysteine, glutathione is able to regulate its own synthesis (Meister, 1992). Buthionine sulfoximine (BSO) is a drug considered to be a highly specific and irreversible inhibitor of γ -glutamylcysteine synthetase. BSO interacts with ATP at the active site of the enzyme, phosphorylating it to buthionine sulfoximine phosphate (Griffith, 1982). Since glutathione is not transported efficiently into cells, and is continually being used up by the cell, BSO treatment results in the depletion of glutathione (Meister, 1992; Meister, 1991).

Nitric oxide serves numerous physiological roles, including acting as a brain signalling molecule, relaxing smooth muscle in blood vessels by activating guanylate cyclase, and mediating the killing of bacteria and tumour cells by macrophages and neutrophils (Snyder, 1992; Gibaldi, 1993; Snyder and Brecht, 1992). In addition, large amounts of nitric oxide can have numerous toxic effects on cells. It and related species have been shown to cause tissue injury, DNA deamination, strand breaks, and point mutations as well as initiate a cascade of cellular events such as lipid peroxidation,

damage to ion transport systems, etc. (see *Section 1*). The effect of glutathione on NO cytotoxicity has been studied previously and the findings suggest that glutathione may protect cells from NO-mediated cytotoxicity (Luperchio *et al.*, 1996). It has been previously shown in our laboratory that NO is mutagenic in the MN-11 system. Treatment of MN-11 cells with the NO donor glyceryl trinitrate (nitroglycerin, GTN) caused a dose-dependent decrease in cell survival, with an accompanying increase in mutation frequency (Sandhu and Birnboim, 1997). One objective of my study was to examine the role of glutathione in protecting against NO induced cytotoxicity and mutagenicity in MN-11 cells. It was expected that glutathione would have a protective role against mutations, as it has been seen that radiation-induced mutations are protected against by glutathione (Dethmers and Meister, 1981). Cells were treated with BSO to deplete them of their glutathione, and the effect on GTN-induced cell killing and mutations was studied.

Recent interest in the numerous biological effects of NO and its related species has led to the development of an array of NO donors. While all compounds purport to all generate NO or related species, the pathways leading to NO formation, and their chemical reactivities differ significantly among the different compound classes (Feelisch and Stamler, 1996). Previously, our laboratory has shown that, at equitoxic doses, various NO-donating drugs differed widely in their mutagenic potential (Sandhu and Birnboim, 1997). A second objective of my study was to further study the effects of other NO-donors on cytotoxicity and mutagenicity in the MN-11 system.

2.2 Materials and Methods

2.2.1 Materials

A list of all chemicals used, suppliers, and solution compositions is found in **Appendix I**.

2.2.2 Cell Culture Conditions

MN-11 cells were cultured in 10 cm plastic tissue culture dishes in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal calf serum (FCS) (non-selection medium) at 37 °C in a 5% carbon dioxide/ 95% air incubator. Stock cultures were maintained between 1 - 5×10^5 cells per ml by trypsinization and dilution with fresh medium.

Prior to the start of new experiments, in order to remove any pre-existing hprt mutants, cells were cultured in non-selection medium plus 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.5×10^{-5} M thymidine (HAT medium) for 7 days, and a further 2 days in non-selection medium plus 1×10^{-4} M hypoxanthine and 1.5×10^{-5} M thymidine (HT medium) to allow for full aminopterin depletion from the cells.

Cell counts were determined using a hemocytometer and trypan blue exclusion to assess cell viability.

2.2.3 Treatment with Nitric Oxide Donors

Exponentially growing cultures were trypsinized and 5×10^5 viable cells per 10 cm dish were allowed to attach overnight in non-selection medium. Cells were then treated for 24 hours with one of the following freshly prepared NO donors: GTN, SNP, SIN-1, GSNO, or DETA/NO (concentrations are shown in figures). For SIN-1, catalase (50 $\mu\text{g/ml}$) and/or SOD (50 $\mu\text{g/ml}$) were added for the 24 hour treatment period to certain groups (refer to figures). After the indicated treatments, cultures were washed twice with PBS (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4), trypsinized and divided into two sets of dishes for the determination of cytotoxicity and mutation frequency (MF) (see below).

2.2.4 Treatment with Agents that Affect Glutathione Levels

Either 2.5×10^5 viable cells (for BSO, OTZ, or NAC pretreatment experiments) or 5×10^5 viable cells (for BSO coincubation experiments) were allowed to attach overnight in non-selection medium. For pretreatment experiments, BSO (50 μM), NAC (10 mM), or OTZ (10 mM) was added to culture dishes for 48 hours. For NAC and OTZ experiments, certain treatment groups also received 50 $\mu\text{g/ml}$ catalase. At the end of the pre-treatment period, cultures were washed twice with PBS and then NO donors (GTN, SNP, or DETA/NO) were added for 24 hours as described above. For coincubation experiments, BSO (50 μM) was added along with an NO donor (either GTN or SNP) for

the 24 hour treatment period, and then washed out, as described above, for the determination of cytotoxicity and MF.

2.2.5 Determination of Cytotoxicity and Mutation Frequency

To measure the cytotoxicity and MF induced by various drug treatments, cells were split into two sets of dishes at the time of the final PBS wash. Cytotoxicity was determined by plating 200 cells (or more, depending on the expected level of cytotoxicity) per 6 cm dish in non-selection medium, and scoring colonies after 8 to 10 days. For the determination of MF, 5 to 10×10^5 cells per 10 cm dish were plated in non-selection medium for 7 days to allow for expression of the mutant phenotype. The cells were subcultured as required, never allowing the dishes to reach confluence or plating at lower than 5×10^5 cells per dish. Mutant cells (*hprt*⁻) were detected by plating 1×10^5 cells per 10 cm dish (in duplicate, or, for some experiments, in triplicate) in non-selection medium supplemented with 5×10^{-5} M 6-TG (6-TG-selection medium), and allowing colonies of 6-TG^R cells to form (approximately 9 days). Plating efficiency was determined by plating 200 cells per 6 cm dish in non-selection medium and scoring the resulting colonies.

Colonies were fixed and scored by rinsing the dishes with PBS, fixing the colonies with methanol, rinsing with PBS again, and staining with Wright's stain for 20 minutes. Excess stain was removed by gentle washing with running water. Colonies

greater than 50 cells were scored. MF was expressed as mutants per 10^5 clonable cells, as determined from the plating efficiency.

2.2.6 Cell Irradiation

MN-11 cells (either in non-selection medium, or BSO pretreated as described above) were irradiated with cobalt-60 γ rays (dose rate 1.8 Gy per minute) using a Theratron 780 (Atomic Energy of Canada Limited) irradiator. Following irradiation, cells were trypsinized, and cytotoxicity and MF were determined as described above, except with a mutant expression period of 9 days.

2.2.7 Measurement of Glutathione Levels

To verify glutathione depletion of cells by BSO and to monitor its replenishment upon removal of BSO, the GSH content of BSO treated and untreated cells was determined using the o-phthalaldehyde (OPT) method described by Akerboom and Sies (1982). Briefly, treatment cells were grown in the presence of 50 μ M BSO for 48 hours as described above. Treatment and control cells were trypsinized and counted. Proteins were precipitated with acid (2 N perchloric acid + 4 mM oxalic acid, PCA reagent). The supernatant was treated with 1 N potassium hydroxide to precipitate perchlorate. The supernatant (sample) was placed in Buffer A (0.1 M potassium phosphate / 1 mM CDTA, pH 8.0) and allowed to react with freshly made OPT reagent (1 mg/ml OPT in methanol).

After 20 minutes incubation, fluorescence was measured (Excitation=350, Emission=420) using a LS5 Perkin Elmer fluorescence spectrophotometer.

2.2.8 Measurement of Nitrite Levels

To quantify the amount of NO released by the various NO donating drugs and to determine the effects of BSO pretreatment or coincubation on NO release, nitrite (a more stable end-product of NO) accumulation in culture medium was measured. BSO treated cells were grown in the presence of 50 μ M BSO for 48 hours. Cells (both BSO pretreated and controls) were plated at a density of 2.5×10^5 cells per 3.5 cm dish in 1 ml phenol red - free DMEM + 10% FCS. Different NO donors (plus 50 μ M BSO for coincubation studies) were added to the medium. At various times up to 24 hours, culture medium was removed and nitrite was measured using Griess reaction (Green *et al.*, 1982). Culture medium was added to 4 \times volume of Griess reagent (freshly prepared from equal volumes of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in water and 1% sulphanilamide in 5% H_3PO_4). After 10 minutes, absorbance at 540 nm was measured using a Perkin Elmer UV/VIS Lambda 14 spectrophotometer. A standard curve using $NaNO_2$ was used to calculate the concentration of nitrite.

2.2.9 In Vivo Experiments

C57BL/6 female mice, 8 to 10 weeks of age (from Charles River Laboratories or Tachonic) were injected subcutaneously in the flank region with 5×10^5 HAT/ HT-cleaned MN-11 cells. A portion of these cells was maintained in culture for the duration of the *in vivo* experiment for the determination of spontaneous MF. Beginning on day 7 after tumour inoculation, mice were injected intraperitoneally (ip) with 300 mg/kg BSO in 400 μ l PBS (2 \times 200 μ l injections, each at a different site) for 7 consecutive days (for BSO alone experiments), or 250 mg/kg BSO in 400 μ l PBS (2 \times 200 μ l injections, each at a different site) for 4 consecutive days. Approximately 6 hours following the last 250 mg/ml BSO injections (day 10 after tumour inoculation), 5 mg/kg GTN (diluted in PBS) was injected ip in two 200 μ l injections, each at a different site. Four days later (on day 14 after tumour inoculation) animals were euthanised by cervical dislocation and tumours were removed aseptically. Cells were prepared as described in detail in **Section 3.2.3**. The number of *hprt*⁻ mutants was scored, corrected for plating efficiency and for G418 resistance (see **Section 3.2.3** and **Appendix II**).

2.2.10 Statistical Analysis

Data were analyzed using one-way ANOVA or a Student's *t* test, as applicable. Following detection of a statistically significant F-value, *post hoc* Tukey-Kramer multiple comparisons test of significance was used to identify which treatment condition was different from the control. For *in vivo* experiments, a non-parametric Mann-Whitney test was used to analyze the data. A value of $p \leq 0.05$ was considered to be significant. The computer statistical package used was InStat2 (GraphPad Software, Inc., 1993).

2.3 Results

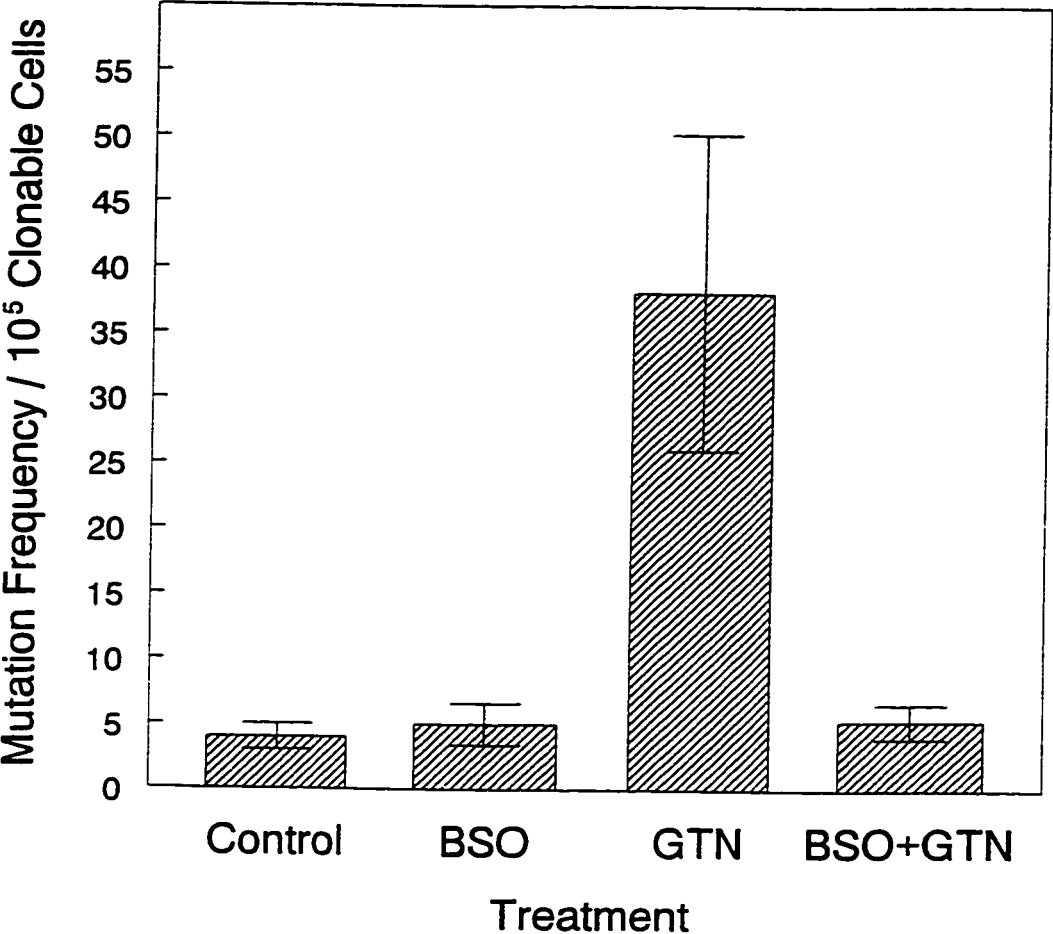
2.3.1 BSO Pretreatment Blocks Mutation Induction by NO Donating Drugs.

Glutathione is considered to be a central figure in a cell's defences against attack by free radicals and electrophiles, conferring resistance to radiation, other oxidizing species and chemotherapeutic drugs (Meister, 1992). GTN, a nitric oxide generating drug, has been shown to be mutagenic in the MN-11 system (Sandhu and Birnboim, 1997). The data in Figure 2-1 confirm our laboratory's earlier findings that *in vitro* treatment of MN-11 cells with 123 μ M GTN for 24 hours induced over 35 mutants per 10^5 viable cells. In order to explore whether glutathione might have a protective effect, glutathione depletion was carried out using buthionine sulfoximine (BSO). It was expected that GSH depletion would increase the MF resulting from treatment with this NO donor. To avoid the possibility that BSO itself might react with GTN or with NO, cells were first pretreated with BSO, which was then washed out before GTN addition. Treatment of MN-11 cells with BSO alone for 48 hours was found to be non-mutagenic. Contrary to what was expected, BSO pretreatment dramatically reduced the induction of mutations (Figure 2-1). To confirm this finding, another NO donor, SNP (which is thought to be metabolised differently than GTN (Feelisch and Stamler, 1996)), was tested.

Figure 2-1. Effect of BSO pretreatment on GTN-induced mutations in MN-11 cells.

MN-11 cells received 24 hour pretreatment with 50 μ M BSO. Control cells were grown in non-selection medium for the equivalent period of time. BSO was washed off, then cells were treated with 123 μ M GTN for a further 24 hours (control cells were grown in non-selection medium). Cells were cultured for 7 days to allow for the expression of mutant phenotype. Mutation frequency is expressed as number of mutant colonies per 10^5 clonable cells. Shown are the mean \pm SEM of 2 independent experiments (n = 15 to 30 for individual groups). Statistically significant differences: control vs GTN $p < 0.01$, GTN vs BSO $p < 0.05$, GTN vs BSO+GTN $p < 0.01$.

Figure 2-1



Treatment of MN-11 cells with 0.5 mM SNP was strongly mutagenic, inducing approximately 120 mutants per 10^5 viable cells. When cells were pretreated with BSO prior to SNP, mutations were completely blocked (Figure 2-2), as was the case for GTN. Thus, for two separate NO donors that are thought to be metabolized differently, GSH depletion by BSO prevented the induction of mutations in MN-11 cells.

2.3.2 The Role of BSO Cytotoxicity in the Prevention of Mutation Induction by GTN and SNP.

To assess the contribution of cytotoxicity by BSO pre-treatment in the dramatic elimination of GTN- and SNP-induced mutations, cell killing caused by BSO and combination drug treatments was assessed. Treatment with increasing concentrations of BSO alone for 48 hours did cause a modest decrease in cell survival, as compared to controls (Figure 2-3). At 50 μ M, the concentration at which all mutagenicity experiments were conducted, survival was above 70%. GTN and SNP, at the concentrations tested (123 μ M and 0.5 mM, respectively), were found to be slightly cytotoxic on their own. The observed cytotoxicity (90% survival for GTN and 70% survival for SNP) are similar to previously found values (Sandhu and Birnboim, 1997). BSO pretreatment of MN-11 cells was found to enhance the NO donor cytotoxicity; however, at the concentration used in experiments (50 μ M), the survival of cells treated with BSO and NO donors was still greater than 50% (Figure 2-3). Thus, the observed elimination of NO donor - induced mutations by BSO cannot be ascribed to excessive cytotoxicity of the combined drugs.

Figure 2-2. *Effect of BSO pretreatment on SNP-induced mutations in MN-11 cells.*

MN-11 cells received 24 hour pretreatment with 50 μ M BSO. Control cells were grown in non-selection medium for the equivalent period of time. BSO was washed off, then cells were treated with 0.5 mM SNP for a further 24 hours (control cells were grown in non-selection medium). Cells were cultured for 7 days to allow for the expression of mutant phenotype. Mutation frequency is expressed as number of mutant colonies per 10^5 clonable cells. Shown are the mean \pm SEM of 2 independent experiments (n = 15 to 25 for individual groups). Statistically significant differences: control vs SNP $p < 0.001$, SNP vs BSO $p < 0.001$, SNP vs BSO+SNP $p < 0.001$.

Figure 2-2

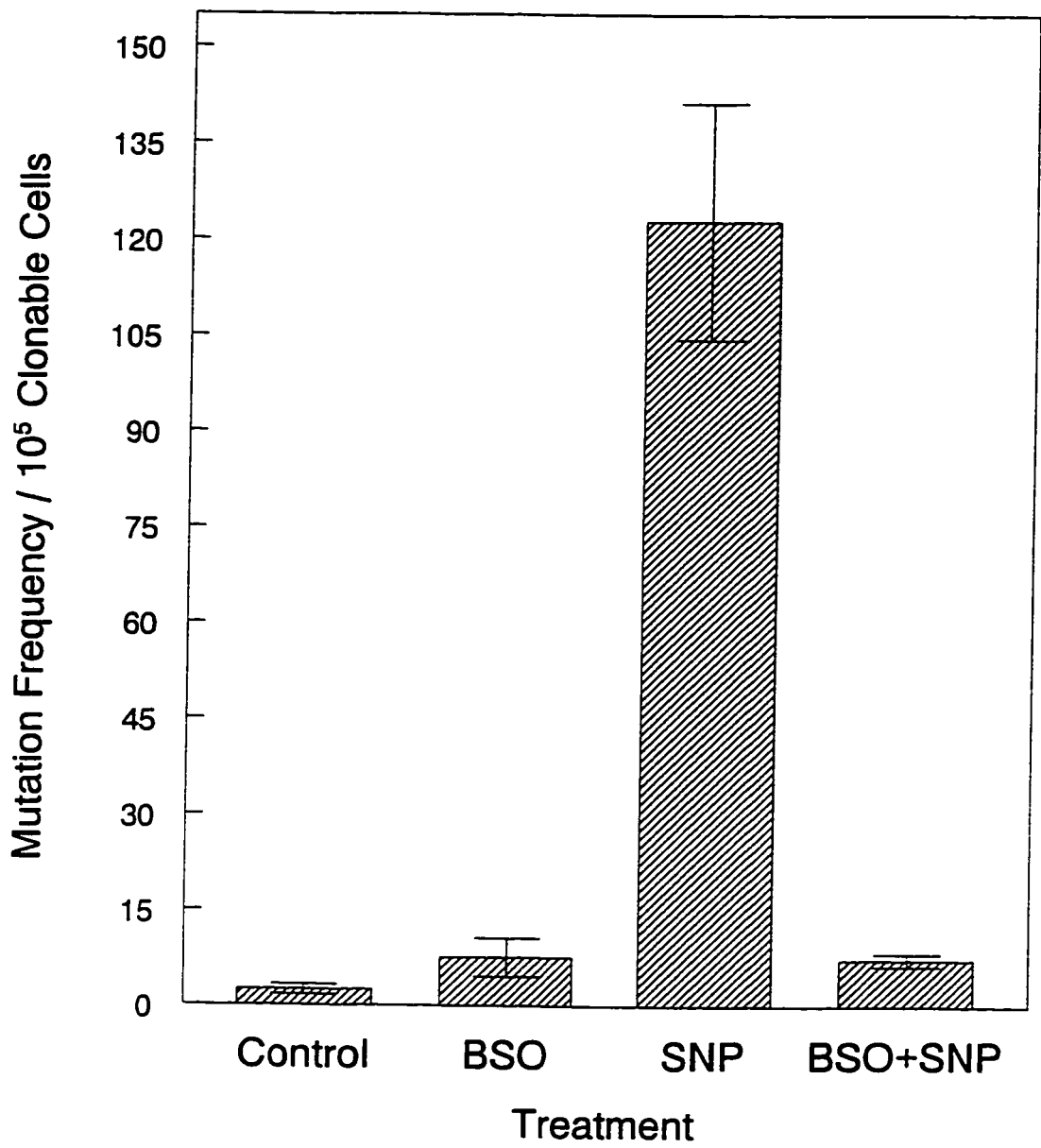
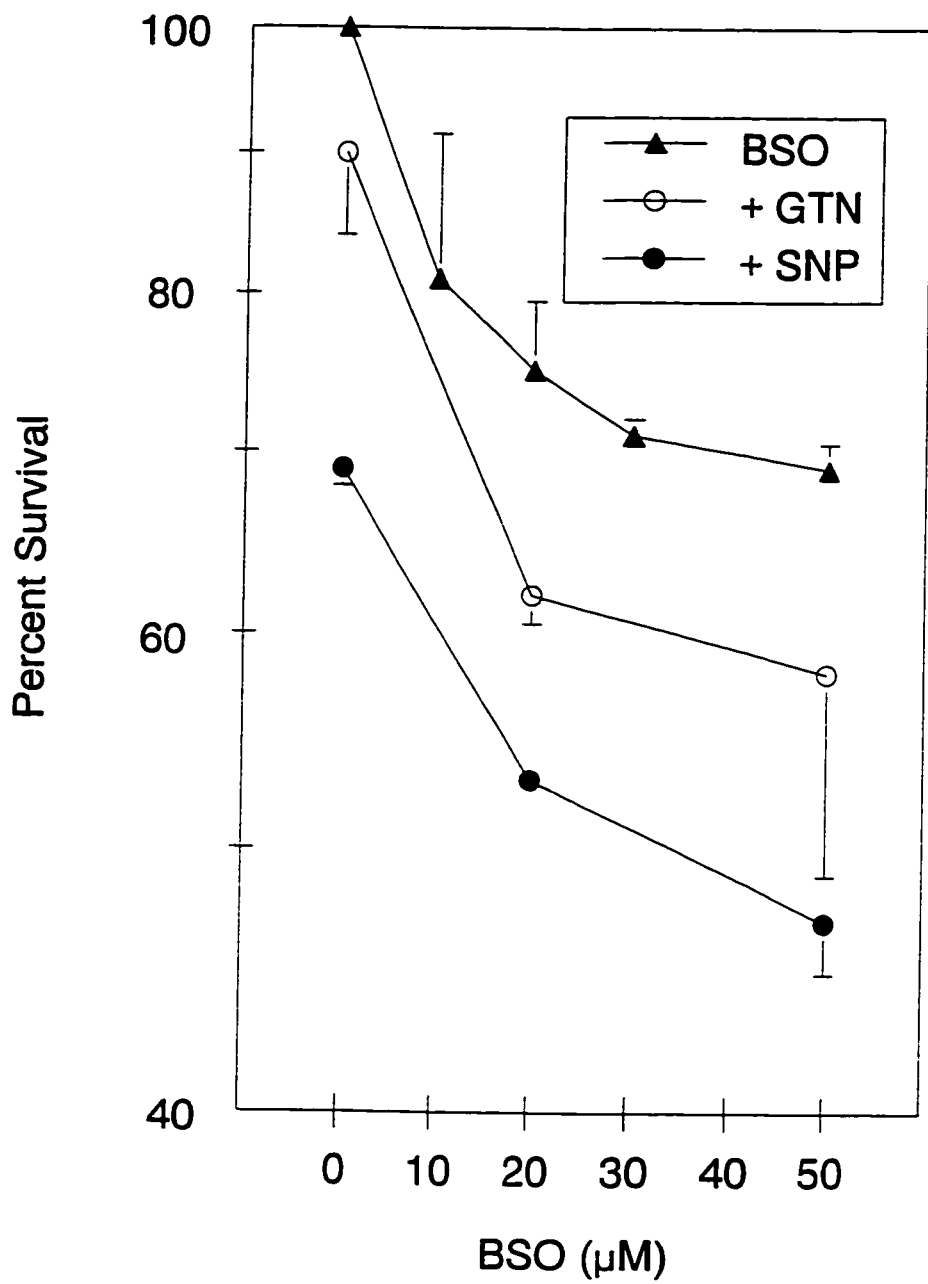


Figure 2-3. Cytotoxicity of BSO and NO donors, GTN and SNP, on MN-11 cells.

Cytotoxicity of various drugs is shown as percent survival of controls. Closed triangles depict percent survival of cells treated for 48 hours with increasing concentrations of BSO alone. The combined effect of 48 hour BSO pretreatment at increasing concentrations, plus 123 μ M GTN (open circles) or 0.5 mM SNP (closed circles) for a further 24 hours following removal of BSO is also shown. Results are expressed as mean \pm SEM for numerous repetitions, for 2 independent experiments.

Figure 2-3



2.3.3 Effect of BSO Pre-treatment on NO release by GTN and SNP.

A common method of measuring NO is to measure nitrite, a more stable end product of NO metabolism. MN-11 cells, in the absence of a NO source, or if pretreated with BSO, do not release NO (Figure 2-4). However, MN-11 cells are capable of metabolizing GTN and SNP, and nitrite accumulates linearly over time in the culture medium. Pretreatment of cells for 48 hours with 50 μ M BSO does not affect nitrite accumulation in culture medium following incubation with GTN (Figure 2-4a); BSO pretreatment followed by SNP results in a slight, statistically non-significant, increase in nitrite accumulation (Figure 2-4b). Thus, the observed elimination of NO donor - induced mutations by BSO pretreatment is not likely attributable to a disruption of GTN or SNP metabolism by BSO.

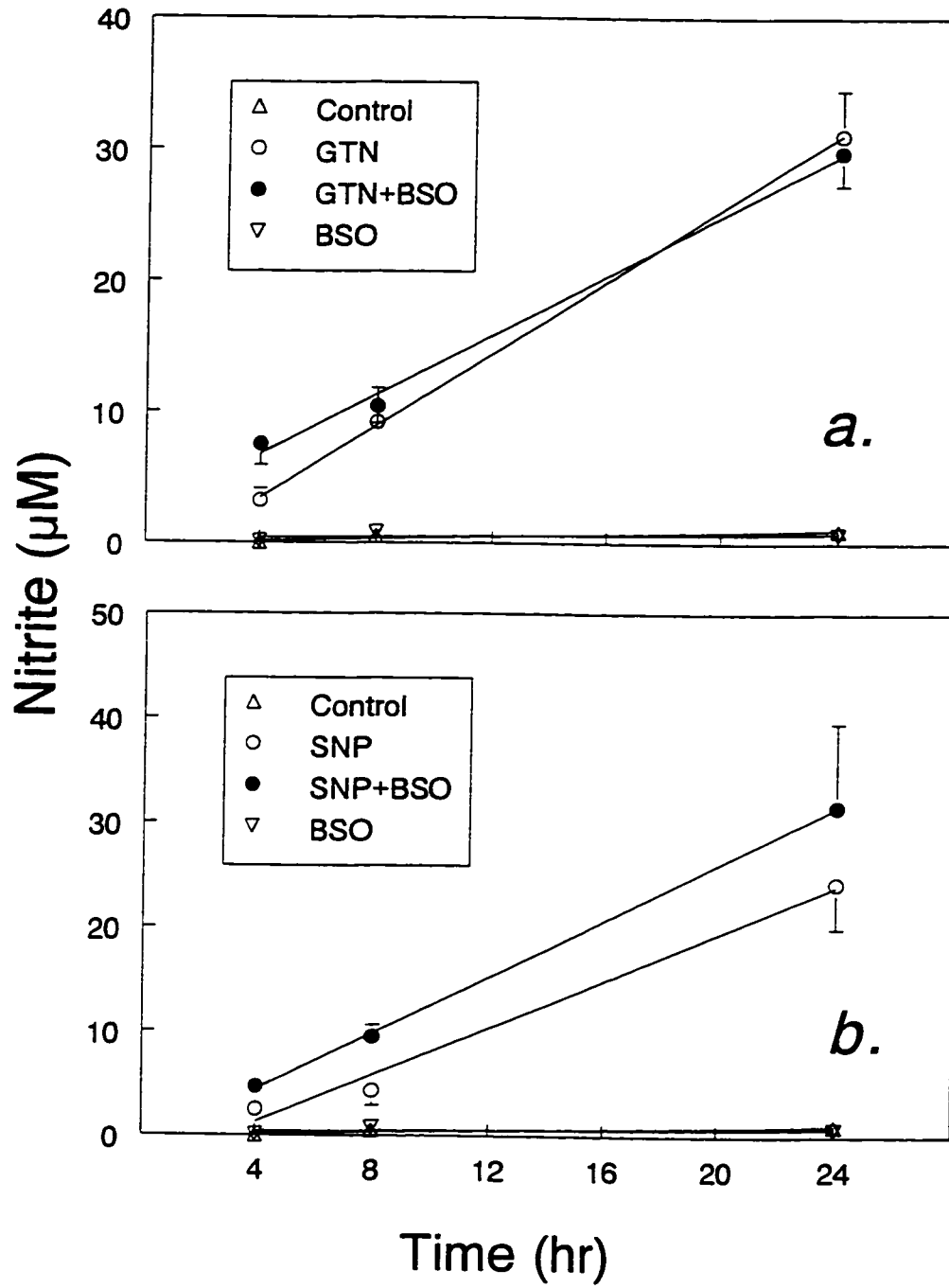
2.3.4 Glutathione Levels Following BSO and NO Donor Treatment.

BSO depletes cells of glutathione by irreversibly inhibiting γ -glutamyl-synthetase, the enzyme involved in the first step of glutathione synthesis. To confirm that BSO was depleting MN-11 cells of glutathione, and to determine the rate of recovery of glutathione following the removal of BSO, the level of glutathione in MN-11 cells was monitored. The OPT method allows the quantification of thiols (glutathione is the predominant acid-soluble thiol in cells) by yielding a fluorescent product. Control MN-11 cells contain 739 ± 39 pmol reduced glutathione per 10^6 cells (mean \pm SEM of 4 independent experiments).

Figure 2-4. Effect of BSO pretreatment on NO release by NO donors GTN and SNP.

NO release by NO donors is measured as nitrite accumulation in cell culture medium. MN-11 cells were pretreated with 50 μ M BSO for 48 hours, which was then washed out ($t = 0$), and cells were given either 123 μ M GTN (shown in *a.*) or 0.5 mM SNP (shown in *b.*). At $t = 4, 8,$ and 24 hours, culture medium was removed and nitrite was measured. Results shown are mean \pm SEM after subtraction of background levels (medium with no cells).

Figure 2-4



Treatment of cells with 50 μ M BSO for 48 hours resulted in the complete depletion of reduced glutathione (less than 2% of controls). Upon removal of BSO, glutathione levels returned to control levels by 8 hours, and after 24 hours, were 50% higher than controls (Figure 2-5). Glutathione levels in MN-11 cells treated with GTN alone (i.e., not BSO pretreated) also overshoot control levels. After 24 hours of GTN treatment, glutathione was greater than 200% of control levels. Recovery of glutathione following removal of BSO and addition of GTN was rapid. By approximately 6 hours, glutathione levels had reached 100%, and after 24 hours levels were over 300% (Figure 2-5a). Similarly, in cells treated with SNP, for 24 hours glutathione levels rose by 30%. In BSO pretreated cells, glutathione returned to control levels by 6 to 8 hours, and, after 24 hours, reached over 200% (Figure 2-5b). Because of the complex changes in glutathione levels in response to these agents, it remained unclear whether BSO pretreatment of MN-11 cells provided protection from GTN or SNP mutations by causing a depletion of glutathione for the first 6 to 8 hours of NO donor treatment (i.e., glutathione is required for mutations), or if the dramatic glutathione overshoot (200 and 300% above controls) was sufficient to provide complete protection from the NO donors.

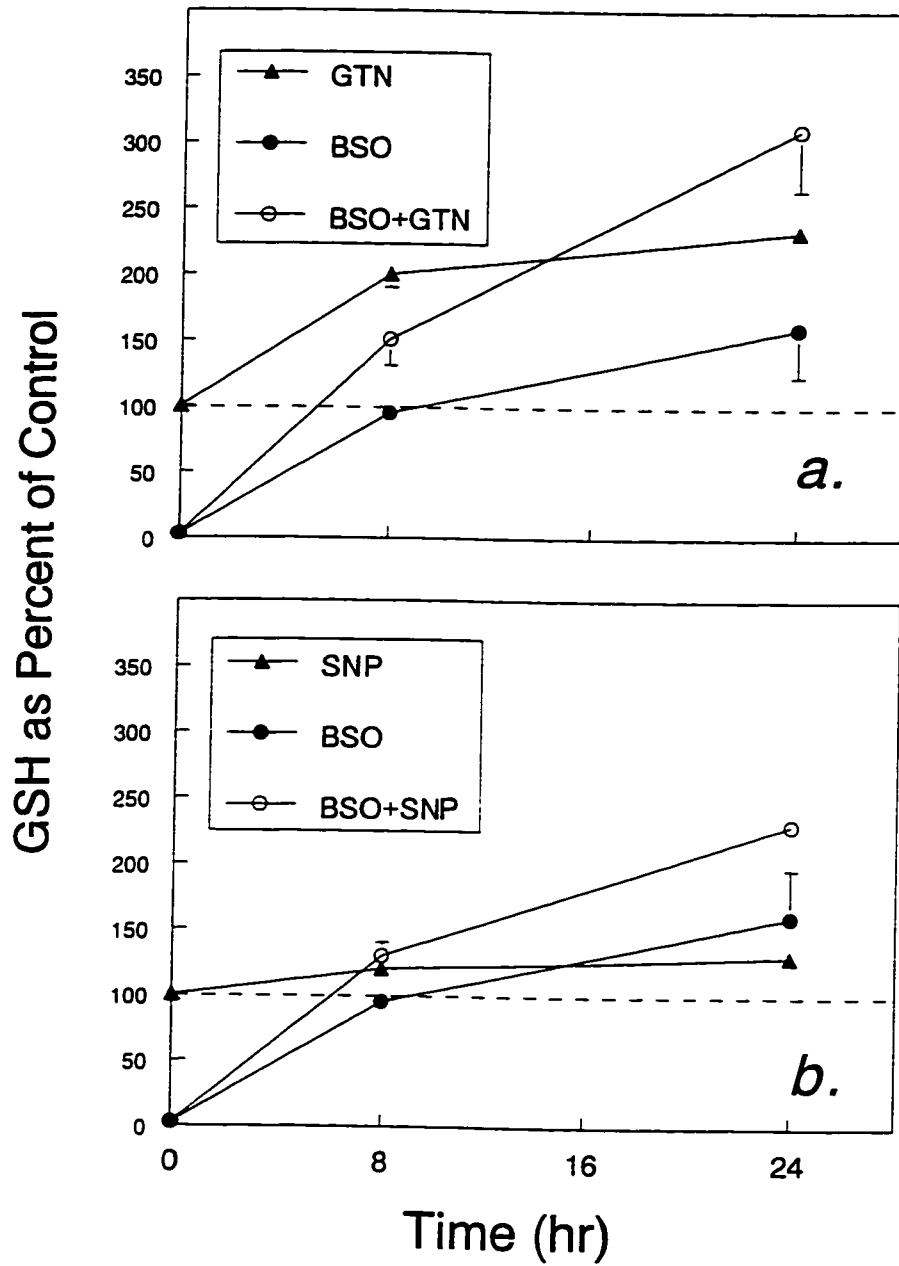
2.3.5 Effect on MF of Coincubation of BSO and NO Donors

To address this question, a modification of the previous experiment was carried out. Instead of receiving a 48 hour BSO pretreatment, MN-11 cells were pretreated for 24 hours with 50 μ M BSO. GTN was then added for a further 24 hours in the continuing

Figure 2-5. Glutathione content of MN-11 cells following treatment with BSO and NO donors GTN and SNP.

Glutathione content of MN-11 cells is shown as percent of control cells. Cells were pretreated with 50 μ M BSO for 48 hours, which was then washed off, or were grown in non-selection medium. Cells then received (t = 0) 123 μ M GTN (shown in *a.*), 0.5 mM SNP (shown in *b.*), or nothing. At t = 0, 8, and 24 hours the glutathione content of cells was measured. Results shown are mean \pm SEM for 2 independent experiments. Glutathione content of control cells is 739 ± 96 pmol per 10^6 cell.

Figure 2-5



presence of BSO. Other experimental details were unchanged. As before, BSO was not mutagenic in the MN-11 system, while 123 μM GTN induced over 50 mutants per 10^5 viable cells. Cells that received 24 hour BSO pretreatment and 24 hour coincubation with GTN had markedly reduced mutation frequency, similar to untreated (control) cells (Figure 2-6). Glutathione content of the cells in the various treatment groups was measured, and is shown in the Figure as a percent of control cells above the bars. The glutathione content of GTN treated cells was, as before, greatly elevated. BSO treatment caused glutathione depletion, both in cells treated with BSO alone and in those that received 24 hours of GTN. Thus, treatment of MN-11 cells with BSO (either pretreatment or coincubation) causes a virtual elimination of GTN - induced mutations.

To address the possibility that co-incubation with BSO was interrupting the cells' ability to metabolize GTN, nitrite accumulation in the culture medium was measured by the Griess reaction (Figure 2-7). As before, nitrite accumulated in cells treated with GTN for 24 hours. Control cells and BSO treated cells did not release nitrite in the absence of GTN. MN-11 cells that received 24 hour BSO pretreatment and 24 hour BSO and GTN coincubation released the same amount of nitrite as cells treated with GTN alone (Figure 2-7). Thus, BSO coincubation does not interfere with GTN metabolism to nitrite by MN-11 cells.

Figure 2-6. *Effect of BSO coincubation on GTN-induced mutations in MN-11 cells.*

MN-11 cells were pretreated with 50 μ M BSO for 24 hours, and coincubated for a further 24 hours with 123 μ M GTN. Control cells were maintained in non-selection medium, GTN alone cells received 123 μ M GTN for 24 hours, and BSO alone cells received 50 μ M BSO for 48 hours. Cells were cultured for 7 days to allow for the expression of mutant phenotype. Mutation frequency is expressed as number of mutant colonies per 10^5 clonable cells. Shown are mean \pm SEM for 2 independent experiments (n = 9 to 17 for individual groups). Statistically significant: control vs GTN $p < 0.01$, GTN vs BSO+GTN $p < 0.01$, GTN vs BSO $p < 0.05$. Also shown is glutathione content of cells for each group, given as percent of control cells, as measured at the end of GTN and BSO treatment.

Figure 2-6

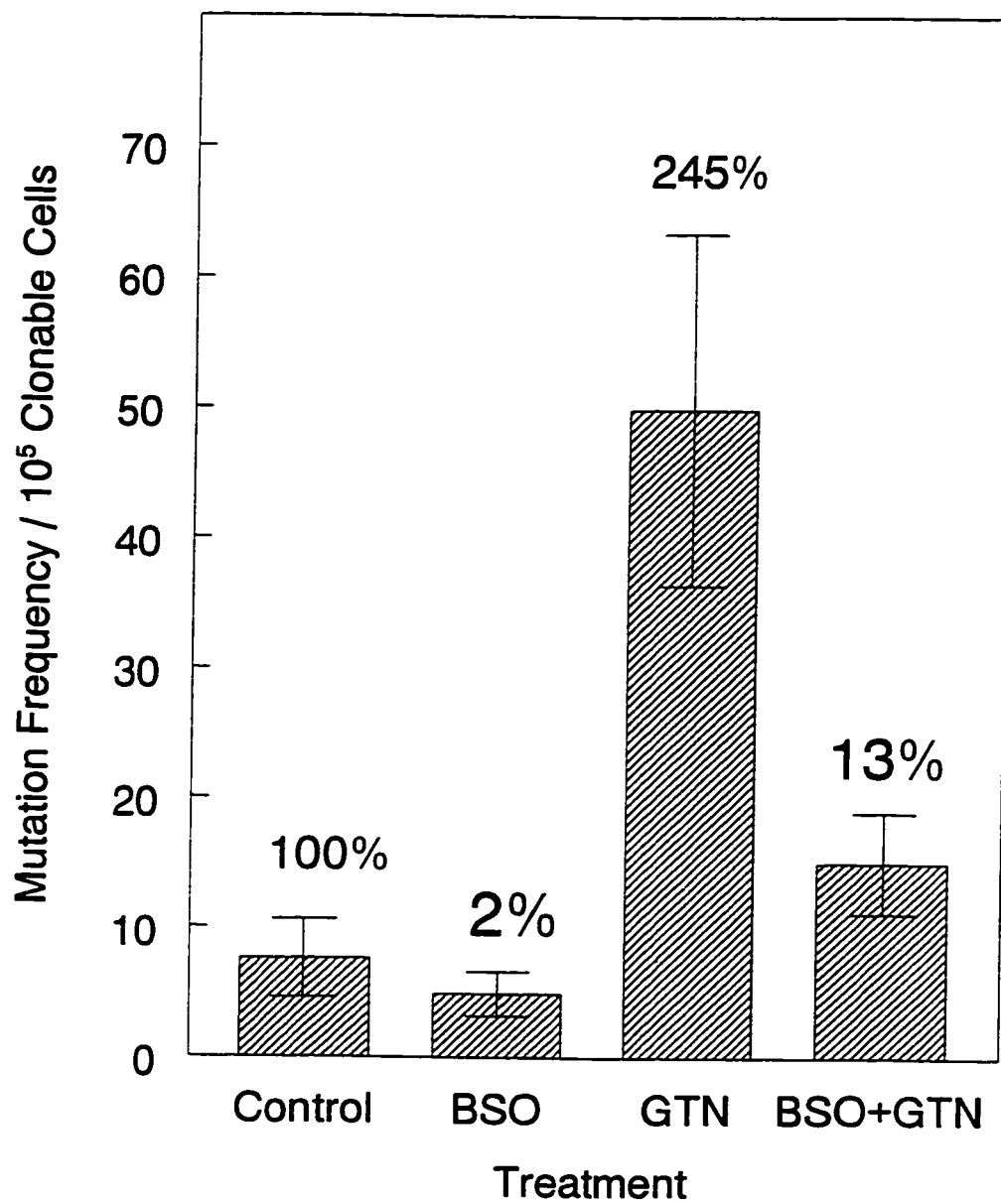
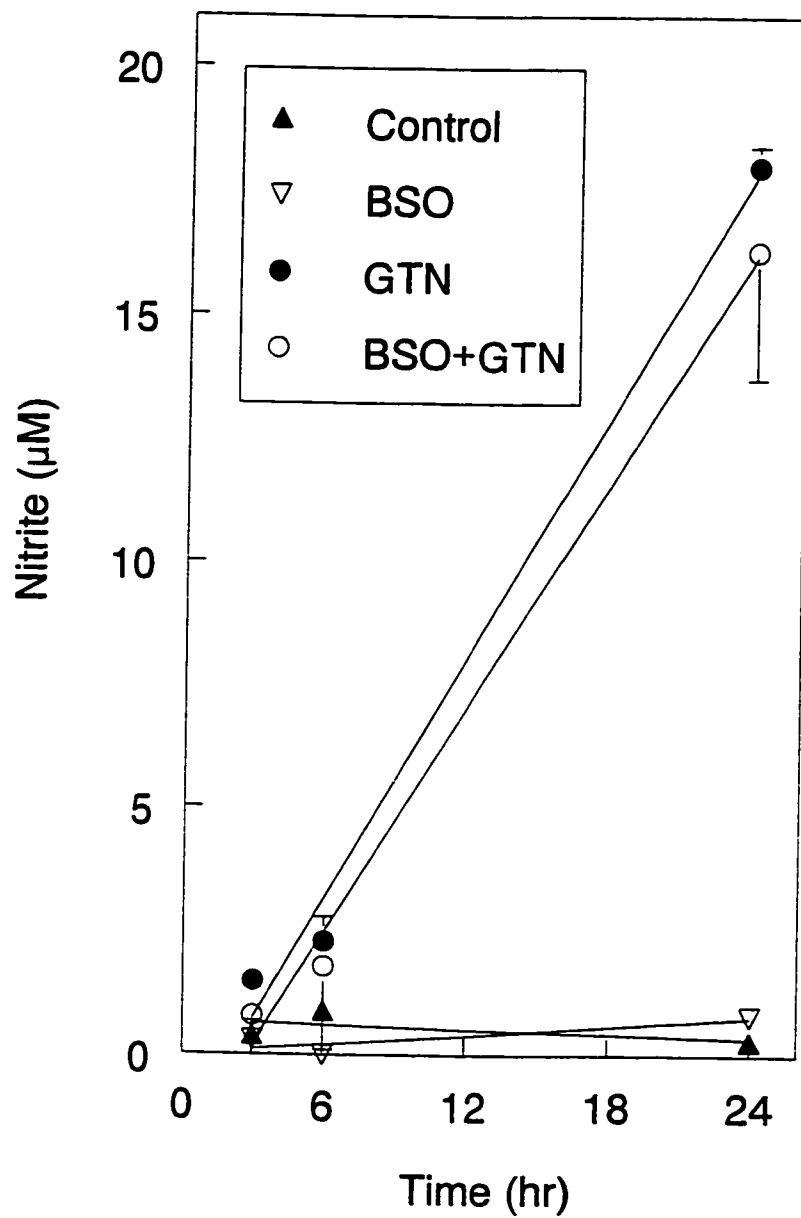


Figure 2-7. Effect of BSO coincubation on NO release by GTN.

NO release by GTN is measured as nitrite accumulation in cell culture medium. MN-11 cells were pretreated with 50 μ M BSO for 24 hours (or non-selection medium for control and GTN alone groups). At this time ($t = 0$), cells were given 123 μ M GTN (or nothing for BSO alone group). BSO was not washed out. At $t = 3, 6,$ and 24 hours, culture medium was removed and nitrite was measured. Results shown are mean \pm SEM after subtraction of background levels (medium with no cells).

Figure 2-7



A similar co-incubation experiment was performed on cells treated with 0.5 mM SNP. As with GTN, elimination of SNP - induced mutations was observed in BSO and SNP coincubated cells (Figure 2-8). The glutathione content of SNP treated cells was elevated slightly over controls, while cells that had received BSO and BSO plus SNP had drastically reduced glutathione levels (as shown above the bars in Figure 2-8). Thus, for both GTN and SNP (chemically unrelated NO donors that are metabolized differently (Feelisch and Stamler, 1996)), treatment of cells with BSO for 48 hours prior to addition of NO donor, and coincubation of BSO with the NO donor, causes an elimination of NO donor - induced mutations to control levels. In the first case (BSO pre-treatment), glutathione content of cells became dramatically elevated over control cell levels (by 100 to 200%), while in the second case (BSO co-incubation), glutathione levels remained low throughout the 24 hour treatment.

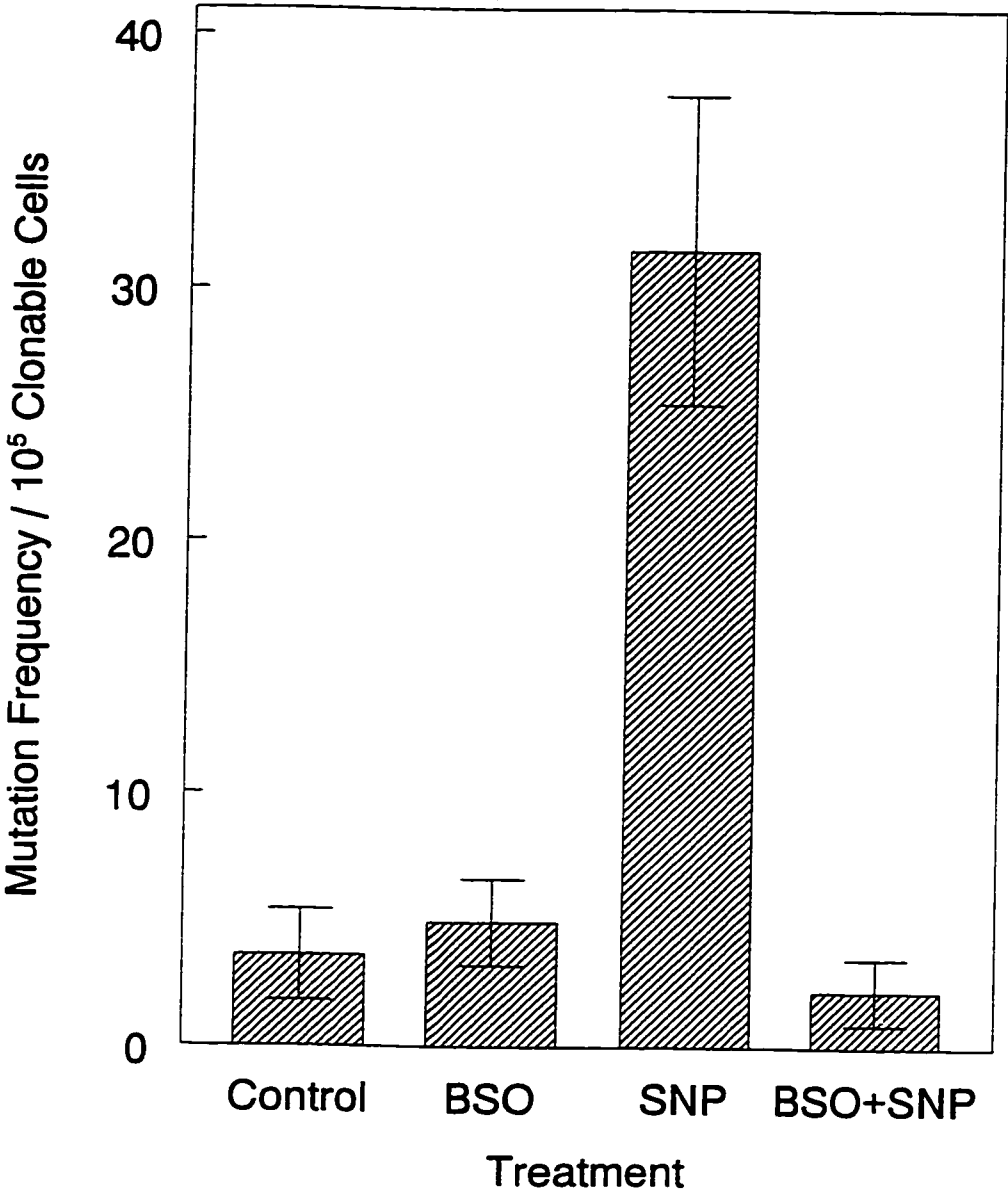
2.3.6 Effect of Elevated Glutathione Levels on GTN - Induced Mutations

BSO is known to very specifically deplete cells of their glutathione by inhibiting its synthesis. There exist a number of compounds that do the opposite - raise cellular glutathione levels. The effect of two of these compounds, N-acetylcysteine (NAC) and L-2-oxo-4-thiazolidine-carboxylic acid (OTZ), on GTN - induced mutations was studied. In each case, MN-11 cells were pretreated for 48 hours with either 10 mM NAC or 10 mM OTZ, with or without 50 µg/ml catalase. Both compounds in their metabolism may release a small amount of hydrogen peroxide, a relatively weak mutagen capable of

Figure 2-8. *Effect of BSO coincubation on SNP-induced mutations in MN-11 cells.*

MN-11 cells were pretreated with 50 μ M BSO for 24 hours, and coincubated for a further 24 hours with 0.5 mM SNP. Control cells were maintained in non-selection medium, SNP alone cells received 0.5 mM SNP for 24 hours, and BSO alone cells received 50 μ M BSO for 48 hours. Cells were cultured for 7 days to allow for the expression of mutant phenotype. Mutation frequency is expressed as number of mutant colonies per 10^5 clonable cells. Shown are mean \pm SEM (n = 4 to 13 for individual groups). Statistically significant: control vs SNP $p < 0.05$, SNP vs BSO+SNP $p < 0.01$, SNP vs BSO $p < 0.05$. Also shown is glutathione content of cells for each group, given as percent of control cells.

Figure 2-8



causing DNA strand breaks (Bradley and Erickson, 1981), and being slightly mutagenic in the MN-11 system (Sandhu and Birnboim, 1997). Neither NAC (Figure 2-9), nor OTZ (Figure 2-10) were mutagenic on their own, or in the presence of catalase. A dramatic decrease in GTN - induced mutations was seen in cells pretreated with both compounds, with or without catalase. In all cases, survival was greater than 90% (data not shown). Thus, NAC and OTZ prevented GTN-induced mutations, presumably by increasing glutathione levels in cells (Meister, 1983).

2.3.7 BSO and Radiation - Induced Cytotoxicity and Mutations

BSO is a known radiosensitizer (Dethmers and Meister, 1981). Its effects on ^{60}Co γ -irradiation - induced cytotoxicity and mutagenicity to MN-11 cells were studied. The generation of mutants in MN-11 cells by ^{60}Co γ -rays has been shown previously (Wilkinson *et al.*, 1995). In my experiment, a similar number of mutants was induced as seen previously (Figure 2-11b). Likewise, the survival of MN-11 cells following radiation was similar to that reported by Sandhu and Birnboim (1997) (approximately 80% survival at 1.5 Gy, and 50% survival at 3.0 Gy) (Figure 2-11a). To test the effects of glutathione depletion on radiation - induced cytotoxicity and mutagenicity, MN-11 cells were pretreated with 50 μM BSO for 48 hours. Immediately upon removal of BSO, treated cells and control cells were irradiated. Figure 2-11a shows that BSO pretreatment did not significantly increase cell killing by radiation. Results have been adjusted for

Figure 2-9. *Effect of NAC, with and without catalase, on GTN-induced mutation frequency in MN-11 cells.*

MN-11 cells were treated for 48 hours with 10 mM NAC, with or without 50 $\mu\text{g/ml}$ catalase (except control and GTN alone groups, which were maintained in non-selection medium). After the NAC was washed out, cells were treated for a further 24 hours with 123 μM GTN (or non-selection medium, as appropriate). Cells were cultured for 7 days to allow for the expression of mutant phenotype. Mutation frequency is expressed as number of mutant colonies per 10^5 clonable cells. Error bars indicate mean \pm SEM for $n = 6$ for each data point. Statistically significant: GTN vs all groups (except NAC + catalase) $p < 0.01$, GTN vs NAC + catalase $p < 0.05$. Cytotoxicity experiments were also performed. In all groups ($n = 5$ per group), survival was greater than 90%, as compared to control (data not shown).

Figure 2-9

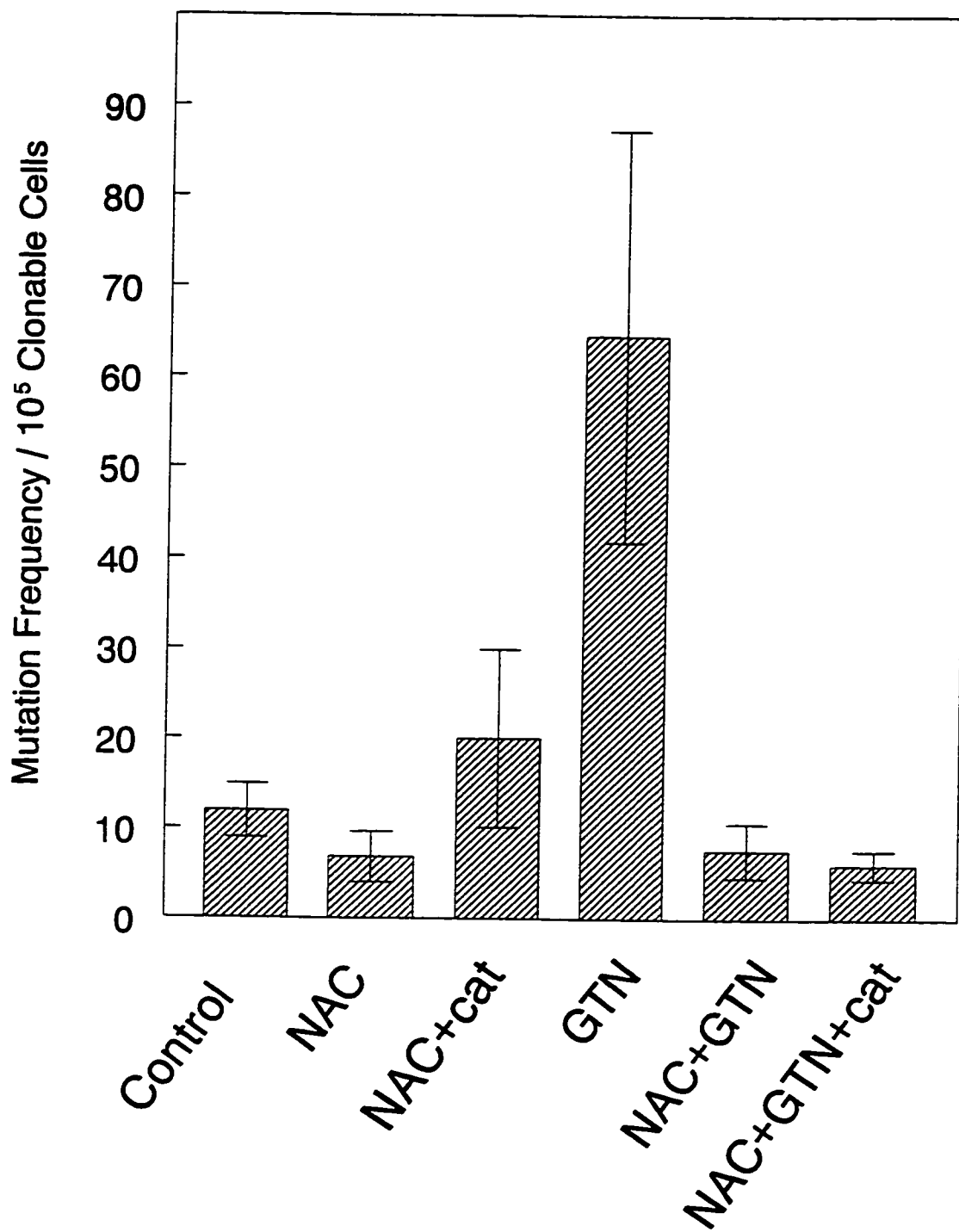


Figure 2-10. *Effect of OTZ, with and without catalase, on GTN-induced mutation frequency in MN-11 cells.*

MN-11 cells were treated for 48 hours with 10 mM OTZ, with or without 50 $\mu\text{g/ml}$ catalase (except control and GTN alone groups, which were maintained in non-selection medium). After the OTZ was washed out, cells were treated for a further 24 hours with 123 μM GTN (or non-selection medium, as appropriate). Cells were cultured for 7 days to allow for the expression of mutant phenotype. Mutation frequency is expressed as number of mutant colonies per 10^5 clonable cells. Error bars indicate mean \pm SEM for $n = 4-6$ for each data point. Statistically significant: GTN vs all groups $p < 0.001$. Cytotoxicity experiments were also performed. In all groups ($n = 5$ per group), survival was greater than 90%, as compared to control (data not shown).

Figure 2-10

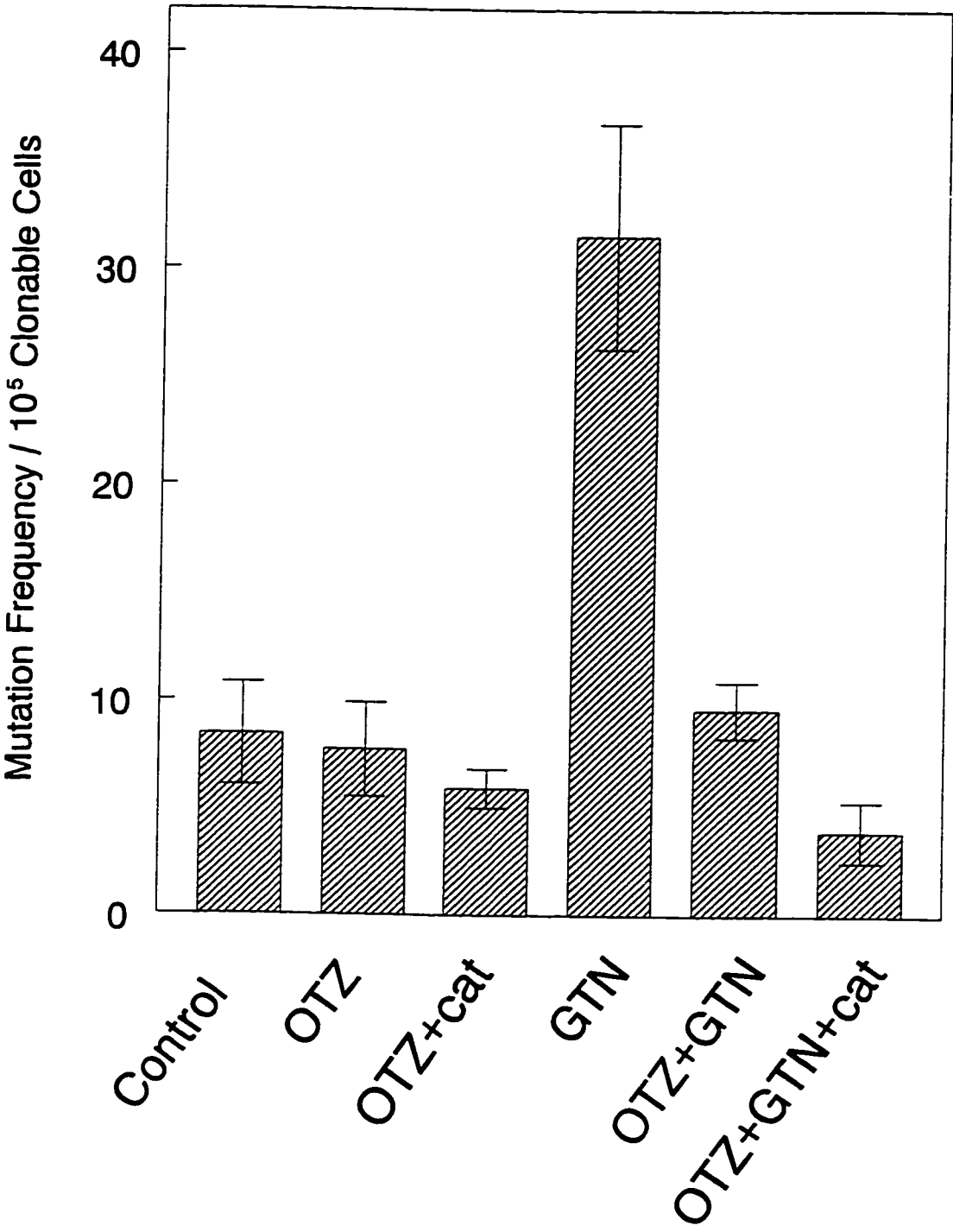
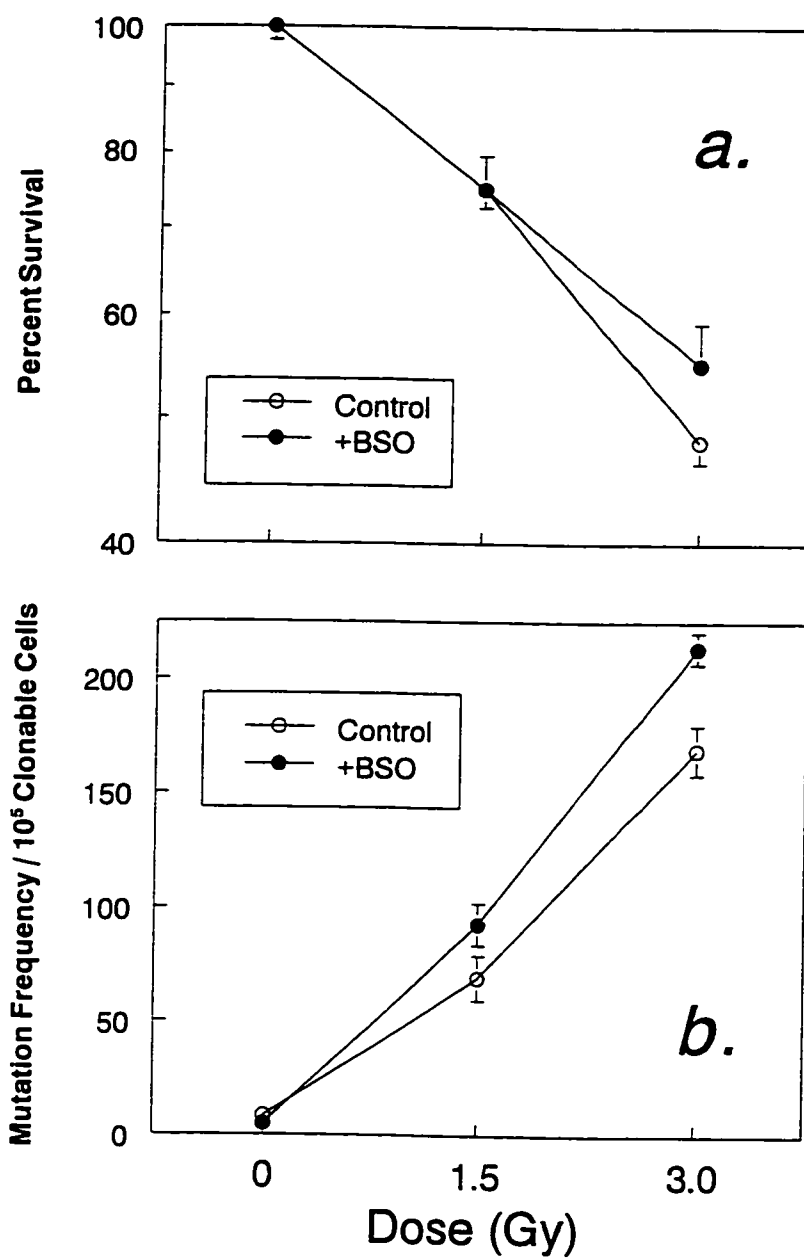


Figure 2-11. Cytotoxic and mutagenic effects of BSO and ionizing radiation on MN-11 cells.

The effect of glutathione depletion by BSO treatment (50 μ M BSO for 48 hours) on cytotoxicity (shown in *a.*) and mutation frequency (shown in *b.*) by ^{60}Co γ -irradiation is given. Error bars indicate mean \pm SEM of a single experiment (n = 5 replicate dishes at each experimental point). *a.* All cytotoxicity values have been adjusted against controls. BSO pretreated cytotoxicity values have been corrected for the cytotoxicity by BSO alone (a difference of 18%). *b.* Cells were cultured for 9 days to allow for the expression of mutant phenotype. Mutation frequency is expressed as number of mutant colonies per 10^5 clonable cells. Statistically significant: Control vs BSO-treatment at 3.0 Gy p=0.008 (in *b.*).

Figure 2-11



initial BSO cytotoxicity of 18%. At the higher dose, BSO appeared to protect slightly against cell killing; however, this difference was not statistically significant. There was a slight increase in radiation - induced MF in BSO pretreated cells (Figure 2-11b), which was found to be statistically significant at the higher dose of 3.0 Gy.

2.3.8 Cytotoxicity and Mutagenicity of Other NO Donors on MN-11 Cells

There exists a large number of compounds of different classes that produce NO in biological systems. The pathways by which they form NO differ significantly, as well as their chemical reactivities (Feelisch and Stamler, 1996). Both GTN and SNP are known to require metabolic processing to release NO. We wanted to determine if NO donors that do not require metabolic processing would also be mutagenic in MN-11 cells. Three drugs that release NO spontaneously, GSNO, SIN-1, and DETA/NO, were given to cells for 24 hours, then washed out. This was followed by a 7 day mutation expression time. Figure 2-12 shows the effects of GSNO on MN-11 cells. While there was increasing cytotoxicity caused by increasing concentrations of GSNO, there was little or no increase in mutation frequency over control cells. The cytotoxicity of various concentrations of SIN-1 was tested, with and without 50 μ g/ml catalase or 50 μ g/ml superoxide dismutase (SOD) (Figure 2-13). While catalase did decrease SIN-1 - induced cytotoxicity (Figure 2-13a), SOD had no effect (data not shown). As for GSNO, SIN-1 caused little or no increase in mutation frequency (Figure 2-13b). The cytotoxic and mutagenic effect of 24 hour treatment of DETA/NO was tested over a wide range of drug doses (Figure 2-14).

Figure 2-12. Cytotoxic and mutagenic effects of NO donor GSNO on MN-11 cells.

The effects of treatment for 24 hours with various concentrations of GSNO are shown. *a.* Cytotoxicity values have been adjusted against controls. *b.* Cells were cultured for 7 days to allow for the expression of mutant phenotype. Mutation frequency is expressed as number of mutant colonies per 10^5 clonable cells. Error bars indicate mean \pm SEM for $n = 5$ for each point.

Figure 2-12

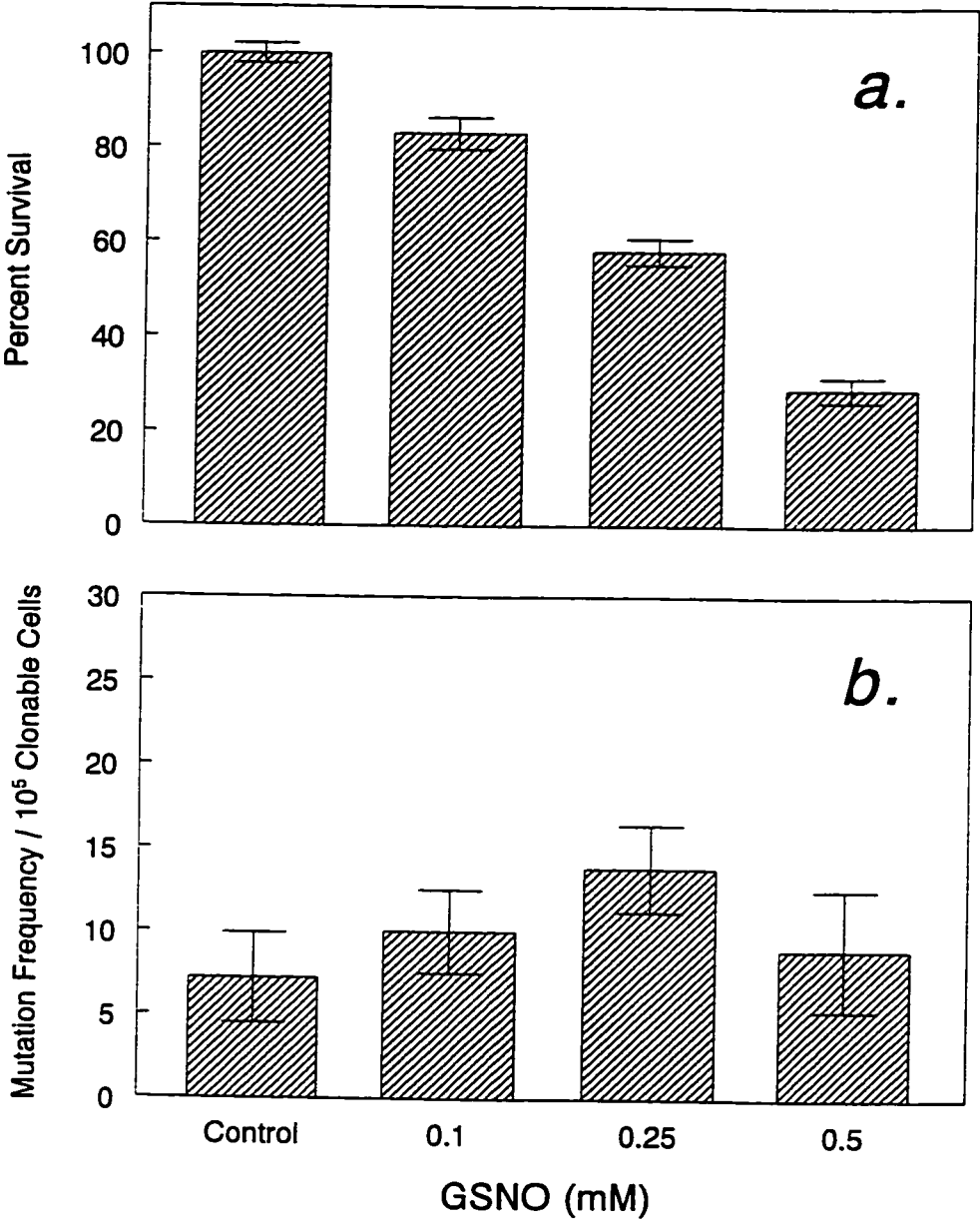


Figure 2-13. Cytotoxic and mutagenic effects of NO donor SIN-1 on MN-11 cells.

The effects of treatment for 24 hours with various concentrations of SIN-1, with and without 50 $\mu\text{g/ml}$ catalase are shown. *a.* Cytotoxicity values have been adjusted against controls. *b.* Cells were cultured for 7 days to allow for the expression of mutant phenotype. Mutation frequency is expressed as number of mutant colonies per 10^5 clonable cells. Error bars indicate mean \pm SEM for $n = 5$ for each point.

Figure 2-13

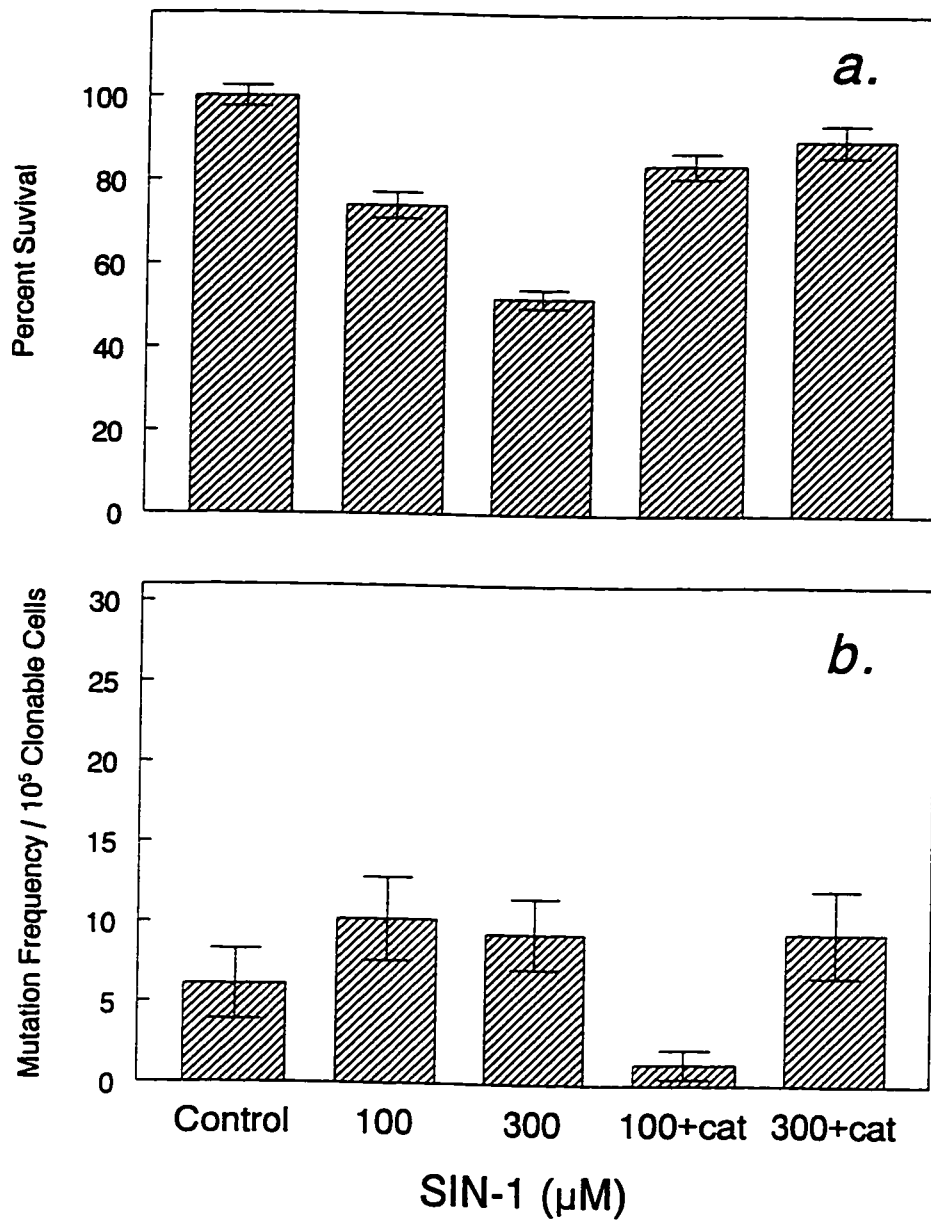
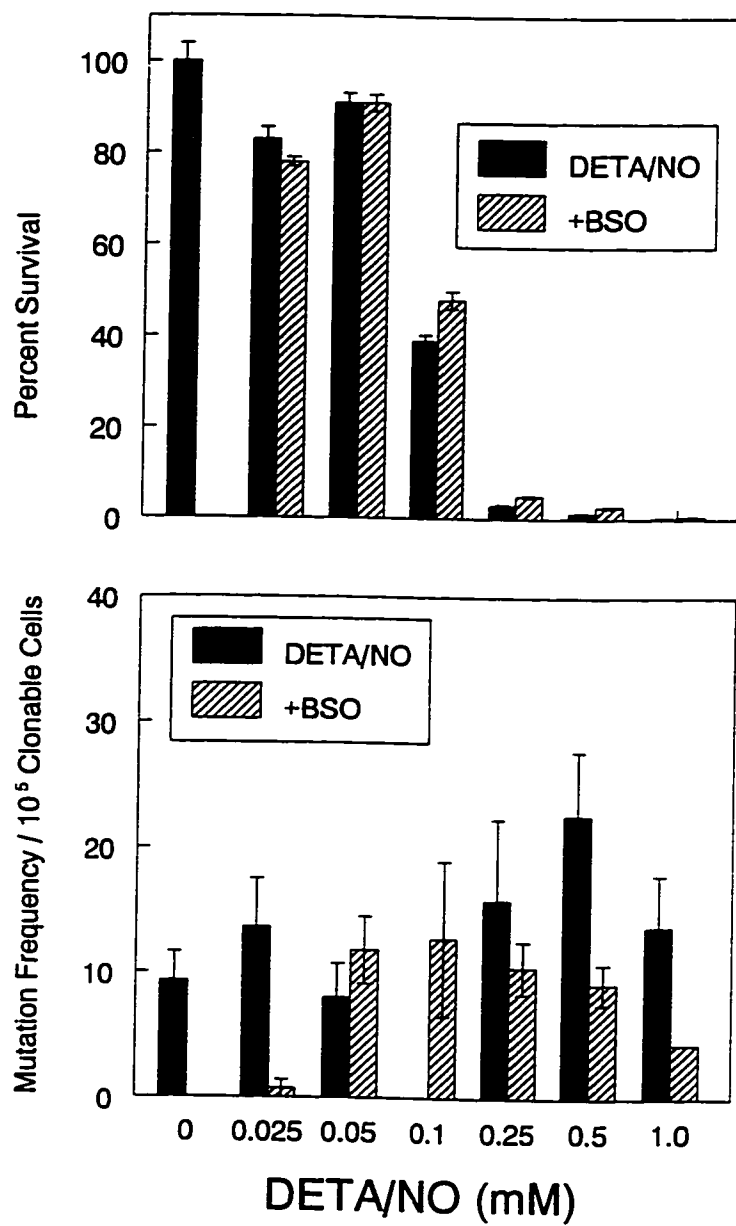


Figure 2-14. *The effect of BSO on the cytotoxicity and mutagenicity of NO donor DETA/NO on MN-11 cells.*

MN-11 cells were pretreated for 48 hours with 50 μ M BSO, or were maintained in non-selection medium. BSO was washed off, and cells then received various concentrations of NO donor DETA/NO for a further 24 hours (control cells were maintained in non-selection medium). *a.* Cytotoxicity values have been adjusted against controls. *b.* Cells were cultured for 7 days to allow for the expression of mutant phenotype. Mutation frequency is expressed as number of mutant colonies per 10^5 clonable cells. Error bars indicate mean \pm SEM for n = 4-9 for each data point.

Figure 2-14



Concentrations of greater than 0.1 mM caused major cytotoxicity to cells (Figure 2-14a). There was, however, little or no increase in mutant frequency (Figure 2-14b). In some cases, 48 hour pretreatment with 50 μ M BSO was carried out. BSO pretreatment had no effect on either cytotoxicity or mutagenicity of DETA/NO on MN-11 cells (Figure 2-14a and b). None of the NO donors tested (GSNO, SIN-1, DETA/NO) caused a significant increase in MF in the MN-11 system.

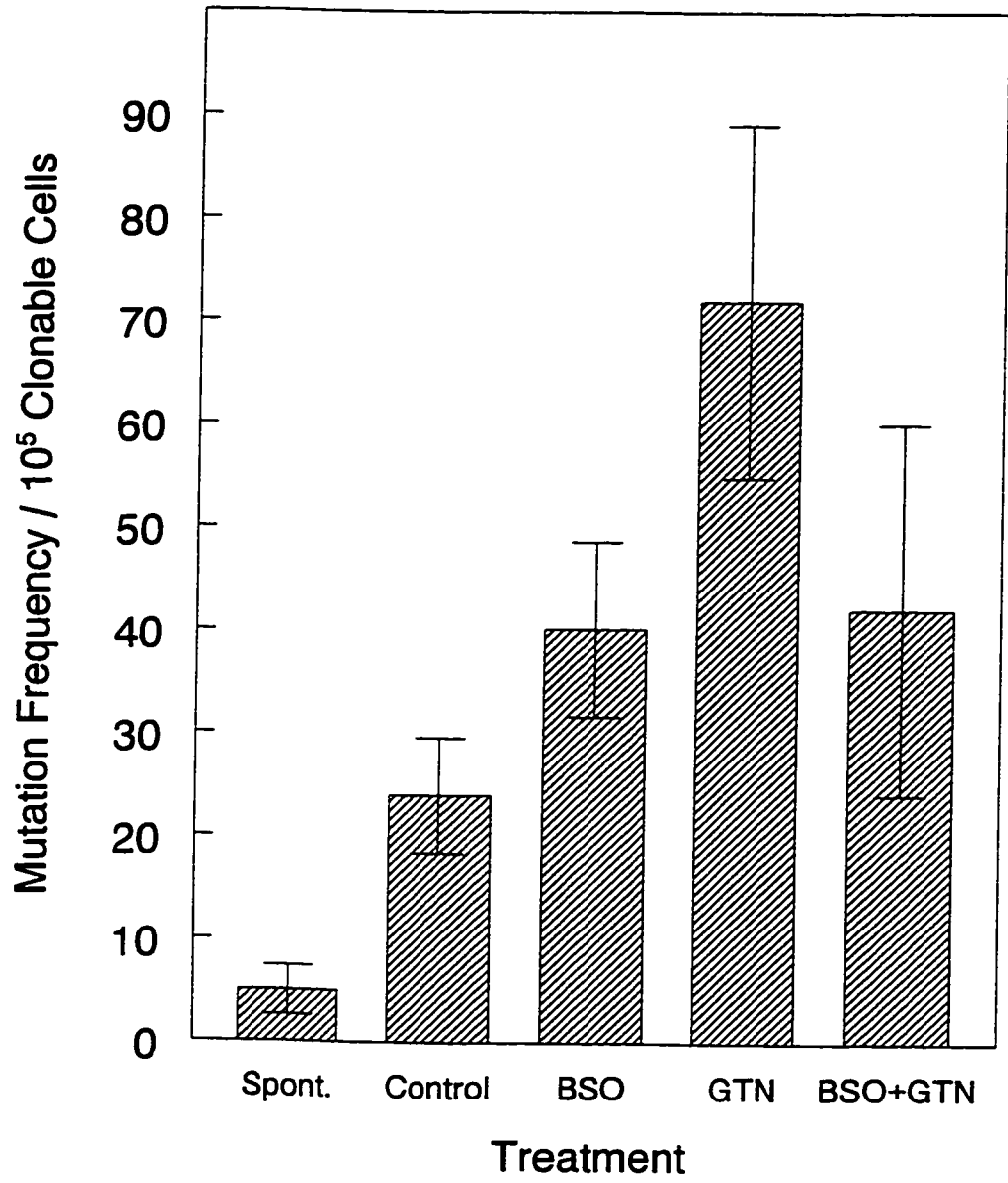
2.3.9 In Vivo GTN and BSO Experiments

It had been shown previously that GTN is mutagenic both to cultured cells, as well as to cells grown as tumours in syngeneic mice (Sandhu and Birnboim, 1997; Sandhu and Birnboim, manuscript in preparation). Since BSO was found to have a marked effect on GTN - induced mutations *in vitro*, its the effect on GTN - induced mutations *in vivo* was studied. Animals received 200 mg/kg BSO ip daily for 4 days just prior to 5 mg/kg GTN injection. Results are shown in Figure 2-15. Control animals (which received PBS injections) had an approximately 5 fold increase in mutation frequency over cells that were cultured for the duration of the *in vivo* experiment. Treatment with GTN *in vivo* caused an additional 3 fold increase in mutation frequency. BSO treatment alone caused a slight but statistically insignificant increase in mutation frequency. Tumours from mice that had received both BSO and GTN had a mutation frequency about the same as tumours from animals treated with BSO alone. One way ANOVA analysis resulted in a *p* value of almost significant 0.053, indicating that the

Figure 2-15. *The in vivo effect of glutathione depletion by BSO on GTN-induced mutations.*

Animals were injected with MN-11 cells, as described *Materials and Methods*. Beginning on day 7 after tumour inoculation for 4 consecutive days, mice received *ip* injections of 200 mg/kg BSO. Approximately 6 hours after the final BSO injection, animals received *ip* injections of 5 mg/kg GTN. Control mice received injections with PBS. On day 14 after tumour inoculation, animals were euthanized, tumours were processed, and cells were cultured as described in *Section 3, Materials and Methods*. Spontaneous group is cells that were maintained in culture for the duration of the *in vivo* experiments. Results are for 2 independent experiments, with n = 6 - 14 animals per group.

Figure 2-15



trend seen in the *in vitro* experiments also exists in the animal, and glutathione may play a role in *in vivo* GTN-induced mutations. In two further experiments, the effect of treatment with BSO alone was further analyzed. Mice received BSO injections for 2, 4 or 7 consecutive days. In all cases, mutation frequency was very slightly higher (statistically not significant) than control tumours (data not shown). Thus, GTN causes a large increase in mutation frequency over control tumours which is lowered by pretreatment with BSO.

2.4 Discussion

It has been shown in our laboratory that some NO donors can cause a significant increase in mutation frequency in the MN-11 system (Sandhu and Birnboim, 1997). Although NO has numerous physiological roles in biological systems, such as a mediator of blood vessel relaxation, a neurotransmitter, and an effector molecule in immunological reactions (Moncada *et al.*, 1991; Gibaldi, 1993), it should also be considered as a reactive and potentially toxic molecule. The targeted bacteriocidal action of NO and related species, when released by macrophages and neutrophils, may also result in concomitant reactions leading to DNA damage in neighbouring cells (Nguyen *et al.*, 1991). It has been recognized for a number of years that nitrite can cause mutations in prokaryotic organisms (Singer and Grunberger, 1983); more recently Wink *et al.* (1991) found that NO was mutagenic in *Salmonella typhimurium* and Nguyen *et al.* (1992) found that NO caused mutations and DNA strand breakage in TK6 human lymphoblastoid cells.

Glutathione is involved as a cofactor in numerous cellular reactions in addition to its action as an important cellular antioxidant. It protects cells against oxidative and free radical damage, both important factors in mutagenesis and carcinogenesis (Ames, 1991). Recently, glutathione has been implicated in providing protection against NO-induced cytotoxicity in CHO and TK6 cells (Luperchio *et al.*, 1996). Our expectation was that glutathione would also protect against both NO-induced cytotoxicity and mutations in the MN-11 system, and therefore we hypothesized that glutathione depletion with BSO

would result in an enhancement of GTN-induced cytotoxicity and mutations. BSO is considered to be a very specific inhibitor of the rate-limiting enzyme in the pathway of glutathione biosynthesis (Meister, 1983). This leads to depletion of glutathione over a several hour period, which has been shown to sensitize tumour cells to radiation (Dethmers and Meister, 1981; Shrieve *et al.*, 1985; Clark *et al.*, 1984; Leung *et al.*, 1993), and to chemotherapeutic agents *in vitro* (Meister, 1983; Arrick *et al.*, 1983) and *in vivo* (Kramer *et al.*, 1987). In Chinese hamster cells depletion of glutathione with BSO to <1% of controls was shown to increase both the cytotoxicity and the mutagenicity of two 2-nitroimidazole-aziridines (Orazio *et al.*, 1992). BSO has been tested as an adjuvant to cancer chemotherapy in clinical trials (Smith *et al.*, 1989; Bailey *et al.*, 1992). However, not all chemotherapeutic compounds are enhanced by BSO. Liebmann *et al.* (1993) found that glutathione depletion by BSO antagonizes taxol cytotoxicity, and suggest that attempting to modulate the activity of taxol with BSO in clinical trials would result in a decreased tumour response to taxol. BSO treatment of MN-11 cells prior to irradiation did not radiosensitize the cells to killing but caused a slight increase in mutation frequency. Since glutathione levels are restored once BSO is washed out, cytotoxicity might have been observed had the BSO not been washed out prior to irradiation. Ali-Osman *et al.* (1996) recently found that a greater level of BCNU cytotoxicity in human glioma cells was achieved with continuous BSO exposure than with BSO preexposure alone.

An increase in GTN- and SNP-induced cytotoxicity was observed in glutathione depleted cells; however, the opposite effect was observed for mutation frequency.

Depletion of glutathione virtually prevented mutations by these two compounds. Both GTN and SNP require cellular metabolism to release NO, but are metabolised differently (Feelisch and Stamler, 1996). GTN is an organic nitrate and requires either enzymatic or nonenzymatic bioactivation; SNP, a transition metal nitrosyl, releases NO after undergoing reduction and loss of cyanide (Feelisch and Stamler, 1996; Bates *et al.*, 1991). To test whether BSO was interfering with the metabolism of these drugs, nitrite release into cell culture medium was measured. Nitrite is a more stable end product of NO that accumulates in aqueous solution (Ignarro *et al.*, 1993). It can be used as a measure of the metabolism of these drugs. BSO pretreatment did not affect nitrite release from either GTN or SNP. Thus the observed elimination of mutations by BSO pretreatment cannot be attributed to interference in metabolism of the NO donors.

BSO is rapidly taken up and eliminated from cells (Malaker *et al.*, 1994; Smith *et al.*, 1989; Thanissar, 1995). The cellular uptake half-life of BSO was found to be 55 min in a human glioblastoma cell line (Malaker *et al.*, 1994). In two human glioma cell lines, treatment with 100 μ M BSO resulted in greater than 90% glutathione depletion after 24 hours (Ali-Osman *et al.*, 1996). However, recovery of glutathione levels after removal of BSO varied greatly between the two cell lines: after 24 hours, one had returned to control levels, while the second had recovered only 50% of its glutathione in that time (Ali-Osman *et al.*, 1996). After 48 hours of BSO treatment, glutathione levels in MN-11 cells were less than 2% of controls. After removal of BSO, glutathione levels quickly recovered, such that after 8 hours glutathione was at control levels (100%), in both

control (BSO alone) and SNP- or GTN-treated cells. In all cases, glutathione levels overshoot control levels, by as much as 200% in GTN-treated cells. The homeostatic mechanisms for glutathione are clearly perturbed by prolonged glutathione depletion. Recent findings have indicated that oxidative stress conditions enhance the expression of genes encoding antioxidant enzyme systems (Pinkus *et al.*, 1996), including γ -glutamyl cysteine synthetase (Shi *et al.*, 1994).

It remained unclear at this point whether the elimination of GTN and SNP-induced mutations was due to the overabundance of glutathione in the latter part of the treatment (i.e., glutathione was acting as predicted to protect against NO-induced mutations), or due to the decreased glutathione levels for the first 8 hours of treatment (i.e., glutathione was required for mutation induction and, in its absence, mutations could not arise). To explore further this issue, cells were maintained in BSO at the same time as they received a NO donor so that glutathione levels remained low throughout treatment. Once again, coincubation of cells with BSO and a NO donor (GTN) prevented mutations. It did not, however, inhibit the accumulation of nitrite in culture medium. From these experiments, it appears that glutathione is required for mutation induction by GTN or SNP. Since, in the BSO preincubation experiments, glutathione levels are low for only the first 8 hours of NO donor treatment (and then greatly overshoot control levels), this suggests that this early time is crucial for mutation induction. Interestingly, 8 hours of GTN treatment is not sufficient to induce mutations in MN-11 cells; it appears that a full 24 hours is necessary (J. K. Sandhu, personal communication). This may

explain why elevated levels of glutathione for the latter 16 hours of treatment are not sufficient to cause mutation induction in BSO pretreated groups.

Cellular glutathione may be manipulated in many ways, including increasing its level by delivering of the rate limiting amino acid in glutathione synthesis, cysteine (Baruchel *et al.*, 1995). However, cysteine is not transported efficiently into cells and, even at moderate doses, L-cysteine is toxic (Meister, 1988), possibly due to H₂O₂ production (Kanabus-Kaminska, 1988). Effective delivery of cysteine requires the administration of a cysteine “prodrug” that is readily transported into cells and converted into cysteine (Meister, 1988). Two such compounds are *N*-acetyl-L-cysteine (NAC) and L-2-oxothiazolidine-4-carboxylate (OTZ) (Meister, 1992). OTZ requires the activity of 5-oxoprolinase, which converts OTZ to L-cysteine (Meister, 1994). To explore further the effect of altering levels of glutathione on NO donor-induced mutations, MN-11 cells were treated with NAC or OTZ for 48 hours. In both cases, GTN-induced mutations were almost reduced to control levels. The addition of catalase to quench any hydrogen peroxide generated from cysteine was unnecessary, as neither compound was cytotoxic or mutagenic on its own. Thus, increased levels of cysteine are protective against NO donor-induced mutations. Whether this is due to reaction of cysteine directly with NO or due to the expected increase in glutathione is uncertain.

My experimental results appear to present a paradox with respect to the question of whether glutathione blocks or facilitates mutations by NO donors. NO donors GTN and SNP are clearly mutagenic in the MN-11 system and alteration of glutathione levels

affects these mutations. GTN and SNP alone can elevate glutathione levels, suggesting some reaction with glutathione. Depletion of glutathione by treatment with BSO prevents mutations. Treatment with compounds that increase cellular cysteine levels also prevents NO donor-induced mutations. Cysteine and glutathione may both react with NO, but may have opposite effects with respect to mutations. It may also be that glutathione behaves in a biphasic manner, perhaps due to two separate reaction pathways: At normal cellular concentrations, glutathione appears to participate in the mutagenicity of GTN and SNP, perhaps through the formation of a mutagenic intermediate (see below). When cellular glutathione is removed, the mutagenic species is not formed, although GTN and SNP are both metabolised to nitrite, perhaps through a different pathway.

Neither GTN nor SNP spontaneously liberates NO in the absence of tissue in the dark (Brien *et al.*, 1988; Bates *et al.*, 1991), though the exact metabolic activation pathways are as yet unknown (Feelisch and Stamler, 1996). GTN requires bioactivation that may occur through two different pathways, one enzymatic, the other non-enzymatic, with both reactions competing with each other (Noack and Feelisch, 1991). Non-enzymatic NO formation requires interaction with thiol groups, releasing nitrite directly as the major nitrogenous metabolite, although certain thiol compounds (not glutathione) promote NO generation as well (Feelisch and Stamler, 1996). Enzymatic bioactivation is believed to involve both denitration by cytosolic glutathione-S-transferase, producing mainly nitrite, and enzymatic reduction of GTN to thionitrite esters, which form NO via nitrosothiol (RSNO) intermediates (Noack and Feelisch, 1991). SNP, a complex of Fe^{2+} , 5 CN^- , and NO^- , requires reductive activation to release NO, for example with thiol

(Lipton *et al.*, 1993). Both GTN and SNP exhibit strong NO⁻ character (GTN by way of its RSNO intermediate, SNP contains an NO⁻), and it has been suggested that some of the biological effects of these compounds may be due to NO⁻ (Lipton *et al.*, 1993). Previously our laboratory has found that, of a variety of NO donors tested (SNAP, SPER/NO, GTN, SNP), the drugs requiring bioactivation through thiol-mediated reactions (i.e. GTN and SNP) had the highest mutation frequency (Sandhu and Birnboim, 1997). This suggests that an intermediate formed in the metabolism of these compounds with thiols, perhaps an NO⁻ - like compound, may be the mutagenic species. At low levels of glutathione, this species may not be formed. However, through alternative pathways, NO may still be released or bioactivation of the NO donor may lead to the direct release of nitrite itself.

Thus, at low levels of glutathione, mutations may be prevented. At high (greater than normal) levels of glutathione, the mutagenic species may be formed. However, with the excess of glutathione, these species are readily quenched through the protective properties of glutathione before they are able to cause damage. A biphasic effect of thiols on cellular DNA phenomenon has been seen in our laboratory before: Cysteamine can have both protective and damaging effects on leukocyte DNA (Kanabus-Kaminska *et al.*, 1988). It was found that cysteamine reacts with oxygen to generate hydrogen peroxide, which leads to DNA strand breakage. Yet, at higher concentrations, cysteamine eliminates hydrogen peroxide by reacting with it, thus protecting the DNA (Kanabus-Kaminska *et al.*, 1988). It is possible that glutathione is exhibiting a similar biphasic character in the MN-11 system.

The *in vivo* effects of BSO were also tested. Since it had been shown previously in our laboratory that GTN is also mutagenic in syngeneic mice (Sandhu and Birnboim, manuscript in preparation), we tested the effect of glutathione depletion on GTN-induced mutations in mice bearing MN-11 tumours. Injections of BSO causes depletion of glutathione in various tissues in laboratory animals (Griffith and Meister, 1979; Lee *et al.*, 1989; Smith *et al.*, 1989; Zimmerman *et al.*, 1989; Thanislass *et al.*, 1995), although some have found that tumours are more susceptible to the effects of glutathione depletion than normal tissues (Revesz *et al.*, 1994; Terradez *et al.*, 1993). In MN-11 tumours, ip injections of 250 mg/kg BSO over 4 days caused a depletion of tumour glutathione levels to less than 20% of controls (J. K. Sandhu, personal communication). There was no indication of an increase in mutation frequency by BSO treatment for 2, 4, or 7 days prior to tumour excision and MF analysis. However, as in the case of *in vitro* experiments, treatment with GTN following BSO injections resulted in a drop in GTN-induced mutations. However, due to the complexity in interpretation of these experiments, this line of research was not pursued further.

NO is involved in a diverse set of biological roles. Since most of our information concerning NO involvement in cellular functions has resulted from our ability to mimic endogenous responses by the application of an NO donor, there is considerable interest in these compounds (Feelisch and Stamler, 1996). All NO donors have the characteristic of producing NO-related activity, yet the pathway through which this happens is different for a given NO donor (Noack and Feelisch, 1991; Feelisch and Stamler, 1996). This multiplicity of pathways, and which form of NO (NO^+ , NO^- , or NO^\cdot) is actually released

may make a significant difference in biological systems (Pan *et al.*, 1996; Lipton *et al.*, 1993; Feelisch and Stamler, 1996). In the MN-11 system, at equicytotoxic doses, the induced mutant frequency varied greatly for the different NO donors tested previously (SNP, GTN, SPER/NO and SNAP) (Sandhu and Birnboim, 1997). In my work, several more NO donors were tested for their mutagenic potential to attempt to elucidate the species involved. SIN-1, the active metabolite of the antianginal drug molsidomine, is a potent vasorelaxant and antiplatelet agent, and releases NO spontaneously (Feelisch and Stamler, 1996). SIN-1 is capable of stoichiometric generation of superoxide along with NO. The reaction product, peroxynitrite, is a potent and destructive oxidant (Koppenol *et al.*, 1992). It would therefore be expected that superoxide dismutase would attenuate SIN-1 cytotoxicity. This was not the case. Rather, NO-mediated cytotoxicity appears to be at least partially dependent on hydrogen peroxide, as catalase was able to protect against SIN-1 cytotoxicity. These findings are consistent with previous work by Farias-Eisner *et al.* (1996) (who reports that NO-mediated loss of cell viability is dependent on both NO and H₂O₂ in a human ovarian cancer cell line) and Ioannidis and de Groot (1993) (who reported that H₂O₂ enhances NO toxicity in a hepatoma cell line). Farias-Eisner *et al.* (1996) suggest that NO and H₂O₂ in the presence of trace metals are capable of generating hydroxy radical (\cdot OH), a highly reactive and potentially damaging species. SIN-1, while cytotoxic in the MN-11 system, did not produce a statistically significant increase in mutation frequency over control levels at any of the concentrations tested. It is possible that, due to its rapid hydrolysis at neutral pH (Feelisch and Stamler, 1996), cells were not exposed to the mutagenic species for a long enough period of time for it to

cause a large increase in mutations. It is important to note that even GTN had to be present for 24 hours to cause a high mutation frequency (Sandhu, 1998).

S-nitrosoglutathione (GSNO) is a member of the thionitrites class of NO donors (S-nitrosothiols, RSNO). It is thought that S-nitrosothiol groups in proteins are involved in the metabolism of NO and in the regulation of cellular functions such as NO transfer reactions (Stamler, 1995). In physiological buffers RSNOs undergo decomposition to yield a disulfide and NO, creating thiyl radicals as intermediates (Feelisch and Stamler, 1996). As well, RSNOs can decompose heterolytically, thus acting as donors of NO, NO[•], or NO⁻ (Feelisch and Stamler, 1996). Since glutathione can react with NO to form GSNO (Feelisch, 1991), and since reactions of GSNO with GSH may result in the formation of numerous radical species (such as glutathionyl radical, GS[•], and peroxysulfonyl radical, GS(OO⁻)[•]) (Singh *et al.*, 1996), this compound was tested for its mutagenicity in the MN-11 system as a possible clue to the mutagenic species involved in GTN and SNP bioactivation. However, although treatment with GSNO caused an increase in cytotoxicity with increasing concentration, there was no statistically significant increase in mutation frequency at any of the concentrations tested. Unlike SIN-1, GSNO has a stability in solution of several hours (Singh *et al.*, 1996). However, it is unlikely that GSNO is able to enter cells readily. Thus, it is possible that the mutagenic species are released but cannot reach the nucleus.

DETA/NO is a member of the NONOates class of NO donors. It was tested for its mutagenic potential since it is thought to release NO spontaneously and has a

relatively long half life of approximately 20 hours (thus mimicking the slow release of NO due to bioactivation of GTN or SNP) (Mooridian *et al.*, 1995; Maragos *et al.*, 1991). A great range in cytotoxicity was seen over the concentration range tested. However, once again, there was no significant increase in mutation frequency at any of the doses, nor did pretreatment with BSO have any significant effect. Yet when nitrite accumulation was measured, it became apparent that, contrary to the literature report (Feelisch and Stamler, 1996), all release of NO occurred within the first two hours of treatment. Therefore, it was not possible to determine if slow release of NO *per se* by GTN and SNP was responsible for their mutagenic activities, or whether it was due to some by-product of their metabolic activation. Although DETA/NO was chosen for this purpose, in our experimental system it proved to release NO too quickly.

It is obvious that a careful selection of the NO donor used in experimentation must be made, as each one is different in its pathway to NO donation and chemical reactivity, and, therefore, in its suitability for each experiment. In the MN-11 system it has proved difficult to differentiate which species is (are) responsible for the mutagenicity of GTN and SNP. Glutathione appears to have a dual role as both protectant and player. Further experiments, such as the direct measurement and application of NO with glutathione manipulation, may help to elucidate the species involved, and thus provide insight into genotoxic reactions that occur *in vivo*.

3. THE EFFECT OF INFILTRATING NEUTROPHILS AND NECROSIS ON IN VIVO MUTATION FREQUENCY

3.1 Introduction

An association between chronic inflammatory states and cancer has been known for a long time for numerous diseases (Weitzman and Gordon, 1990) (refer to *Section 1* for examples). Strong evidence suggests that stimulated inflammatory cells, at least in part through their release of reactive oxygen and nitrogen species which subsequently damage host cells, might contribute to the development of many types of cancer (Ohshima and Bartsch, 1994).

In the MN-11 system, it was previously found that the mutation frequency of cells grown as tumours was higher than that of cells grown in culture dishes for the equivalent period of time, suggesting that something in the tumour environment in the animal was mutagenic. MN-11 tumours are infiltrated with inflammatory cells, as solid tumours typically are (Lee *et al.*, 1996; Weitzman and Gordon, 1990; Rosin *et al.*, 1994). In MN-11 tumours the infiltrate consists primarily of neutrophils, with small numbers of macrophages, mast cells, and lymphocytes (see Sandhu, 1998 for a full description). The presence of this infiltrate suggests an inflammatory reaction, and the observed increase in MF in cells recovered from tumours may be due to reactive species produced by these cells. The objective of this part of my thesis was to determine whether the number of

neutrophils correlates with the frequency of mutation, thus lending support to the above hypothesis.

In order to investigate the role of infiltrating neutrophils on MF, large numbers of neutrophils were attracted to the tumour using local injections of interleukin-8 (IL-8). IL-8, also known as neutrophil attractant/activating protein (NAP-1), monocyte-derived neutrophil-activating peptide (MONAP), monocyte-derived neutrophil chemotactic factor (MDNCF), and neutrophil-activating factor (NAF), and by several other names, is a known chemoattractant for neutrophils, inducing chemotaxis, exocytosis, and the respiratory burst Colditz *et al.*, 1989). IL-8 is produced by phagocytes and a variety of tissue cells upon exposure to infection, ischemia, and trauma (Baggiolini and Clark-Lewis, 1992). It is thought to play a causal role in the establishment of inflammation (Harada *et al.*, 1994), and has been shown to elicit massive neutrophil accumulation at the site of injection (Baggiolini and Clark-Lewis, 1992). Elevated IL-8 levels have been reported in a number of inflammatory diseases including inflammatory bowel disease, rheumatoid arthritis (Schuerer-Maly *et al.*, 1994), ulcerative colitis (Mahida *et al.*, 1992), and acute alcoholic hepatitis (Sheron *et al.*, 1993). Melani *et al.* (1995) analyzed two human carcinoma cell lines for the constitutive expression of various cytokines, and found that both produced IL-8, supporting their hypothesis that neutrophils can favour the growth of tumours. In my study, I used IL-8 injections into palpable MN-11 tumours to attract neutrophils to the tumours, allowing a comparison of MF in relation to the amount of infiltration.

3.2 Materials and Methods

3.2.1 Materials

A list of all chemicals used, suppliers and solution compositions is found in **Appendix I**.

3.2.2 Cell Culture Conditions

Cell culture conditions are described in detail in **Section 2.2.2**.

3.2.3 Tumour Induction and Injections

C57BL/6 female mice, 8 - 10 weeks of age (Charles River Laboratories or Tachonic) were injected subcutaneously on the flank region with 5×10^5 MN-11 cells in 0.1 ml PBS. The MN-11 cells had been grown in HAT medium for 7 days followed by 2 days in HT medium to first remove any pre-existing *hprt* mutants. A portion of these cells were maintained in culture for the duration of the experiments to determine spontaneous *in vitro* mutation frequency.

On day 11 following tumour inoculation, when tumours were approximately 5 mm in diameter, mice received intra-tumour injections of interleukin-8 (IL-8) alone or in conjunction with prostaglandin E2 (a vasodilator, PGE2), or with fMLP (a bacterial

chemotactic peptide) in conjunction with PGE2, or with endotoxin-free sterile saline as a control. Amounts injected were 1×10^{-12} mol IL-8, 3×10^{-10} mol PGE2, and 1×10^{-10} mol fMLP, in a total volume of 6 μ l using an ethanol-sterilized glass Hamilton syringe capable of delivering multiple 2 μ l injections (to obtain 6 μ l, the syringe was depressed 3 times). Prior to injection, the flank was thoroughly swabbed with alcohol.

On day 15 following tumour inoculation (4 days after IL-8/PGE2 injections), animals were euthanised by cervical dislocation and tumours were removed under sterile conditions. A section of the tumour (from the middle portion) was removed for fixation or freezing (described below). The remainder of the tumour was used to prepare cell suspensions by trituration with a 3 c.c. syringe, followed by low speed (1000-1200 rpm) centrifugation of the supernatant at 4 °C using a Sorvall RT 6000D centrifuge to collect suspended cells. Cell pellets were resuspended in non-selection medium and cells were incubated for 3 - 6 days to allow cell establishment and expansion, and to remove tumour debris. Using trypan blue exclusion as a measure of cell viability, 1×10^5 viable cells per 10 cm dish were plated in triplicate in 6-TG-selection for scoring *hprt*⁻ mutants. As well, 200 cells per 6 cm dish were plated in triplicate in non-selection medium for determination of plating efficiency. MN-11 cells contain a *neo* gene, which confers G418 resistance and allows distinction of tumour cells from host cells. Thus, the percentage of G418 resistant cells was determined by plating in triplicate 200 cells per 6 cm dish in non-selection medium supplemented with 500 μ g/ml G418 (G418 medium) (typically 80

- 90%). The number of 6-TG resistant mutants was corrected for plating efficiency and for G418 resistance. A sample calculation is shown in **Appendix II**.

3.2.4 Tumour Preparation for Histology

Different methods of fixation were tried for the 4 independent experiments. In experiment 1, tumour slices were fixed in an alcohol-based fixative, GenoFix, for a minimum of 24 hours. In experiment 2 tumour slices were fixed in 10% formalin for 20 - 24 hours. In both cases the slices were transferred to 70% ethanol. Paraffin embedding and sectioning were performed by Zaida Ticas and Louise Kobylinsky (Department of Pathology, University of Ottawa). Detection of neutrophils by staining for endogenous peroxidase using the diaminobenzidine (DAB) method (see below) did not work for paraffin-embedded sections. Subsequently, frozen sections were used. Tumour slices were first fixed in LANA's fixative (modified Zamboni's fixative) for 2 hours at 4 °C. The slices were then rinsed twice (for 5 minutes each) in chilled 100 mM phosphate buffer, pH, 7.2 (PB). Tumours were stored in 1:1 (v:v) mixture of 10% sucrose (containing 0.1% sodium azide) and 3% Triton-X 100 in PB, at 4 °C, for a minimum of 24 hours. Fixed tumours were embedded and frozen in Optimal Cutting Compound (OCT). Tumours were blotted and placed in a plastic tissue - freezing 'boat' filled with OCT until submerged. The boat was held so just touching nearly frozen isopentane (isopentane in a container was held in liquid nitrogen until it became a frozen 'shell' with

liquid centre). Care was taken that freezing of OCT was slow and uniform to ensure preservation of tissue. Once fully frozen, blocks were stored at -80 °C.

Six to eight hours before sectioning, blocks were placed at -20 °C to prevent shattering. Tumours were sectioned using a Microm HM 500 OM cryostat to a thickness of 5 µm, mounted on aminoethylcarbazole (AEC) coated slides (prepared by Z. Ticas) and stored at -80 °C for staining.

Tumour sections which had been embedded in paraffin (from experiments 1 and 2) were deparaffinized and stained with hematoxylin and eosin (H&E). Frozen tumour sections were air-dried at room temperature for 30 min, fixed in absolute acetone for 30 min, air-dried for a further 20 min, stained for endogenous peroxidase with DAB, and counterstained with hematoxylin. Detailed staining procedures are given in **Appendix III**.

3.2.5 Scoring of Neutrophils and Necrosis

The method used for scoring the number of neutrophils and percent necrosis was developed with the assistance of Dr. George Wenckebach (Pathologist, Ottawa General Hospital). Both counts (neutrophil and percent necrosis) were performed “blindly”, on coded tumour sections. A 2 mm grid was drawn directly onto the coverslip using a fine-tipped marker over the tumour section to be analyzed. The two top corners of each

square (or all 4 corners for small tumour sections) were analyzed for the number of neutrophils and percent necrotic area using a microscope ocular containing a 10 by 10 grid. The number of neutrophils within the grid was counted, and the neutrophil count for the tumour was the average number of neutrophils in all the 2 mm by 2 mm squares. Percent necrosis was determined by counting the number of times a necrotic zone passed through the crosses of the ocular's 10 by 10 grid (ignoring the first row and the first column, so that the total number of crosses was 100). The percent necrosis of a tumour was the average percent necrosis for all the 2 mm by 2 mm squares. All scoring was carried out under 400× magnification using an Olympus BH-2 microscope. At least 10 sections were scored per tumour.

3.2.6 Statistical Analysis

Data were analyzed using the Mann-Whitney U test for non-parametric data, and by linear regression analysis or Pearson r correlation analysis followed by ANOVA to determine significance (on log transformed data, as required). Calculations were carried out using GraphPad InStat version 2.0. ANCOVA was carried with the assistance of Dr. Hans Damman (Carleton University), using the statistical package SuperANOVA. A *p* value of ≤ 0.05 was considered to be significant.

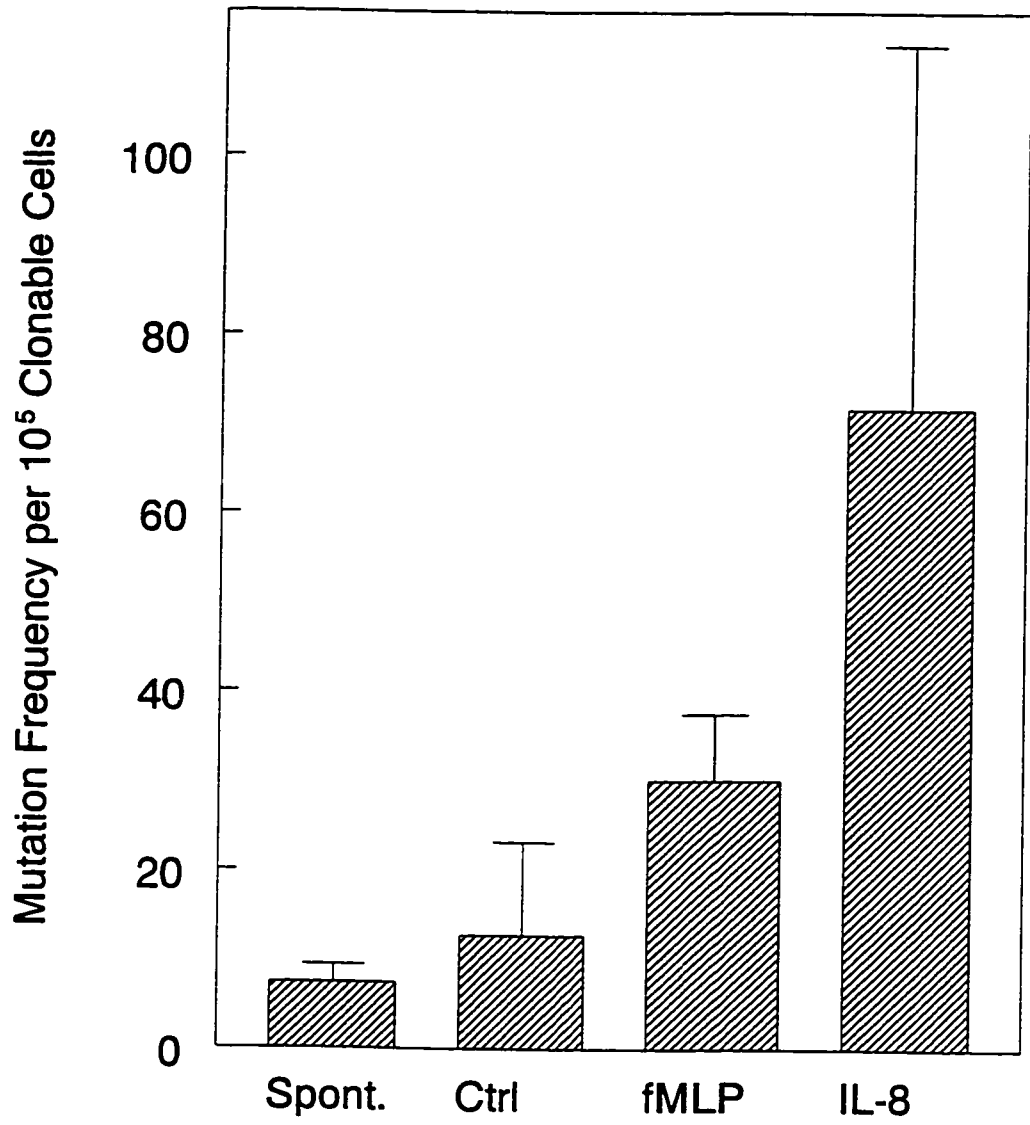
3.3 Results

A method of increasing the number of infiltrating neutrophils was devised to investigate the effects of tumour-infiltrating neutrophils as potential contributors to the previously observed 3.4 fold increase in mutation frequency in MN-11 cells grown as subcutaneous tumours compared to cells cultured for the equivalent period of time. As a pilot experiment to optimize conditions, the effects on mutation frequency of localized injections of fMLP and IL-8 into the tumours was investigated. fMLP is a frequently used chemotactic peptide, and IL-8 is a known chemoattractant for neutrophils. Prostaglandin E2 was coinjected with both IL-8 and fMLP as it has been shown to increase local neutrophil infiltration (Rampart *et al.*, 1989). Injection of these compounds into tumours was performed once tumours were palpable (day 11 following tumour inoculation). Four days later, tumours were removed and cultured for MF. Sections of tumours were fixed and stained, and qualitative measurements of amount of neutrophil infiltration were made. In general, control tumours had very little neutrophil infiltration. Tumours that had received either fMLP or IL-8 had visibly greater amounts of infiltration, while a few of the IL-8 injected tumours had a vast number of neutrophils. The effect of the various treatments on MF is depicted in Figure 3-1. Injection of fMLP + PGE2 caused a modest, but insignificant increase in MF. Injection of IL-8 + PGE2 caused a greater (6 fold) increase in MF that, too, was statistically insignificant due to the large variation in values and small sample size. However, the increase in MF plus the observed influx of neutrophils in tumours injected with IL-8 appeared promising, and so

Figure 3-1. Effect of intra-tumour injections of Interleukin-8 and fMLP (in conjunction with PGE2) on mutation frequency in MN-11 tumours.

On day 11 following tumour inoculation, mice received intra-tumour injections of IL-8 +PGE2, fMLP + PGE2, or nothing (controls), as described in *Materials and Methods*. Tumours were excised on day 15, and MF was determined. MN-11 cells were maintained in tissue culture for the duration of the experiment for the determination of spontaneous MF (spont.). Shown are mean \pm SEM for each group. All data are from a single experiment, with n = 7 in both IL-8 and fMLP injected groups.

Figure 3-1



all further experiments used IL-8 treatment as a means of increasing neutrophil infiltration.

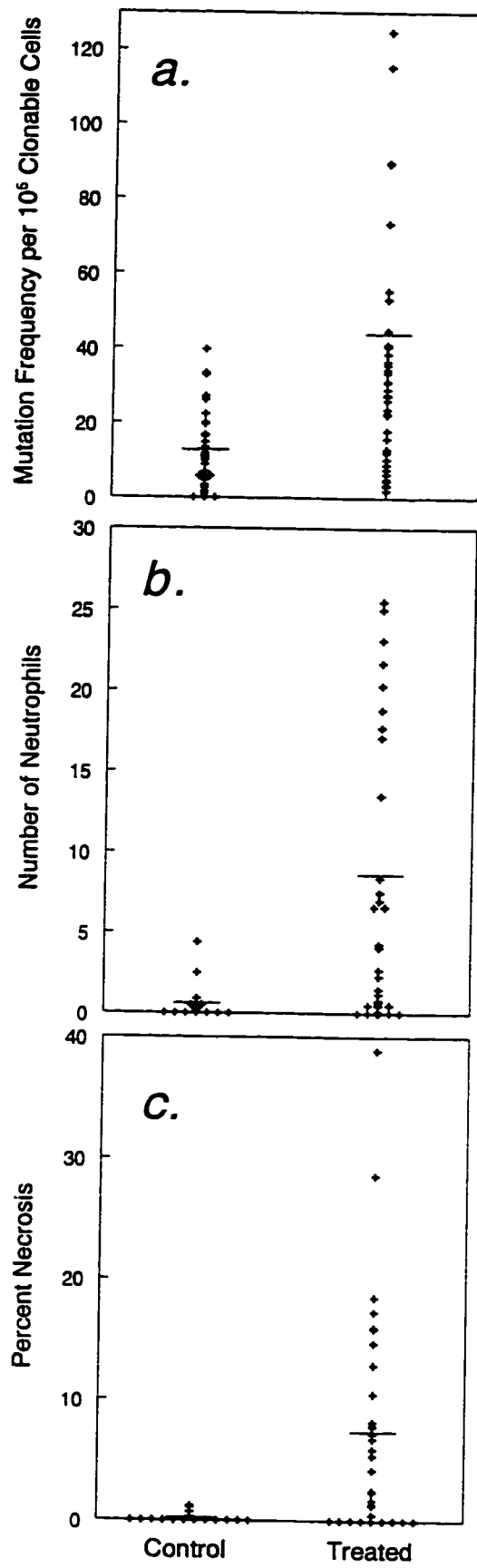
In all, four independent experiments were performed, with a total of 79 mice (31 controls and 48 treated). For most, at the time of tumour extraction, sections of the middle of the tumour were frozen or fixed for neutrophil counts. Only in instances when the tumour was very small, and all cells were needed for culture, were sections not taken. Likewise, for a few tumours, sections were taken and neutrophil counts were made, but corresponding MFs are not known. Although care was taken to recover tumour cells under sterile conditions, it was performed on an open bench, and occasionally bacterial contamination occurred. For the vast majority of tumours, both neutrophil counts and MF were performed. At the time the procedure for quantifying neutrophil infiltration was developed, it was also noted that the amount of tumour necrosis varied from tumour to tumour. Thus, a procedure for quantifying tumour necrosis was developed, and performed simultaneously with neutrophil counts.

To verify that the treatment used to increase neutrophil infiltration (i.e., IL-8 + PGE2 injections) worked, a comparison of the MF, neutrophil counts, and percent tumour necrosis between the two groups was made (Figure 3-2). Tumours from the 'Control' group received either injection of PBS or no injection (MFs are not statistically different: no injection 7.3 ± 2.9 mutations per 10^5 cells; PBS injections 9.8 ± 2.0 mutations per 10^5 cells). Tumours from the 'Treated' group received either injection of IL-8 alone or IL-8

Figure 3-2. Effect of IL-8 treatment on MN-11 tumour mutation frequency, number of infiltrating neutrophils, and percent tumour necrosis.

On day 11 following tumour inoculation, mice received intra-tumour injections of IL-8, or IL-8 + PGE2 (TREATED group), or injections of PBS or nothing (CONTROL group). Following tumour excision on day 15, MF was determined. A section of each tumour was fixed or frozen and stained for the determination of neutrophil counts and percent tumour necrosis. Shown are the means and individual data points for 4 independent experiments. **a.** Effect of treatment on MF, with $n = 31$ controls and $n = 42$ treated mice. The difference in MF between the groups is extremely significant ($p=0.0001$). **b.** Effect of treatment on number of infiltrating neutrophils, with $n = 17$ controls and $n = 32$ treated mice. The difference between the groups is extremely significant ($p=0.0005$). **c.** Effect of treatment on percent tumour necrosis, with $n = 17$ controls and $n = 32$ treated mice. The difference between the groups is extremely significant ($p=0.0003$). The horizontal bar in each graph represents the median value.

Figure 3-2



+ PGE2 (MFs are not statistically different: IL-8 alone 24.6 ± 6.2 mutations per 10^5 cells; IL-8 + PGE2 injections 25.5 ± 5.0 mutations per 10^5 cells). The difference in the MF between the control and treated groups is considered extremely statistically significant, with a p value of 0.0001 (Figure 3-2a). Likewise, the difference in the number of infiltrating neutrophils (Figure 3-2b) and the difference in the percent tumour necrosis (Figure 3-2c) between the control and treatment groups are both considered extremely statistically significant ($p=0.0005$, and $p=0.0003$, respectively).

Since it was clear that the IL-8 treatment was effectively attracting neutrophils to the tumour, the correlation between MF and number of neutrophils and percent tumour necrosis was examined. Figure 3-3 shows cross-sections of two typical tumours. Figure 3-3a is a tumour with no necrosis and little neutrophil infiltration. Figure 3-3b shows a tumour with massive neutrophil infiltration and necrosis. In Figure 3-4 the relationship between the number of infiltrating neutrophils and MF in individual tumours ($r = 0.4554$) is shown. Linear regression analysis following log transformation of the dependent variable shows that the relationship is significantly different from zero ($p=0.0034$). Similar results were obtained for percent tumour necrosis and MF (Figure 3-5). The correlation coefficient (r value) was 0.4928 and linear regression analysis following log transformation revealed a highly significant non-zero relationship ($p=0.0007$). Finally, the correlation between the number of neutrophils and percent necrosis was analyzed (Figure 3-6). A very strong correlation was identified following log transformation ($r = 0.8416$), which is extremely significantly different from zero ($p<0.0001$).

Figure 3-3. Typical cross sections of MN-11 tumours.

Tumours were fixed in LANA's fixative, frozen to -80 °C, sectioned, and stained with DAB and hematoxylin. *a.* Tumour section with little neutrophil infiltration or necrosis. *b.* Tumour section with massive neutrophil infiltration (N) and large areas of necrosis. Photography by J.K. Sandhu.

Figure 3-3

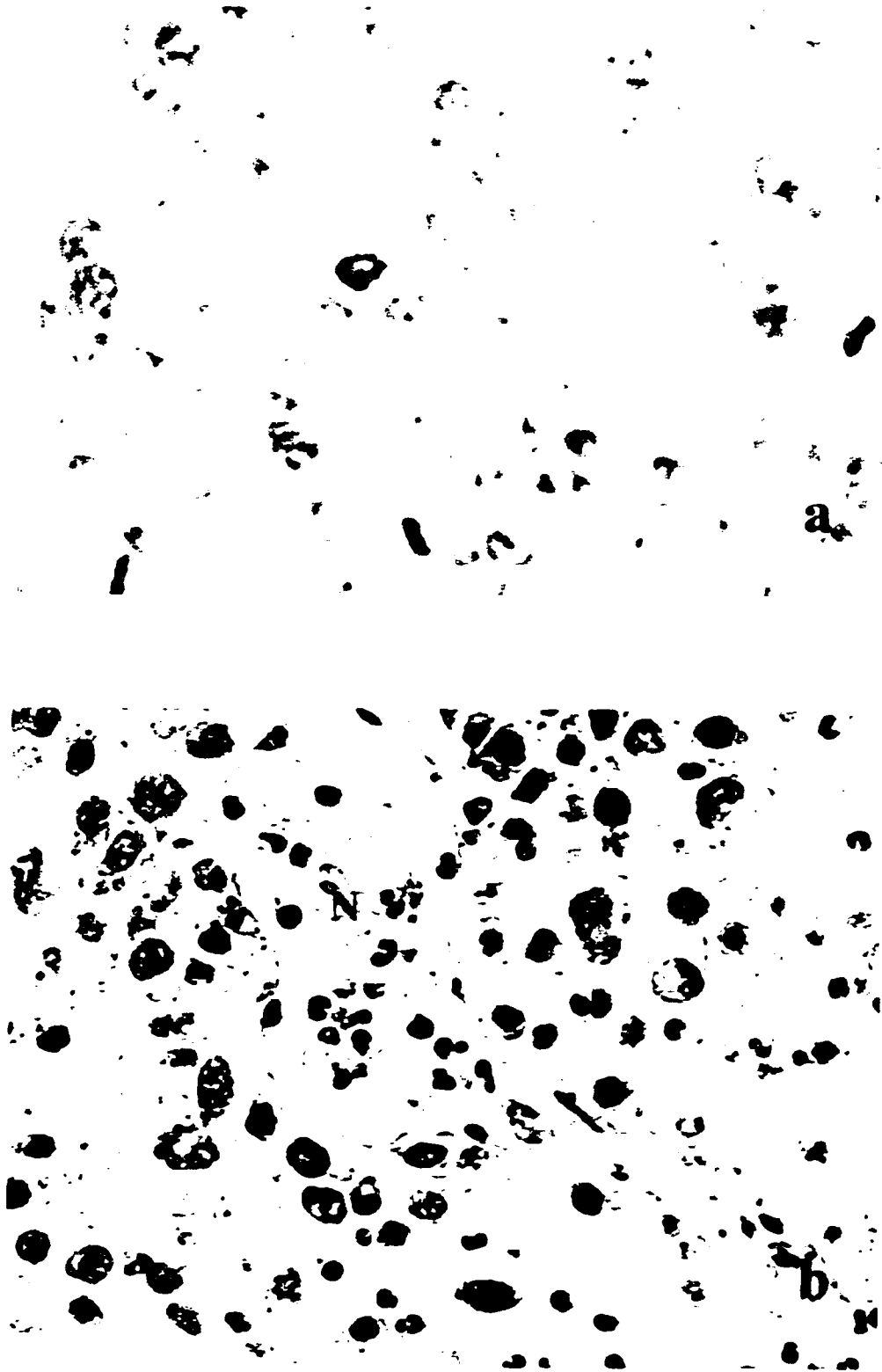


Figure 3-4. *The relationship between neutrophil infiltration and mutation frequency in the MN-11 tumour.*

Scatter plot depicts the corresponding MF at each neutrophil count, regardless of treatment, for n = 43 tumours (from 4 independent experiments). Data were analyzed using linear regression analysis, using log transformed dependent variable (log MF): $r = 0.4373$, which is very significant ($p=0.0034$).

Figure 3-4

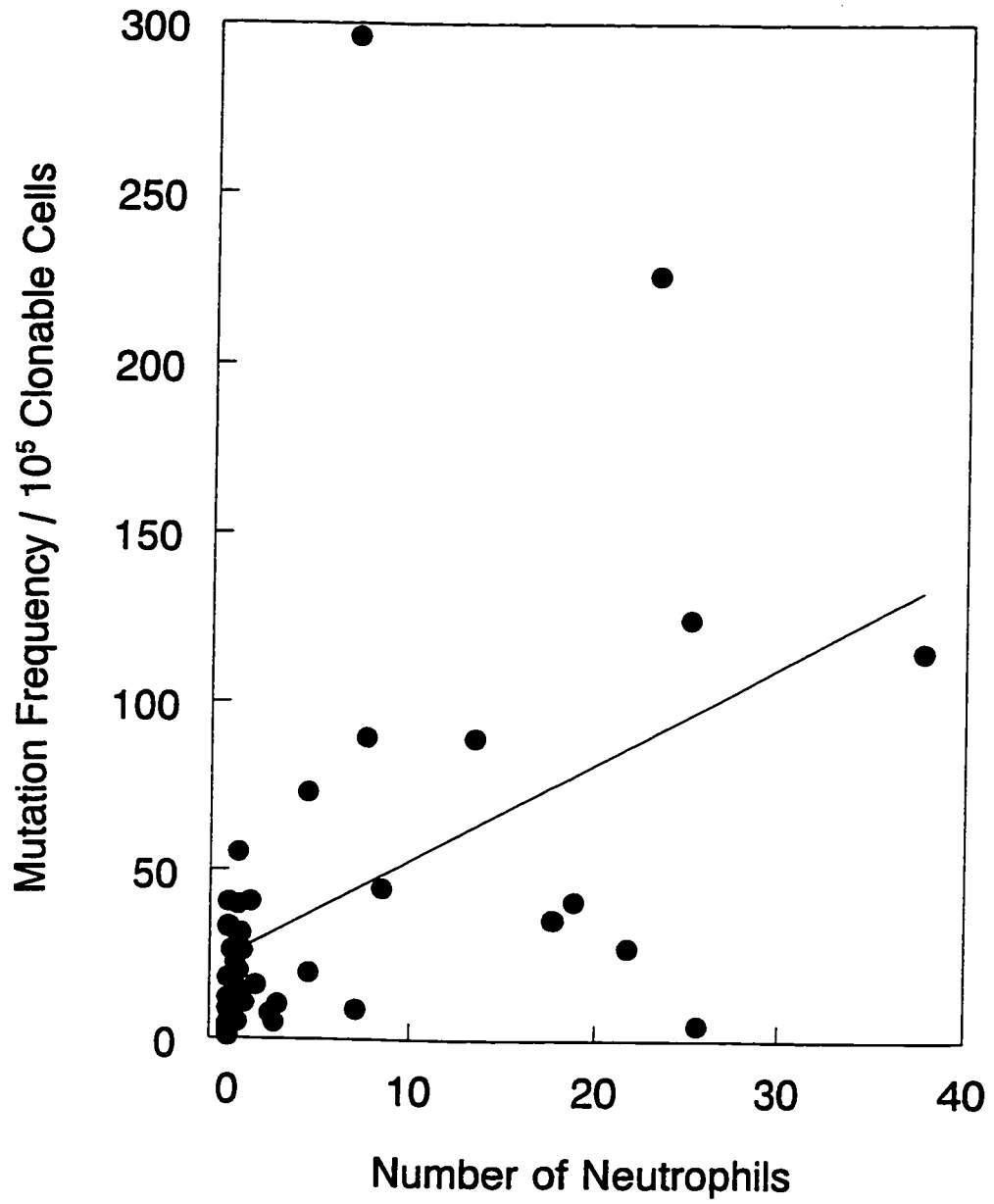


Figure 3-5. *The relationship between amount of tumour necrosis and mutation frequency in the MN-11 tumour.*

Scatter plot depicts the corresponding MF for each value of percent necrosis, regardless of treatment group, for n = 43 tumours (from 4 independent experiments). Data were analyzed using linear regression analysis, using log transformed dependent variable (log MF) $r = 0.4985$, which is extremely significant ($p=0.0007$).

Figure 3-5

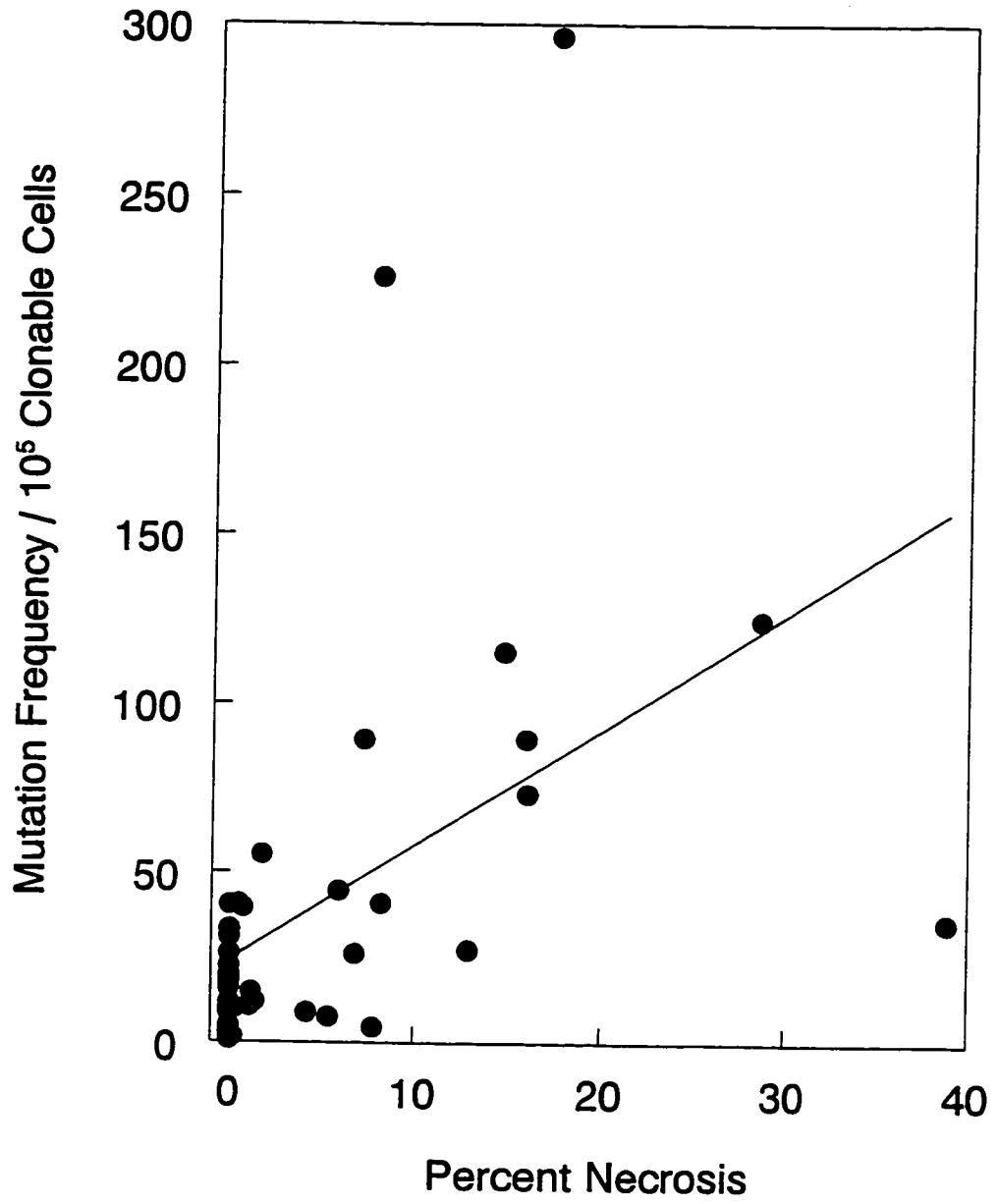
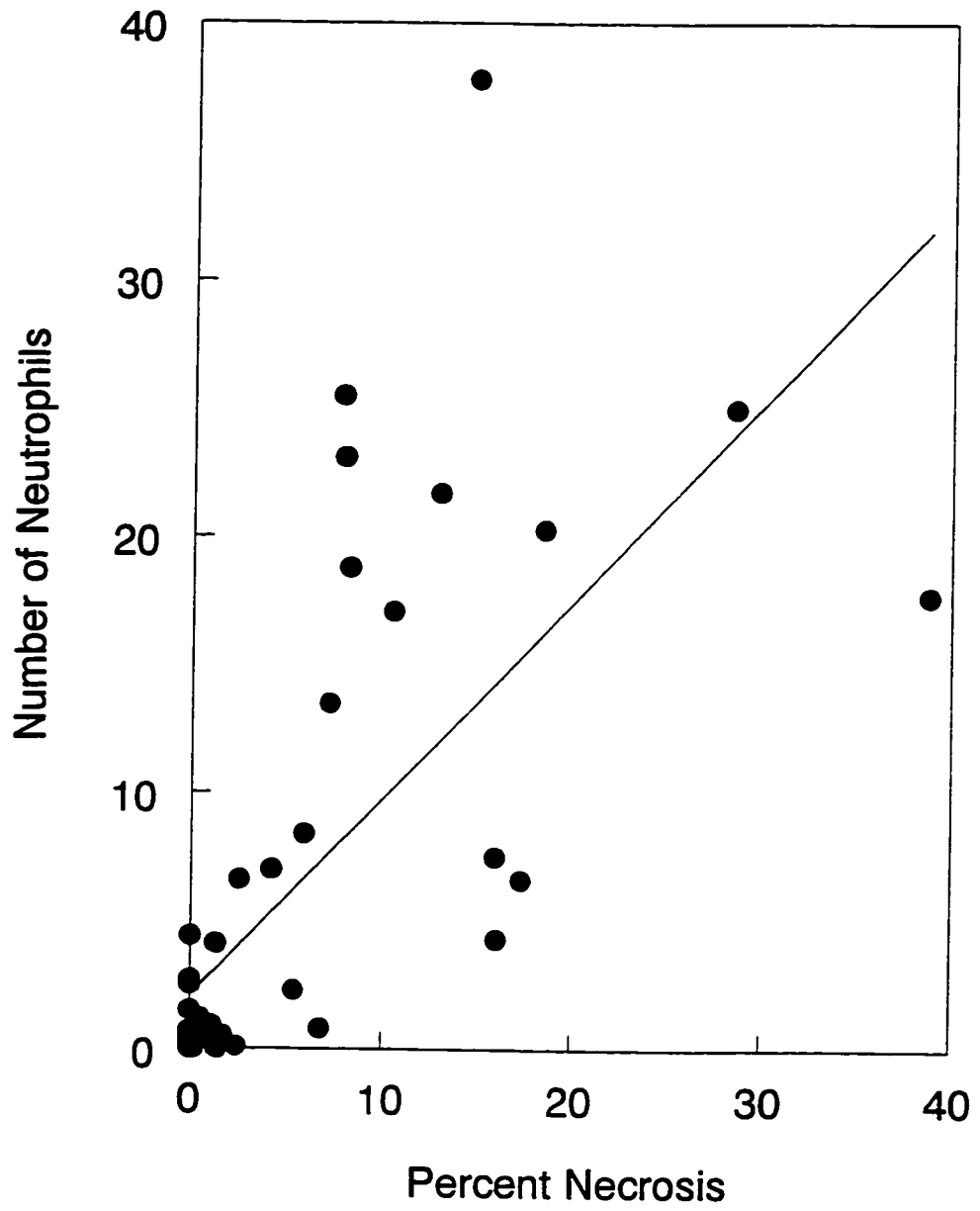


Figure 3-6. *The relationship between amount of tumour necrosis and number of infiltrating neutrophils in the MN-11 tumour.*

Scatter plot depicts the correlation between percent tumour necrosis and number of neutrophils, regardless of treatment group, for $n = 49$ tumours. Data were analyzed using correlation analysis on log transformed variables ie. $\log (\% \text{necrosis} + 1)$ and $\log (\# \text{neutrophils} + 1)$: $r = 0.8416$, which is extremely significant ($p < 0.0001$).

Figure 3-6



Analysis of Covariance (ANCOVA) was used to produce a model of the relative predictive powers of the two variables (number of neutrophils and percent necrosis) for MF. Type I sum of squares was used in this analysis. This indicates the amount of remaining variation explained by a variable entering into the model after the variation explained by the preceding variables has been accounted for. If the number of neutrophils is entered into the model first, it explains a significant amount of variation ($p=0.0034$). If percent necrosis is then added into the model, it still explains a significant amount of variation after the number of neutrophils have been accounted for ($p=0.043$). However, if the variables are entered into the model in the opposite order, a different effect is seen. Entering percent necrosis into the model first explains a significant amount of variation ($p=0.0007$). Adding the number of neutrophils into the model next does not explain significantly more of the variation than does percent necrosis alone ($p=0.304$). This suggests that, in the interest of developing the simplest model, percent tumour necrosis is a better predictor of MF than is the number of neutrophils.

3.4 Discussion

Our laboratory has been interested in studying factors in the tumour environment that may be genotoxic and thus lead to tumour progression. For a number of years, the link between chronic inflammatory conditions and cancer has been recognized. This has lead to the hypothesis that inflammatory cells, such as activated macrophages and neutrophils, attracted to and infiltrating the site of infection or injury may release factors that are genotoxic, and thus lead to the development of cancer.

In the past, it had been observed that MN-11 tumours become infiltrated with inflammatory cells, in particular neutrophils. Since MN-11 cells grown as tumours had a 3.4 fold increase in MF over cells grown in culture for the equivalent period of time, it was hypothesized that infiltrating neutrophils may be, at least in part, responsible for this increase in MF. To test this, experiments were carried out to attract large numbers of neutrophils to the tumour, score the resulting MF, and determine the relationship between neutrophil number and MF.

A preliminary experiment was first devised to test the ability of intra-tumour injections of two compounds, fMLP and IL-8, to attract large numbers of neutrophils to the tumours. fMLP, a tri-peptide containing the bacterial formylated methionine, has been used extensively as a chemotactic agonist for neutrophil infiltration, as have complement factor C5a, platelet-activating factor (PAF), and leukotriene B₄ (LTB₄)

(Baggiolini and Clark-Lewis, 1992; Rampart *et al.*, 1989). IL-8 is naturally released by phagocytes and a wide variety of tissue cell types in response to inflammatory stimuli (Baggiolini and Clark-Lewis, 1992; Harada *et al.*, 1994). The sequence, 3D structure, and profile of biological activity are known for human IL-8. Human IL-8 has been shown to act on mouse, guinea pig, rat, and rabbit neutrophils (Oppenheim *et al.*, 1991) and therefore human IL-8, which is commercially available, was used for all mouse experiments. For both fMLP and IL-8 treatments, PGE2 was co-injected. Several studies have shown that PGE2 acts as a vasodilator and, when combined with a chemoattractant (either fMLP or IL-8), causes a synergistic increase in neutrophil infiltration (Foster *et al.*, 1989; Rampart *et al.*, 1989). PGE2 on its own does not induce neutrophil migration (Rampart *et al.*, 1989). My preliminary experiment suggested that IL-8 was a better chemoattractant for neutrophils than fMLP in the MN-11 system, as seen from the greater MF in IL-8 injected tumours, and from qualitative inspection of accompanying tumour sections showing great numbers of neutrophils in several IL-8 injected tumours. This is in agreement with Colditz *et al.* (1989), who found that neutrophil-activating factor (IL-8) was 3 times more potent than fMLP at inducing plasma leakage and neutrophil accumulation following intradermal injection in rabbits.

My results demonstrate that localized injections of IL-8 cause a significant increase in neutrophil infiltration over control (saline or uninjected) tumours. Accompanying this, IL-8 injected tumours are also significantly more necrotic than control tumours. The MF between the two groups is also significantly different, with IL-8 injected tumours having a much higher MF than controls. These results indicate that

the experimental protocol was effective at increasing neutrophil infiltration into the tumours. As controls, most mice received injections of endotoxin-free saline, while some received no injections at all. There was no difference in the MF between these two groups. However, it cannot be ruled out that in the injection of some mice, bacteria from the animal's fur may have been co-injected with the solution (although fur was first swabbed with an alcohol pad), and thus may have been contributing to the attraction of neutrophils independently of the IL-8.

Linear regression analysis demonstrated a highly significant non-zero relationship between the number of neutrophils present in a tumour, and the MF of the MN-11 cells comprising the tumour. To my knowledge, no demonstration of such a direct relationship *in vivo* has ever been previously demonstrated. The genotoxic capabilities of neutrophils have been shown *in vitro*. For example, stimulated neutrophils were shown to cause cytogenetic damage in CHO cells (Weitberg *et al.*, 1983), and DNA strand breaks in murine erythroleukemia cells (Birmboim, 1983). My findings provide further support for the hypothesis that neutrophils have the potential for contributing to host tissue damage through the generation of mutations, and thus contributing to the carcinogenic process.

During the course of the experiments, it was noted that varying levels of necrosis were evident in the different tumours. Upon formal quantification of percent tumour necrosis, highly significant relationships between the number of neutrophils and percent necrosis, and percent necrosis and MF, emerged. ANCOVA was used to develop the simplest model to predict MF, given the two variables: percent necrosis and number of

neutrophils. The analysis suggested that necrosis on its own is capable of accounting for all the variation explained by number of neutrophils, indicating that percent necrosis is a better predictor of MF than either the number of neutrophils alone, or the number of neutrophils and percent necrosis together. These results do not, however, indicate which variable is the causative factor. Necrosis commonly occurs in both human and experimental solid tumours, and is probably the result of a lack of nutrients and oxygen due to impaired tumour vasculature (Tannock, 1992). Necrosis has been shown to be involved in tumour regression (Colombo *et al.*, 1996); however, hypoxia (associated with necrosis) is also associated with resistance to radiation therapy and disease progression (Maxwell *et al.*, 1997).

Although a statistically significant relationship between the number of neutrophils and MF, and percent necrosis and MF, was found, there was much variation in the results from individual animals. Inherent differences due to injections may explain this variation. MN-11 tumours are a heterogeneous mixture of cell-types and the microenvironment of the injection site could not be controlled. For example, it is possible that some injections may have been into blood vessels, with the result being the rapid removal of IL-8 from the tumour. Further work is currently underway to create more controlled delivery of IL-8, and therefore better regulation of neutrophil infiltration.

4. OVERALL DISCUSSION AND CONCLUSION

The link between chronic inflammation and cancer has been recognized for a long time. It has been proposed that inflammatory cells themselves and their products may be contributing to the carcinogenic process. Our laboratory has developed an *in vivo/in vitro* tumour model system, MN-11, which allows for the identification and study of factors in the tumour environment that may contribute to tumour development and progression. The previously observed 3.4 fold increase in mutation frequency in cells grown as tumours over cells cultured for the equivalent period of time implicates the importance of these factors, and experiments with various NO donors suggests that this molecule, released from infiltrating inflammatory cells, may, be at least in part, responsible.

In *Section 2* of this thesis, I have described experiments aimed at identifying the nature of the mutagenic NO-related species released by the metabolism of NO donors GTN and SNP by MN-11 cells. It was hypothesized that glutathione would have protective effects against NO donor- (GTN and SNP) induced mutations. Instead a paradoxical role for glutathione became evident. Glutathione appears to play a dual role of both providing protection against, and being required for, GTN- and SNP-induced mutations, perhaps through involvement in the formation of a mutagenic intermediate species. It has been suggested that glutathione has a similar role in the activation of murine bone marrow-derived macrophages for nitrite secretion and leishmanicidal activity. In these cells, glutathione is involved both in the synthetic processes that generate NO and toxic oxygen metabolites, and in the protection of macrophages from

the harmful effects of these toxic molecules (Buchmuller-Rouiller *et al.*, 1995). Our *in vivo* experiments showed a similar involvement of glutathione in GTN-induced mutations; however due to the increased complexity of the system these experiments were beyond the scope of this thesis, and therefore not pursued. Yet, the involvement of glutathione in the generation of mutagenic NO related species should be kept in mind during treatment of patients with vasodilators GTN or SNP.

Section 2 also describes the results of treatment of MN-11 cells with several other NO donors, in an attempt to identify the nature of the mutagenic NO-related species. Previous work suggested that continuous, slow-release of NO species over a 24 hour period (i.e. by GTN and SNP) resulted in a higher mutation frequency than donors that release NO in a rapid, “bolus” fashion. None of the NO donors tested (SIN-1, GSNO, and DETA/NO) increased mutation frequency. Even DETA/NO, an NO donor with a reported long half-life of around 24 hours that releases NO spontaneously, was not mutagenic. However, analysis indicated that in our system NO release had occurred within the first few hours of DETA/NO treatment. Thus, treatment of MN-11 cells with SIN-1, GSNO, and DETA/NO did not reveal anything about the nature of the mutagenic NO species. Therefore, it is still uncertain whether NO or an intermediate species in the metabolism of SNP and GTN is the mutagenic species in the MN-11 system. Further work will be required to identify the species responsible for the mutagenicity of these NO donors. These experiments reaffirm the importance of careful selection of an appropriate NO donor for each specific application.

NO, when produced biologically, has a very short half-life, and rapidly reacts with other molecules, including glutathione, forming S-nitrosoglutathione, or oxygen metabolites, forming a series of toxic and potentially damaging species. It has been suggested that S-nitrosoglutathione is the transport form of NO within cells, allowing for its far-reaching effects. Several cell-types contain nitric oxide synthases and generate NO endogenously. Immune system cells, macrophages and neutrophils, generate large amounts of NO as part of their armament against invading microbes. In *Section 3*, I describe experiments that implicate neutrophils, the most abundant inflammatory cell infiltrating MN-11 tumours, in generating an elevated mutation frequency, perhaps, at least in part, due to their production of NO and related species. Intra-tumour injection of IL-8 caused significant infiltration of neutrophils to the injection site, as had been previously shown by others. The highly statistically significant relationship between neutrophil numbers and mutation frequency is a novel indication that neutrophils may be mutagenic in a tumour, and thus contribute to tumour progression. Work by others thus far has been contradictory. While tumour-associated leukocytes are seen within most human and experimental tumours, their effects on tumour progression and on prognosis are still unclear (Colombo *et al.*, 1996). Neutrophils and macrophages have been shown to deeply infiltrate tumours and kill tumour cells in response to various cytokine-induced reactions (Colombo *et al.*, 1991; Colombo *et al.*, 1992). Yet, it has been shown by others that neutrophils can favour the growth of certain tumours, and that their elimination results in inhibition of tumour growth (Pekarek *et al.*, 1995). The ultimate effect on tumour growth by infiltrating neutrophils may depend on a balance between inhibitory and stimulatory signals produced both by tumour cells and the neutrophils. My work

indicates that the presence of neutrophils may be mutagenic. Even if they act to kill tumour cells, some tumour cells may escape killing and may have an increased malignant potential.

A high correlation between neutrophil numbers and tumour necrosis was found. Widespread necrosis is seen in human solid tumours (Michal *et al.*, 1996) and may result from hypoxia due to loss of tumour vasculature (Colombo *et al.*, 1996). Hypoxia has been associated with resistance to radiation therapy, metastasis, and tumour progression (see Maxwell *et al.*, 1997). Recently Colombo *et al.* (1996), using a mouse tumour model, showed that neutrophils attracted to tumours release IL-1 α , IL-1 β , and TNF- α , which, through a cascade of events, attract IFN- γ producing T-cells. This results in damage to the vascular endothelium, restriction of vasculature function, hypoxia, widespread necrosis, and tumour regression (Colombo *et al.*, 1996). It is possible that in my experiments, necrosis (through the release of toxic compounds, and thus injury of neighbouring cells) may have been the mutagenic source. Tumour regression was not noted; however, the 4 days between IL-8 injection and mouse sacrifice may have been insufficient time for it to take place. ANCOVA analysis implicated the importance of necrosis to mutation frequency, although whether it, or NO, is the primary causative source is still unknown.

In this thesis I provide evidence that neutrophils, potentially through their release of NO, are mutagenic in a tumour, thus supporting the laboratory's working model.

Further experimentation is being carried out to substantiate these findings, and to identify the mutagenic species released by infiltrating neutrophils, or in necrotic tumours. Currently, work is underway to create an MN-11 cell-line into which has been cloned a regulatable IL-8 gene. This would allow the regulation of IL-8 secretion, and give better control of neutrophil infiltration, thus overcoming the variability inherent to injections. In future experiments, a NO synthase “knock-out” mouse could be created, which could be used, in combination with IL-8 secreting MN-11 cells, to demonstrate unequivocally the relationship between NO released by infiltrating neutrophils and tumour mutation frequency. It is hypothesized that mutation frequency would be lower in tumours infiltrated with neutrophils lacking a NO synthase (in the NO synthase knock-out mouse) than in tumours infiltrated with neutrophils containing a fully functional NO synthase. It is hoped that results contained in this thesis, and work that follows from it, will aid in understanding of the mechanism of genotoxicity believed to underlie the link between inflammation and cancer.

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APPENDIX I: LIST OF MATERIALS, SUPPLIERS, AND SOLUTION COMPOSITIONS

absolute acetone	prepared by adding 5 ml sodium sulphate per litre acetone, and allowing to settle; stored at 4°C
acid alcohol	0.2% HCl in 70% ethanol.
aminopterin stock	Sigma Chemical Co.; 4×10^{-5} M solution prepared by dissolving 1.76 mg in 100 ml ddH ₂ O, adding 2-3 drops NaCl, filter sterilizing, and storing at -20°C.
BSO	ICN Biomedicals Inc.; prepared fresh as 1 mM stock in PBS, added to non-selection medium to a final concentration of 50µM.
Buffer A	0.1 M potassium phosphate / 1 mM CDTA, pH 8: made by preparing 0.5 M K ₂ PO ₄ , adjusting to pH 8 with 0.5 M KH ₂ PO ₄ , diluting to 0.1 M, and adding 0.5 M CDTA to 1 mM.
C57BL/6 mice	Charles River Laboratories or Tachonic.
catalase (bovine liver)	Sigma Chemical Co.; freshly prepared in non-selection medium, filter sterilized, added to final concentration of 50 µg/ml.
CDTA	Sigma Chemical Co.
DAB	Sigma Chemical Co.; stock concentration of 2.4% DAB prepared in TBS, aliquoted and frozen; final working concentration was 0.02% DAB.
DETA/NO	Toronto Research Chemicals; freshly prepared 0.1 M stock in 0.01 M NaOH, filter sterilized.
DMEM	GIBCO BRL
eosin	Fisher Scientific
fetal calf serum	Immunocorp
fMLP	stock solution prepared in 20 mM in DMSO, diluted to working concentration in sterile, endotoxin-free saline.
formalin	BDH
G418 (Geneticin)	GIBCO BRL; stock concentration prepared in PBS, added at a final concentration of 50 mg/ml to medium.
Genofix	fixative developed by Dr. H. C. Birmboim.
glutathione (reduced)	Sigma Chemical Co.
Griess reagent	prepared by mixing equal volumes of 0.1% <i>N</i> -(1-naphthyl)-ethylenediamine dichloride in water and 1% sulphanilamide in 5% H ₃ PO ₄ immediately before use.
GSNO	Sigma Chemical Co.; freshly prepared 0.05 M stock in non-selection medium, filter sterilized.
GTN	David Bull Laboratories; supplied as 22 mM sterile solution in saline, added directly to medium to final concentration.

HAT medium	non-selection medium supplemented with HT stock to make 1×10^{-4} M hypoxanthine and 1.5×10^{-5} M aminopterin stock to make it 4×10^{-7} M in final solution.
hematoxylin	Fisher Scientific.
HT medium	non-selection medium supplemented with HT stock to make 1×10^{-4} M hypoxanthine and 1.5×10^{-5} M thymidine final concentration.
HT stock	1×10^{-2} M hypoxanthine and 1.5×10^{-3} M thymidine, prepared by dissolving 0.1361 g hypoxanthine in 40 ml ddH ₂ O and combining with 0.036 g thymidine dissolved in 40 ml ddH ₂ O, adding 2-3 drops NaCl, filter sterilizing, and storing at -20°C.
hypoxanthine	Sigma Chemical Co.
IL-8	Endogen; prepared 10 μ M stock in sterile endogen-free saline, frozen at -100°C in 10 μ L aliquots, diluted to working concentration in same sterile saline.
LANA's fixative	modified Zamboni's fixative: 4% paraformaldehyde, 0.4% picric acid in 160 mM phosphate buffer, pH 6.9.
N-acetyl-cysteine	ICN; freshly prepared in non-selection medium, filter sterilized, added to final concentration of 10 mM.
non-selection medium	DMEM + 10% FCS
OPT	Sigma Chemical Co.
OPT reagent	1 mg/ml OPT in methanol
OTZ	Sigma Chemical Co.; freshly prepared in non-selection medium, filter sterilized, added to a final concentration of 10 mM.
PBS	2.7 mM KCl, 1.5 mM KH ₂ PO ₄ , 140 mM NaCl, 8 mM Na ₂ HPO ₄ , pH 7.4.
PCA reagent	2 N perchloric acid plus 4 mM oxalic acid.
PGE2	Sigma Chemical Co.; prepared 10 mM stock in ethanol, stored at -20°C, diluted to working concentration in endogen-free sterile saline.
SIN-1	Sigma Chemical Co.; freshly prepared 10 mM stock in non-selection medium, filter sterilized.
SNP	Aldrich Chemical Company; stock in non-selection medium prepared fresh in the dark, filter sterilized.
SOD (bovine liver)	DDI Pharmaceuticals; freshly prepared in non-selection medium, filter sterilized, added to final concentration of 50 μ g/ml.
TBS (working)	50 mM TBS; 1:10 dilution of stock TBS in dH ₂ O.
TBS (stock)	0.5 M stock made by combining 242 g tris, 340 g NaCl, and 2 L dH ₂ O, and adjusting pH to 7.6 with 12 N HCl.
6-TG	Sigma Chemical Co.; 10mM stock prepared by dissolving 0.0835 g in 50 ml ddH ₂ O with 2-3 drops NaCl, filter sterilizing through 0.2 μ m filter, and storing at -20°C.

6-TG-selection	non-selection medium plus 5×10^{-5} M 6-TG.
thymidine	Sigma Chemical Co.
trypan blue stain	4 parts 0.2% trypan blue mixed with 1 part 4.25% NaCl prior to use; mixed with cells in a 1:1 (v/v) ratio.
10× trypsin-EDTA	GIBCO BRL; 0.5% trypsin, 5.3 mM EDTA · 4 Na
trypsin-EDTA	10× trypsin-EDTA + PBS (1:10)
(working)	
Wright's stain	BDH; 1 g Wright's stain powder per 600 ml methanol.

APPENDIX II: SAMPLE CALCULATIONS OF MUTATION FREQUENCY CORRECTED FOR PLATING EFFICIENCY AND G418 RESISTANCE

Calculation shown for IL-8 + PGE2 injected tumour number I+P3 (extracted 97/07/10):

- 6-TG resistant colonies (/10⁵): 5, 8, 9 mean = 7.3
- plating efficiency colonies (/200): 43, 45, 59 mean = 49 = 25%
- G418 resistant colonies (/200): 42, 42, 43 mean = 42 = 21%

$$\% \text{ G418 resistant} = 42 / 49 = 86\%$$

$$\text{MF corrected for \% G418 resistance} = (7.3 \times 100\%) / 86\% = 8.5$$

$$\text{MF corrected for plating efficiency} = (8.5 \times 100\%) / 25\% = \underline{34}$$

Therefore, MF corrected for plating efficiency and G418 resistance is 34 mutants per 10⁵ clonable cells.

APPENDIX III: STAINING PROCEDURES

A] Paraffin-Embedded Sections

1) *Deparaffinization*

5 min toluene (× 2)
↓
10 dips 100% alcohol (× 3)
↓
2 min running H₂O
↓
dH₂O

2) *Hematoxylin Staining*

5 min hematoxylin
↓
running H₂O
↓
2 min acid alcohol
↓
running H₂O
↓
1 dip LiCO₃

3) *Eosin Staining*

running H₂O
↓
30 sec eosin
↓
20 dips alcohol (× 4)
↓
20 dips toluene (× 4)
↓
coverslip with Permount

B| Frozen Sections

1) Fixation (post-cutting)

30 min air dry slides at RT
↓
30 min in absolute acetone
↓
30 min air dry slide
↓
Equilibrate in TBS

2) Endogenous Peroxidase Staining

stain 10 min in dark, while stirring, in:

188 ml 50 mM TBS
40 μ l H₂O₂
0.8 ml stock DAB

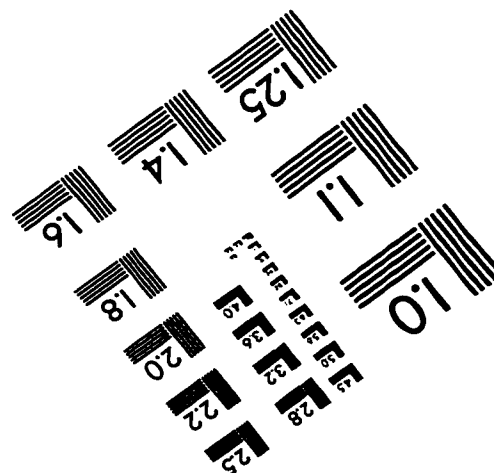
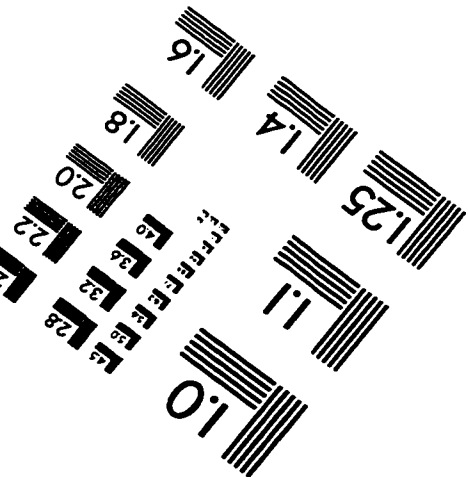
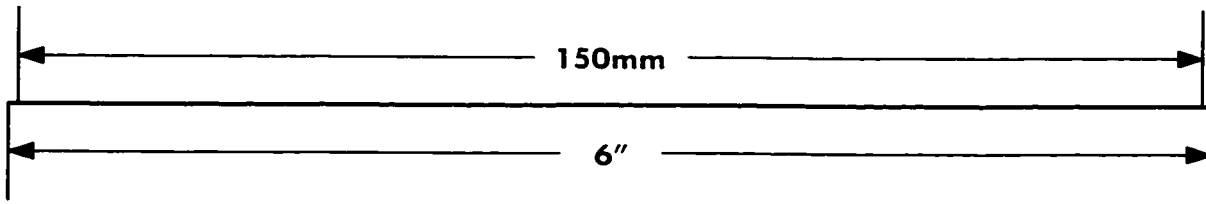
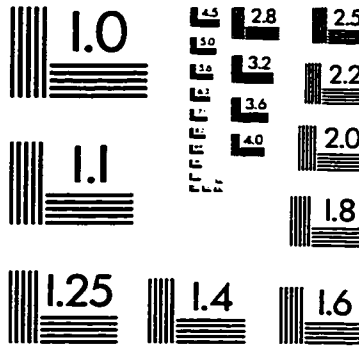
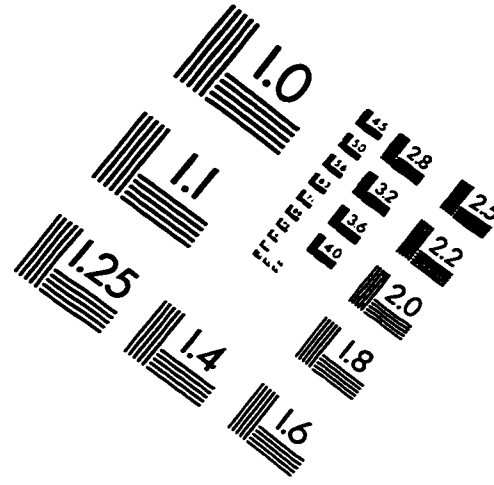
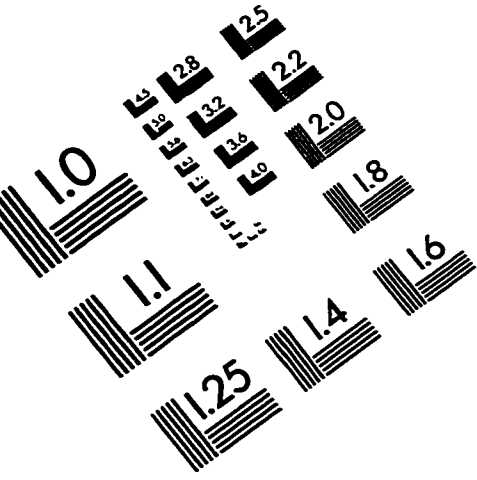
rinse in running water 5 min

3) Hematoxylin Staining

procedure as for paraffin-embedded sections, followed by:

10 dips alcohol (\times 3)
↓
20 dips toluene (\times 3)
↓
coverslip with Permount

IMAGE EVALUATION TEST TARGET (QA-3)



APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

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