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Mutations in the hypoxanthine phosphoribosyltransferase (*hprt*) gene in T lymphocytes from arthritis patients and in human B lymphoid cell lines exposed to nitric oxide-donating drugs

A thesis submitted to the School of Graduate Studies
University of Ottawa

In partial fulfilment of the requirement for the degree of
Master's of Science
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine

Abstract

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial joints. Infiltrating and resident cells participate in a sustained inflammatory response within the joint and release multiple mediators of inflammation, among them species with mitogenic and mutagenic potential. The frequency with which somatic mutations occur in cells within the inflamed joint is elevated. For example, the mutation frequency (MF) of a gene used as a marker of genetic damage, the hypoxanthine phosphoribosyltransferase (*hprt*) gene, was shown to be elevated in peripheral T cells from RA patients and was increased further in T cells from the synovial tissue of these patients. In addition, synoviocytes from RA patients have been reported to have a high frequency of p53 mutations. Therefore, we postulate that a mutagenic environment exists within the inflamed joint.

There is evidence that nitric oxide (NO), a reactive free radical produced in large quantities by resident synoviocytes, chondrocytes and infiltrating macrophages, is elevated in the synovial fluid and serum of RA patients. NO and its related nitrogen species have been shown to induce DNA damage in the form of small mutational events such as deaminations, leading to base-pair changes. However, observations within our lab suggest that NO may be capable of causing mutations of significant size, including large-scale deletion (LSD) mutations. We define LSD mutations as events that cause the loss of several hundred to several thousand DNA base-pairs. These mutations are therefore

detectable by PCR. Since NO has been shown to cause DNA damage and since genetic changes also occur in the inflamed joints, we hypothesize that NO may participate in the genotoxic environment of RA joints.

To determine if LSD mutations contribute to the increase in T cell *hprt* MF observed earlier, *hprt* mutant (*hprt*$^-$) T cells were isolated from RA patients and screened by multiplex-PCR (MP-PCR) for deletion of *hprt* exons. Fifty-four *hprt*$^-$ T cell clones were isolated from 16 RA patients and 5 (9.3%) clones had substantial deletions. Although the ends of the LSD mutations were not mapped precisely, these mutations included 2 clones missing *hprt* exons 2, 3 and 7/8, 1 clone missing *hprt* exons 2 and 3, 1 clone lacking *hprt* exon 2 and another missing *hprt* exon 3. From 6 patients with osteoarthritis (OA), a non-autoimmune joint disease of a less inflammatory nature than RA, 19 *hprt*$^-$ T cell clones were isolated and screened by MP-PCR. One clone lacked *hprt* exon 2; no mutations were detectable by MP-PCR in the remaining 18 clones.

To determine if NO is capable of inducing LSD mutations such as those found in RA and OA T cells, two human B lymphoblastoid cell lines (TK6 and WIL2-NS) were exposed to NO-donating drugs and the resulting *hprt*$^-$ clones were examined for *hprt* LSD mutations by MP-PCR. Both cell lines were shown to weakly metabolize two NO-donating drugs, nitroglycerin (NG) and sodium nitroprusside (SNP). Although the *hprt* MF of TK6 cells was elevated in cultures treated with NG or SNP, results were inconsistent, likely due to the tendency of this cell line to readily undergo apoptosis. Furthermore, in spontaneously arising

hprt⁻ TK6 clones, a high background rate of large-scale *hprt* deletions (80%) was observed by MP-PCR. Therefore, experiments were repeated using WIL2-NS cells. This cell line was derived from the same patient as TK6 cells, but expresses mutant p53 protein and is less prone to apoptosis. *Hprt* mutations could be induced in WIL2-NS cells by treatment with NG and SNP, an effect that could be reduced by exhausting these drugs of NO prior to treatment. Few spontaneously arising *hprt*⁻ WIL2-NS clones (11.8%) had LSD mutations. In *hprt*⁻ WIL2-NS clones isolated after treatment with NG or SNP, a significant increase in both the number and size of *hprt* deletion mutations was observed. These deletions ranged from 1.6 Kb, found also in spontaneous *hprt*⁻ WIL2-NS clones, to larger than 1.2 Mb.

In summary, LSD mutations were observed in RA peripheral T cells and, to a lesser extent, in OA peripheral T cells. The frequency of these mutations, however, was similar to the frequency of large *hprt* mutations reported to occur in T cells from normal individuals. Although LSD mutations did not predominate in *hprt*⁻ T cell clones from this small number of patients, *in vitro* evidence suggests that NO is capable of inducing such mutations. Deletion mutations of > 826 Kb and > 1.2 Mb were present in WIL2-NS cells treated with NG or SNP, respectively, indicating that NO is capable of causing genetic damage that far exceeds previously reported NO-induced mutations. Since DNA damage may contribute to RA pathology, this study provides additional evidence that inhibition of NO may have therapeutic benefits in this disease. This study also provides

additional information about the genotoxic potential of NO which may help explain the link that exists between inflammation, genetic damage and increased cancer risk.

Acknowledgments

I wish to thank my supervisors, Dr. H.C. Birnboim, Dr. R. Goldstein and Dr. J. Karsh, for allowing me the opportunity of working on this project. Their time and help are much appreciated. I would also like to thank the other members of my thesis advisory committee, Dr. E. Tackaberry and Dr. W. Ross, for their input.

Many thanks to Nancy Biggs for growing the T cell clones necessary for this project. I wish also to thank all the members of the Birnboim laboratory for their technical assistance and encouragement.

Dedication

I would like to dedicate this thesis to my parents, who taught me by example that the joy of learning never fades.

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List of abbreviations

bp	DNA base-pairs
cMF	mutation frequency, corrected for plating efficiency
cNOS	constitutive nitric oxide synthase
FCS	fetal calf serum
hprt	hypoxanthine phosphoribosyltransferase
iNOS	inducible nitric oxide synthase
LSD	large-scale deletion
MF	mutation frequency
MNC	mononuclear cell
MP-PCR	multiplex-polymerase chain reaction
NG	nitroglycerin
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase
OA	osteoarthritis
OH^-	hydroxyl radical
PBMC	peripheral blood mononuclear cell
PE	plating efficiency
PHA	phytohemagglutinin
RA	rheumatoid arthritis

SF	synovial fluid
SNP	sodium nitroprusside
TCR	T cell receptor
6-TG	6-thioguanine
WBC	white blood cell

Chapter 1: General introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial joints and synovial hyperplasia. Following initiating events that remain unclear, inflammatory cells including T and B lymphocytes, monocytes and neutrophils migrate to the synovium and synovial fluid of the affected joints. Here they participate in a sustained inflammatory response, causing tissue injury. Infiltrating and resident cells contribute to the local increase of inflammatory products such as IL-1, TNF-α and reactive oxygen and nitrogen species. Since the etiology of RA remains unclear, understanding the role of inflammatory products in RA pathology may lead to more targeted therapies with fewer toxic side effects than current treatments.

1.2 Nitric oxide

Nitric oxide (NO) is a reactive free radical produced by many cells present within the inflamed joints of RA patients, including resident synoviocytes, chondrocytes and infiltrating macrophages (McInnes et al. 1996). NO reacts quickly with species of oxygen to form other reactive nitrogen compounds. It should be noted that reference to NO also includes the related nitrogen species derived therefrom. NO is very short-lived and is therefore detected by indirect methods. For example, the presence of NO is often detected by assaying for a more stable product, such as nitrite. Nitrite levels have been found to be

elevated in the synovial fluid and serum of patients with RA as measured by the presence of nitrite, and correlate with disease activity (Ueki et al. 1996). Inducible nitric oxide synthase (iNOS) is one of three enzyme isoforms responsible for the production of NO and is capable of producing large amounts (μmolar) of NO. iNOS is expressed by several cell types within the inflamed joints of RA patients but is not detected in the joints of normal individuals (Sakurai et al. 1995; St Clair et al. 1996). Inhibition of iNOS ameliorates disease in many animal models of RA (McCartney-Francis et al. 1993; Connor et al. 1995; Miesel et al. 1996). Methotrexate and dexamethasone, drugs used in the treatment of RA, have been found to have beneficial effects due in part to their ability to inhibit iNOS (Radomski et al. 1990; Murrell et al. 1996).

1.3 Nitric oxide and rheumatoid arthritis

NO contributes to RA pathogenesis in several ways: by inhibiting chondrocyte collagen and proteoglycan production (Amin and Abramson, 1998; Clancy et al. 1998), by enhancing vascular permeability and prostaglandin formation within the synovium, and by altering bone remodelling. NO has also been found to cause genetic damage in a variety of bacterial and mammalian cell types *in vitro*. These NO-induced mutations include DNA deaminations (Wink et al. 1991; Routledge et al. 1993) and single-strand breaks (Nguyen et al. 1992). Observations in our laboratory suggest that NO may be capable of inducing more substantial DNA damage, including large-scale deletion (LSD)

mutations, in a murine tumour cell line. For the purposes of this study, LSD mutations are defined as events that cause the loss of several hundred to several thousand DNA base-pairs (bp) and are therefore detectable by PCR. Small mutational events generally result from a localized alteration of the DNA that, when repaired, causes a mutation of one or several bases. In contrast, LSD mutations likely result from two genetic "hits" that cause DNA double-strand breaks at sites separated by a significant length of genomic sequence. Ligation of these breaks then results in a LSD mutation. However, the ability of NO to cause LSD mutations *in vitro* or *in vivo* has not been addressed to date.

A recent study from our lab has shown that mutations can be detected in T lymphocytes from patients with RA (Cannons et al. 1998). The mutation frequency (MF) of a marker gene, the hypoxanthine phosphoribosyltransferase (*hprt*) gene, commonly used as a marker of somatic mutations *in vivo*, was significantly increased in RA peripheral T cells compared to age and sex matched controls. The *hprt* MF of synovial T cells from the same patients was 10-fold higher than that of peripheral T cells, suggesting that a mutagenic environment exists within the inflamed joints of RA patients. The *hprt* MF of T cells from patients with osteoarthritis (OA), a joint disease of a less inflammatory nature than RA, fell in an intermediate range between the *hprt* MF of T cells from controls and RA patients. The nature of the mutagenic events giving rise to the increase in *hprt* MF in RA and OA T cells was not examined in this earlier study.

The subject of this thesis was to determine the extent that LSD mutations

may be responsible for the observed increase in the *hprt* MF of RA and OA T cells. T cells mutated at the *hprt* locus (*hprt*$^-$) were isolated from the peripheral blood of RA and OA patients, and *hprt*$^-$ T cell clones were screened by multiplex-PCR (MP-PCR) for LSD mutations that included one or more *hprt* exons. To determine if NO is capable of producing LSD mutations, two human B lymphoblastoid cell lines, TK6 and WIL2-NS, were exposed to the NO-donating drugs nitroglycerin (NG) and sodium nitroprusside (SNP) *in vitro*. The ability of these drugs to cause mutations was assessed by measuring the *hprt* MF of TK6 and WIL2-NS cells following drug treatment. TK6 and WIL2-NS *hprt*$^-$ clones that arose spontaneously or after drug treatment were then screened for *hprt* LSD mutations by MP-PCR.

1.4 Hypothesis

In RA, cells present within the inflamed joints are exposed to a mitogenic and mutagenic inflammatory environment, where they sustain genetic damage. Genotoxic species, such as NO and reactive oxygen and nitrogen species, are responsible for DNA damage and may produce a distinctive pattern of mutations at the genomic level, including large-scale deletions. If NO is responsible for the genetic damage believed to occur within the inflamed joints, then cells exposed to NO-donating drugs *in vitro* will develop mutations similar to those found in RA T cells.

1.5 Objectives

a) To determine if LSD mutations occur in T cells from RA patients by isolating T cells mutated at the *hprt* locus (*hprt*$^-$) from the peripheral blood of these individuals, and by screening *hprt*$^-$ T cell clones by MP-PCR for large deletions.

b) To determine if NO is capable of producing LSD mutations *in vitro* by exposing two human B lymphoblastoid cell lines to NO-donating drugs and analysing the resulting *hprt*$^-$ clones for deletion mutations by MP-PCR.

1.6 Significance

The present study is novel in that it represents the first attempt to determine whether or not LSD mutations occur preferrentially within the inflamed joints of RA patients. Genetic damage may contribute to RA pathology and therefore a better understanding of the mutations that occur in the RA joint may be of value. Synoviocytes from RA patients have been found to express high levels of mutated p53 protein (Firestein et al. 1997; Aupperle et al. 1998). p53 is a tumour suppressor gene that, when dysfunctional, may promote synovial hyperplasia, which is a hallmark of RA. Mutational events that affect oncogenes could be partially responsible for the link that exists between RA and certain cancers of the hematopoieitic system (Laakso et al. 1986; Cibere et al. 1997). A better understanding of the genotoxic potential of NO and its role in RA pathology may lead to therapies that target this molecule to reduce inflammation

and prevent the outcome of DNA damage. This study is also the first to determine if NO is capable of causing genetic damage other than small mutational events, information that may contribute to our current understanding of the link between inflammation, genetic damage and increased cancer risk.

Chapter 2: *Hprt* deletion mutations in T lymphocytes from patients with arthritis

2.1 Introduction

2.1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an often debilitating autoimmune disease that affects approximately 1% of the world's population (Maini and Zvaifler, 1994). This disease is characterized by chronic inflammation of the synovial joints that leads to the loss of joint structure and function. Although the events that cause the initiation of RA are not clear, there is a link between the development of disease and an HLA-DR allele, suggesting that MHC-Class II restricted T cells may be involved. Depletion of T cells by means of anti-CD4 antibodies is of limited therapeutic benefit in RA since T cells within the inflamed joints remain relatively unaffected by treatment (Jendro et al. 1995). For example, treatment of RA patients with monoclonal antibodies against the CD4 molecule has resulted in depletion of naive peripheral $CD4^+$ T cells, while primed, IFN-γ-producing T cell numbers remain constant (Rep et al. 1997). Therefore, cells thought to participate in the autoimmune response of RA were least affected by anti-CD4 antibody treatment.

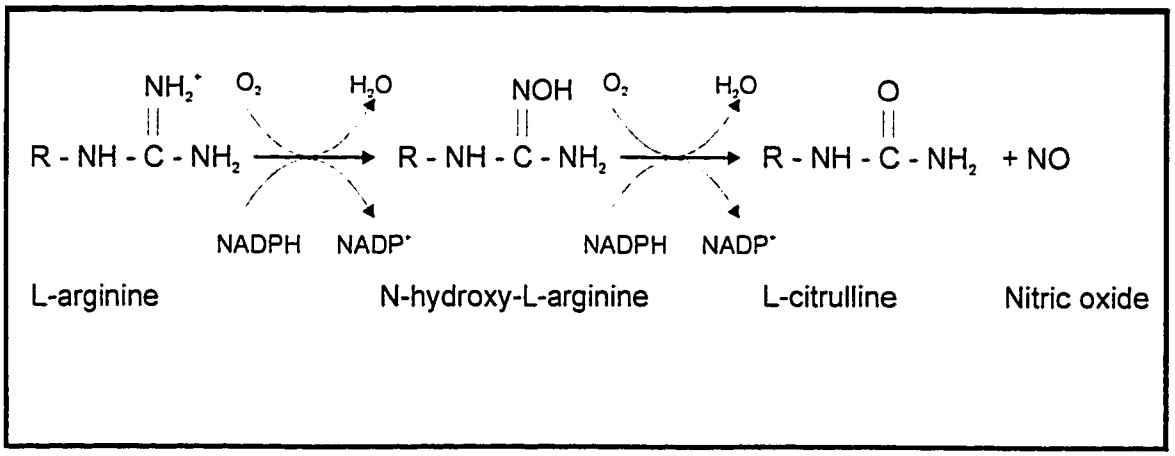
The progression of RA, however, is better characterized. Following initiation, inflammatory cells such as T lymphocytes, B lymphocytes, mast cells, macrophages and neutrophils are found in the synovium and synovial fluid (SF) of the affected joints. The synoviocytes that make up the synovial membrane

become hyperplasic and phenotypically resemble transformed cells (Muller-Ladner et al. 1995). The synovial membrane increases in thickness from 1-3 cell layers in normal individuals to greater than 10 cell layers in RA patients. Metalloproteinases are produced by chondrocytes, leading to degradation of the protective cartilage, and eventually the underlying bone. Inflammatory products such as IL-1, TNF-α and reactive oxygen and nitrogen species are produced by infiltrating and resident cells, causing pain, swelling, tissue damage and further recruitment of inflammatory cells. The cycle of inflammation continues until the synovial cartilage is destroyed. Identifying key players in the perpetuation of RA may lead to new therapies that more effectively reduce inflammation while limiting toxic side effects.

2.1.2 Nitric oxide

Nitric oxide (NO) is a short-lived free radical produced in large quantities by many cell types found within the inflamed joints of RA patients. NO is formed from L-arginine and oxygen by the nitric oxide synthase (NOS) family of enzymes (Figure 2-1). Immunohistochemical staining reveals that the inducible form of NOS (iNOS) protein is expressed by several cell types within the RA synovium. These include the synovial lining cells, endothelial cells, chondrocytes and infiltrating mononuclear cells (Sakurai et al. 1995). Synovium from patients with osteoarthritis (OA), a non-autoimmune joint disease of a less inflammatory nature than RA, also expresses iNOS, although to a lesser extent

Figure 2-1. The conversion of L-arginine to L-citrulline and nitric oxide by nitric oxide synthase.

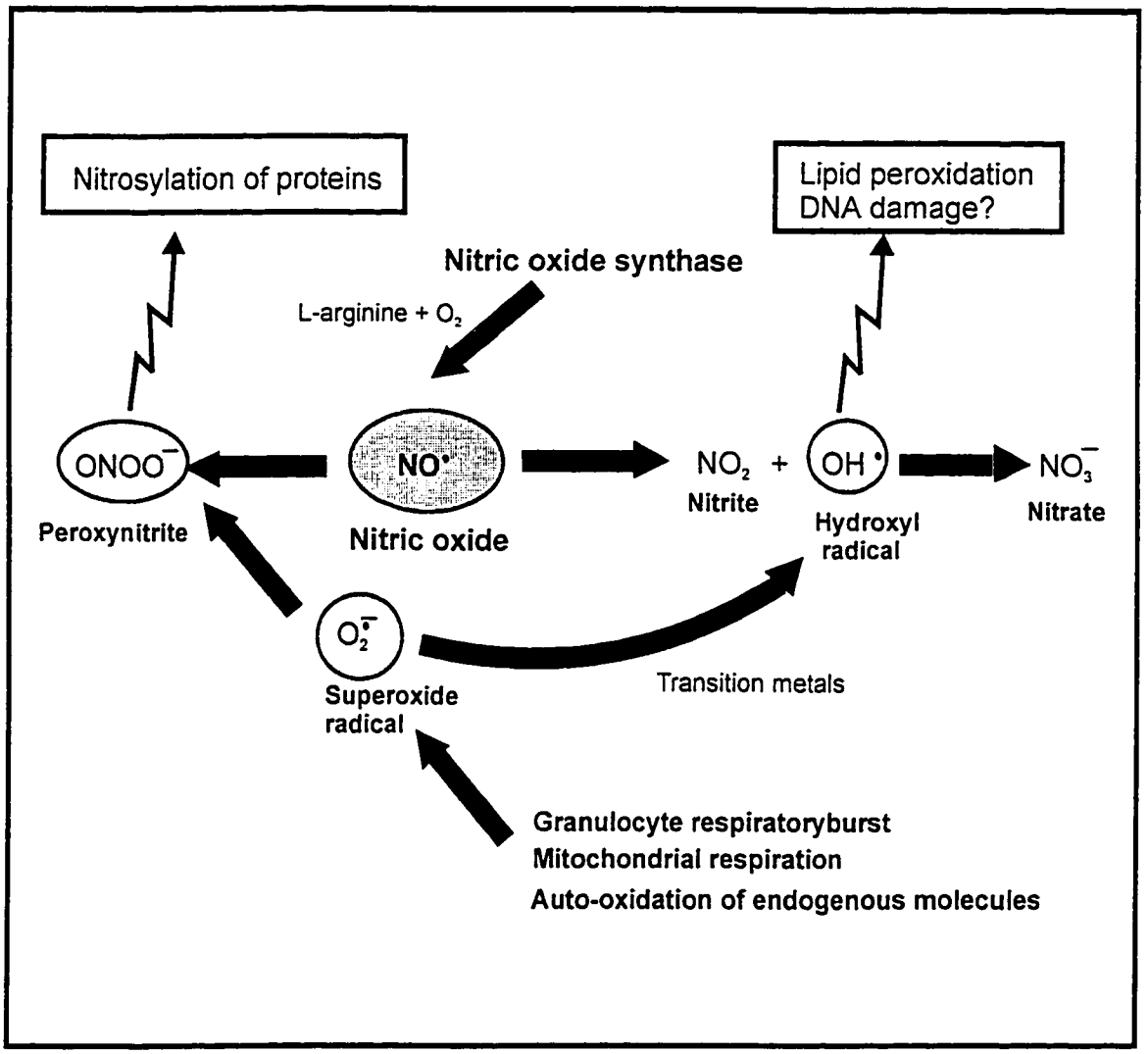

NH2+
O2
H2O
NOH
O2
H2O
O
R - NH - C - NH2
R - NH - C - NH2
R - NH - C - NH2
+ NO
NADPH
NADP+
NADPH
NADP+
L-arginine
N-hydroxy-L-arginine
L-citrulline
Nitric oxide

than RA synovium. Normal synovium shows no sign of iNOS expression (Grabowski et al. 1997). In many animal models of RA, the inhibition of iNOS can prevent the onset of RA-like disease or reduce symptoms of established disease (McCartney-Francis et al. 1993; Connor et al. 1995; Miesel et al. 1996; Tanaka et al. 1998). The beneficial effects of methotrexate and dexamethasone, drugs commonly used to treat RA, may be due to their ability to inhibit iNOS (Radomski et al. 1990; Murrell et al. 1996). In addition to expressing iNOS protein, synovial tissue samples from RA patients spontaneously produce large quantities of NO in culture. NO reacts rapidly with oxygen species to form more stable compounds such as nitrite. Nitrite concentrations have been shown to be elevated in RA patient serum and SF compared to controls, and correlate with periods of acute disease activity (Ueki et al. 1996). Lower nitrite levels are found in the serum and SF of OA patients. Therefore, evidence suggests that high levels of NO are formed within the joints of patients with RA.

2.1.3 Nitric oxide and rheumatoid arthritis

NO may contribute to RA pathogenesis in several ways. NO reacts quickly with oxygen species also present within the inflamed joint to form other reactive nitrogen compounds (Figure 2-2). For example, NO reacts rapidly with superoxide (O_2^-) to form peroxynitrite ($OONO^-$) that can have a half-life of several minutes. Peroxynitrite causes the nitrosylation of protein residues such

Figure 2-2. A schematic of interactions between NO and other reactive oxygen species.


Nitrosylation of proteins
Lipid peroxidation
DNA damage?
Nitric oxide synthase
L-arginine + O_2
$ONOO^-$
Peroxynitrite
$NO^\bullet$
Nitric oxide
NO_2 + $OH^\bullet$
Nitrite
Hydroxyl radical
NO_3^-
Nitrate
$O_2^{\bullet-}$
Superoxide radical
Transition metals
Granulocyte respiratoryburst
Mitochondrial respiration
Auto-oxidation of endogenous molecules

as tyrosine. 3-nitrotyrosine levels are elevated in the serum and SF of RA patients, suggesting that NO plays a role in the oxidative damage seen in the inflamed joint (Kaur and Halliwell, 1994). In animal experiments, NO causes increases in enzymes that degrade the protective cartilage matrix of the synovial joint, while NO inhibits the formation of new cartilage (Amin and Abramson, 1998; Clancy et al. 1998). For example, incubation of bovine chondrocytes with donors of NO was shown to increase the expression of collagenase mRNA while the addition of an inhibitor of NOS attenuated the IL-1-mediated increase in collagenase expression (Lo et al. 1998). It has been suggested that NO may act as an intracellular messenger in response to extracellular stimuli, transducing signals to the cell nucleus to increase gene expression. NO has been shown to increase matrix metalloproteinase (MMP-2 and MMP-9) production in rabbit articular chondrocytes while decreasing collagen and proteoglycan formation (Tamura et al. 1996). In human neutrophils, MMP-8 activity is enhanced by incubation with peroxynitrite (Okamoto et al. 1997). NO also promotes vascular permeability (St.Clair, 1998), mediates the formation of other pro-inflammatory molecules such as prostaglandins (de Mello et al. 1997) and may contribute to bone resorption (Hukkanen et al. 1995; Ralston and Grabowski, 1996).

2.1.4 Nitric oxide and genetic damage

In addition to contributing directly to the symptoms of RA, NO is capable

of causing genetic damage that may contribute further to RA pathology. *In vitro*, NO has been shown to cause DNA deaminations that lead to C to T transition mutations (Wink et al. 1991; Routledge et al. 1993), and to induce DNA strand breaks (Nguyen et al. 1992). The ability of NO to produce mutations that exceed base-pair changes has not been addressed in the literature to date. Data also suggests that NO may be responsible for genetic damage *in vivo*; DNA single-strand breaks are found to be elevated in mononuclear cells (MNCs) from RA patients but not in MNCs from healthy individuals (Bhusate et al. 1992). Our laboratory has also demonstrated that genetic damage occurs in T lymphocytes isolated from RA patients (Cannons et al. 1998). The frequency with which a marker gene, the hypoxanthine phosphoribosyltransferase (*hprt*) gene, was mutated was found to be elevated in peripheral T cells from RA patients but not in the peripheral T cells of age and sex-matched controls. In addition, the *hprt* mutation frequency (MF) of T cells from the synovial tissue of the same RA patients was 10 times higher than that of peripheral T cells, suggesting that a genotoxic environment exists within the inflamed joints. The *hprt* MF of peripheral and synovial T cells from patients with OA was intermediate between controls and RA. The nature of the mutagenic events giving rise to the increased *hprt* MF of RA or OA T cells was not examined.

The genetic damage that occurs within the inflamed joint can affect cell types other than lymphocytes and may contribute directly to RA pathology. For

example, high levels of mutant p53 protein are expressed by synoviocytes from RA patients but not in normal synoviocytes (Firestein et al. 1997). p53 is a tumour suppressor gene that when dysfunctional, may permit the destructive synovial hyperplasia that is characteristic of RA (Aupperle et al. 1998). Many of the p53 mutations identified in synoviocytes from RA patients have also been identified in human cancers. Mutation of oncogenes other than p53 by products of inflammation may partially explain the correlation that exists between RA and cancers of the hematopoietic system (Laakso et al. 1986; Cibere et al. 1997). A better understanding of the genotoxic potential of NO and its role in RA pathology may lead to therapies that target this molecule to reduce inflammation and prevent the outcome of DNA damage.

Although NO has not previously been shown to cause genomic mutations of significant size, indirect evidence from our lab suggests that NO may be capable of causing large-scale deletion (LSD) mutations that result in the loss of several hundred to several thousand DNA base-pairs. For example, mouse tumour cells exposed to NO-donating drugs both *in vitro* and *in vivo*, occasionally produced clones with mutations at the *hprt* locus that were slow growing. Although these mutations have not been fully characterized at the DNA level, the possibility remains that the slow-growing phenotype of these clones was due to large clastogenic events affecting genes neighbouring the *hprt* locus. In addition, the MFs induced by clastogenic agents known to cause LSD mutations, and by NO-donating drugs, were similar at isocytotoxic doses in this mouse cell

line.

2.1.5 The hypoxanthine phosphoribosyltransferase gene

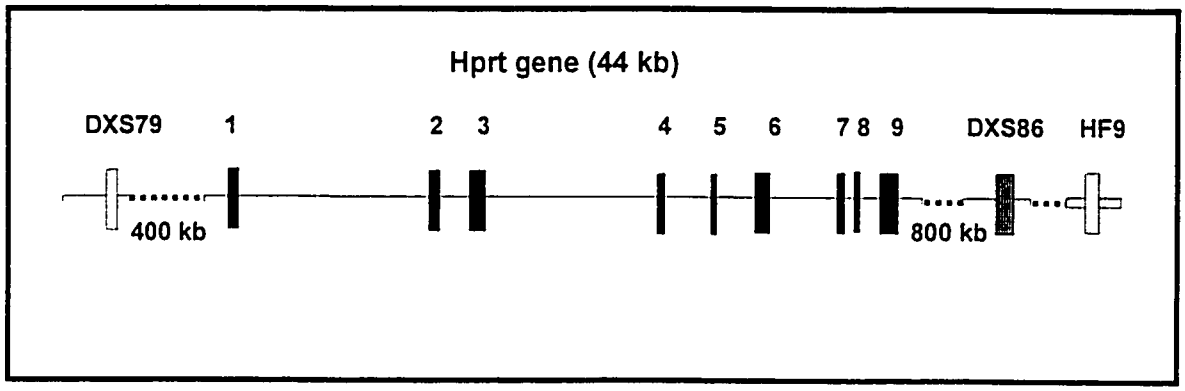
The *hprt* gene has been used extensively as a marker of genetic damage to characterize mutational events occurring both *in vitro* and *in vivo*. The *hprt* enzyme is involved in the purine salvage pathway and, upon mutation, confers resistance to the cytotoxic drug 6-thioguanine (6-TG). *Hprt*$^+$ (non-mutant) cells are able to metabolize 6-TG to its toxic metabolite, which is incorporated into DNA and RNA, while *hprt*$^-$ (mutant) cells are unable to produce this toxic metabolite and consequently they survive. The mechanism of 6-TG-induced death in *hprt*$^+$ cells, although not fully understood, is thought to involve postreplicative DNA mismatch repair (Swann et al. 1996).

The use of the *hprt* gene as a marker of genetic damage has many advantages; this system has the ability to detect as few as 1 *hprt*$^-$ cell in 1×10^6 cells, large deletions (>2 Mb) are tolerated near this locus without interfering with cell viability (Fuscoe et al. 1992; Fuscoe et al. 1994) and many different types of mutations, such as transitions, transversions, deletions and insertions, can be induced. The *hprt* gene has therefore been used in many studies to assess somatic mutations induced by disease and exposure to environmental pollutants. T lymphocytes are commonly used to study *hprt* mutations since they are readily obtained from peripheral blood. The *hprt* MF of T lymphocytes from normal individuals is approximately 1-5 mutants per 1×10^6 cells (Nicklas et al. 1987;

Nicklas et al. 1988), although this increases slightly with age (+1.3 *hprt*$^-$ cells per 1×10^6 cells every 10 years) (Akiyama et al. 1995). The *hprt* MF of T cells has been shown to be elevated not only in RA, but also in other inflammatory diseases such as scleroderma (Sfikakis et al. 1994), multiple sclerosis (MS) (Sriram, 1994) and systemic lupus erythematosus (SLE) (Dawisha et al. 1994; Theocharis et al. 1995). Exposure to cigarette smoke (Vrieling et al. 1992; Ammenheuser et al. 1997), radiation (Skandalis et al. 1997) and other pollutants also increases T cell *hprt* MF.

Molecular analysis of the *hprt* gene (Figure 2-3) can sometimes be used to provide evidence of exposure to a particular mutagen, as some mutagens produce a distinct "fingerprint" of DNA lesions. For example, ionizing radiation tends to produce *hprt* LSD mutations that are observed less frequently in unexposed individuals (Da Cruz and Glickman, 1997). In contrast, exposure to cigarette smoke causes an increase in T cell *hprt* MF without producing a specific mutational spectrum (Vrieling et al. 1992). To determine if NO is capable of producing *hprt* deletion mutations that exceed those arising spontaneously, the normal "spontaneous" spectrum of T cell *hprt* mutations must first be examined. Several groups have isolated *hprt*$^-$ T cells from the peripheral blood of healthy individuals and screened the resulting clones for large deletions by means of Southern blot or multiplex-PCR (MP-PCR). *Hprt* mutations involving the deletion of 1 or more exons at the genomic level have been reported to occur in anywhere from 0.5% to 19% of T cell clones studied (Albertini et al. 1985;

Figure 2-3. Schematic of the *hprt* gene and flanking regions. The *hprt* gene (not shown to scale) is located on the X chromosome and consists of large intronic sequences and 9 small exons. The flanking regions DXS79 and DXS86 are approximately 400 Kb and 800 Kb from the 5' and 3' ends of the *hprt* gene, respectively. The HF9 gene is found distal to the *hprt* gene on the X chromosome.


Hprt gene (44 kb)
DXS79
1
2
3
4
5
6
7 8
9
DXS86
HF9
400 kb
800 kb

Turner et al. 1985; Nicklas et al. 1987; Fuscoe et al. 1992; Steingrimsdottir et al. 1993; Osterholm et al. 1995; Burkhart-Schultz et al. 1996; Huttner and Holzapfel, 1996). In contrast, a study of individuals accidentally exposed to ^{137}Cs radiation has shown that large *hprt* deletions occur in 36.7% of peripheral *hprt*$^-$ T cell clones, a 2-fold increase above background (Da Cruz and Glickman, 1997). If the elevated *hprt* MF observed in RA T cells is largely due to LSD mutations, then these should be detected more frequently in *hprt*$^-$ T cells from RA patients than in controls.

Since the *hprt* gene is located on the X chromosome, DNA studies must be conducted using cells from male individuals. Cells from females can be selected for the *hprt*$^-$ phenotype in 6-TG but will contain the mutated *hprt* gene on the active X chromosome while the *hprt* gene present on the inactivated X chromosome need not be mutated. PCR amplification of the *hprt* gene using female DNA therefore results in two different products: one derived from the mutant form of the *hprt* gene and the second, from a potentially non-mutated gene present on the inactivated X chromosome. If deletion mutations do occur in the mutated *hprt* gene, they are "covered" by positive PCR signals from the intact gene. Therefore, DNA from male T cells must be used to detect LSD mutations.

2.1.6 Hypothesis

During the course of RA, inflammatory cells migrate through the inflamed joints where they are exposed to mitogenic and mutagenic species and subsequently incur genetic damage (Figure 2-4). NO and other reactive oxygen and nitrogen species are able to contribute to this DNA damage and may produce LSD mutations.

2.1.7 Specific Objectives

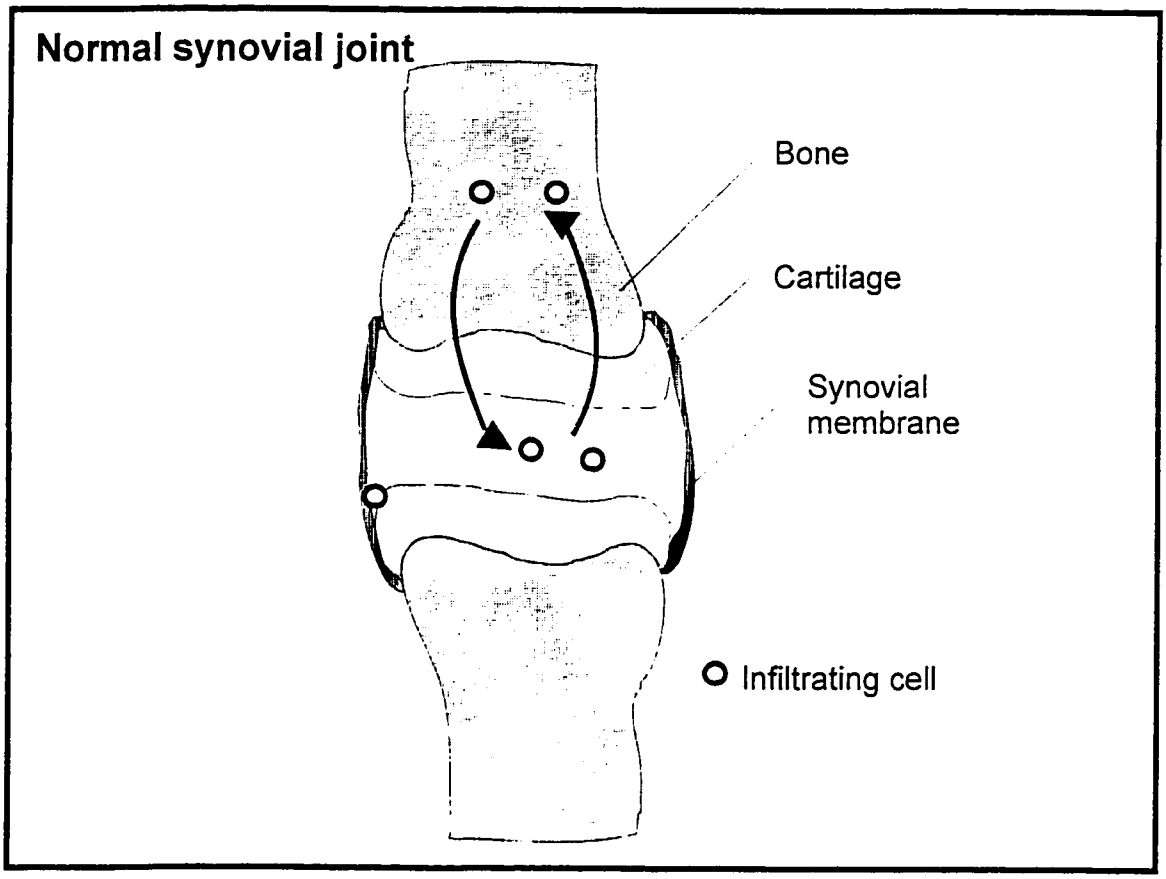
a) To determine if LSD mutations occur in T cells from RA patients, by analysing *hprt*$^-$ T cell clones from male patients for large mutations at the *hprt* locus by MP-PCR.

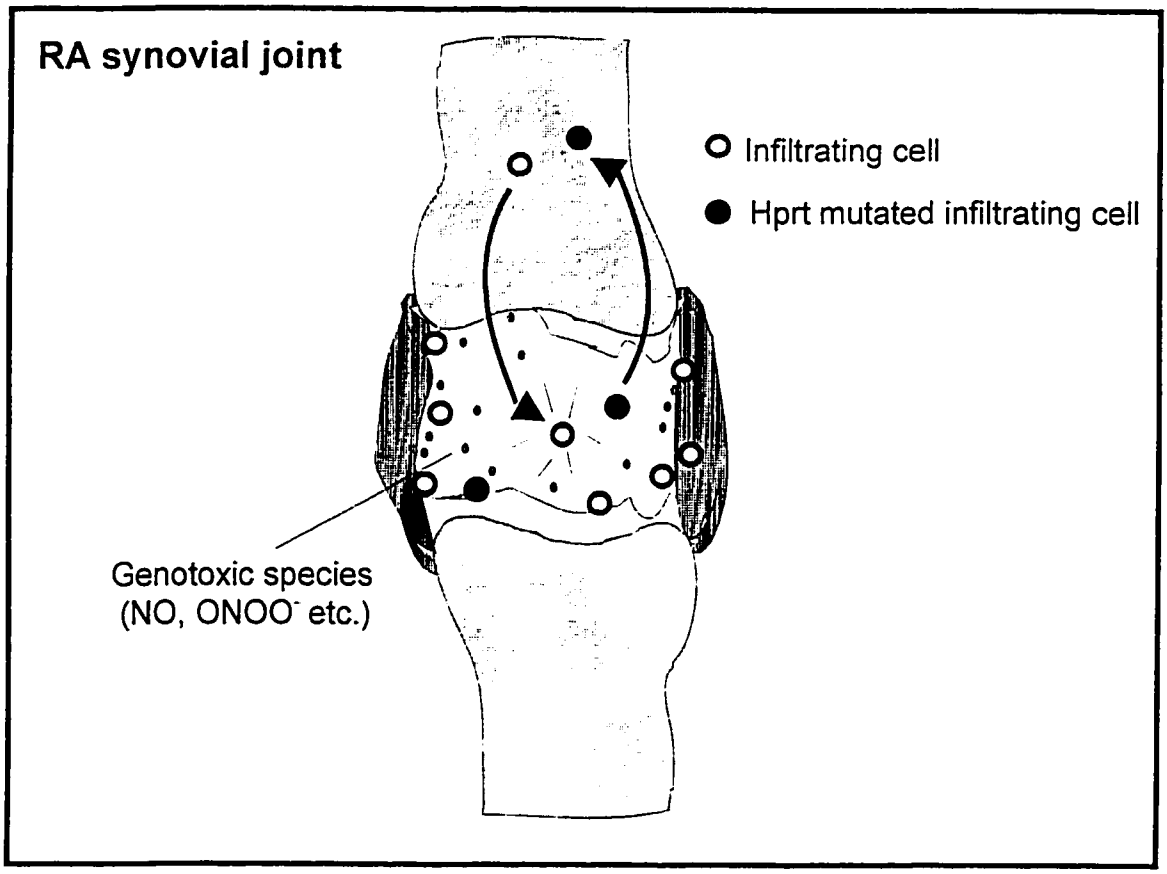
b) To determine if NO may be responsible for LSD, by analysing *hprt*$^-$ T cell clones from OA patients, presumably exposed to lower levels of NO than RA T cells, for large *hprt* mutations.

Significance

This research is of significance since it represents the first attempt to characterize the genetic damage that occurs in RA T cells, and potentially in any cell exposed to the mutagenic environment of the inflamed joint. Somatic mutations may contribute to RA pathology and, if NO is identified as a significant mutagen, then new drugs that target this molecule may be useful in therapy.

Figure 2-4. Hypothetical model of a normal synovial joint and a synovial joint from a patient with RA. Cells trafficking to and from the inflamed joints of RA patients become exposed to the mutagenic environment and incur genetic damage, such as *hprt* mutations. Reactive oxygen and nitrogen species, such as NO, may be responsible for genetic damage since they are prevalent in the inflamed joint.


Normal synovial joint
Bone
Cartilage
Synovial membrane
Infiltrating cell


RA synovial joint
Infiltrating cell
Hprt mutated infiltrating cell
Genotoxic species
(NO, ONOO- etc.)

2.2 Materials and Methods

2.2.1 Criteria for the selection of arthritis patients

RA is an autoimmune disease characterized by high levels of NO within the inflamed joints, whereas OA is a non-autoimmune disease of the synovial joints in which NO levels are lower. Therefore, mutations that occured in RA and OA T cells was compared. Peripheral blood (PB) samples were obtained from 16 male patients diagnosed with RA and from 6 male patients diagnosed as with OA. Informed consent was obtained prior to PB collection and this study was approved by the Ottawa Hospital's Research Ethics Boards. RA patients fulfilled the criteria of the American College of Rheumatology for the classification of RA (Arnett et al. 1988). The patient's age, disease duration and disease severity were recorded. The patients' previous and current use of medication was also noted when available.

2.2.2 Isolation of *hprt*$^-$ T cell clones from the peripheral blood of arthritis patients

Approximately 20 ml of heparinized venous PB was obtained from RA and OA patients. From this, T lymphocytes were isolated and grown in medium selective for the generation of *hprt*$^-$ clones according to a modified protocol (Cannons et al. 1998) of Albertini et al. (Albertini et al. 1982). In brief, peripheral blood mononuclear cells (PBMCs) were obtained from blood centrifuged over Ficoll-Hypaque. The number of viable PBMCs was determined by trypan blue

exclusion and cells were seeded in 96-well plates at $2x10^5$ viable PBMCs per well, along with $1x10^5$ lethally irradiated TK6-D1 "feeder" cells in a total of 200 µl complete selection medium (RPMI 1640 medium containing 10% Fetal Calf Serum (FCS), 20% Hybridoma Serum-Free Medium, 2 mM L-glutamine, 1 µg/ml phytohemagglutinin (PHA), 30 U/ml IL-2, and 100 µM 6-TG to select for cells expressing the *hprt*$^-$ phenotype). TK6-D1 is an *hprt*$^-$ cell line derived from the TK6 B lymphoblastoid cell line (ATCC CRL 8015) following exposure to 1 Gy of ionizing radiation and was provided by D. Wilkinson (Wilkinson, unpublished work). This cell line was found by PCR to lack *hprt* exons 2, 3, 7/8 and 9, along with the anonymous flanking regions, DXS79 and DXS86. Therefore, these cells were chosen as "feeder" cells since the would not produce a PCR background signal at the time of *hprt*$^-$ T cell clone analysis. TK6-D1 cells were irradiated with a total of 80 Gy using the ^{137}Cs Gamma Cell-40 at the University of Ottawa and served as a source of feeder cells during T cell growth. After 1 week, plates were examined for clonal growth; "positive" wells contained visibly viable *hprt*$^-$ T cells while "negative" wells contained only dead feeder cells and dead and dying *hprt*$^+$ PBMCs. When, after 2 weeks of growth, *hprt*$^-$ T cell clones consisted of approximately 200 live *hprt*$^-$ cells, DNA was isolated for PCR analysis.

Non-mutant (*hprt*$^+$) T cell clones were also generated from each patient by plating 1 or 10 PBMCs per well in 96-well plates along with $1x10^5$ irradiated TK6-D1 feeder cells in a total of 200 µl complete selection medium lacking 6-TG. When peripheral white blood cell (WBC) DNA was not available as a positive

control for PCR analysis, DNA was isolated from several non-mutant clones after 2 weeks of growth.

2.2.3 Isolation of DNA from patient peripheral white blood cells

Approximately 5 ml of patient PB was collected, using EDTA as a anticoagulant. DNA was isolated from the buffy coat using a phenol/chloroform extraction-EtOH precipitation method (Appendix II). DNA concentrations were determined using the diaminobenzoic acid (DABA) fluorometric method (Appendix III).

2.2.4 PCR strategy for the detection of LSD mutations in *hprt*$^-$ T cell clones

To detect LSD mutations by MP-PCR, DNA from *hprt*$^-$ T cell clones was used to amplify regions spanning the *hprt* gene (exons 2, 3 and 7/8) as well as the flanking regions DXS79 and DXS86. A portion of the HF9 gene, distal to the *hprt* locus on the X chromosome and not expected to be co-deleted with the *hprt* gene, was co-amplified as a positive internal control. To ensure that PCR amplification failure was not due to allelic variation between patients, WBC DNA from each patient was also analysed by MP-PCR. If WBC DNA was not available for this purpose, 2-3 non-mutant (*hprt*$^+$) T cell clones from the patient were used instead. *Hprt*$^-$ T cell clones that failed to amplify one or more PCR bands while amplifying the HF9 fragment, were considered deletion mutants. When LSD mutation occurred and when 2 or more PCR products failed to

amplify, the intervening DNA was assumed to have been deleted, although future sequencing will be needed to confirm this and to map the exact ends of the deletion.

2.2.5 Hypotonic shock/micrococcal nuclease treatment of T cell clone lysates prior to PCR analysis

In order to detect deletion mutations, DNA from *hprt*$^-$ T cell clones must be analysed in the absence of an *hprt*$^+$ background PCR signal. Although *hprt*$^+$ PBMCs are killed by the cytotoxic actions of 6-TG, these cells remain sufficiently intact such that their DNA could be amplified by PCR even after several weeks in culture. To circumvent this problem it was therefore necessary to devise a method to eliminate the DNA associated with dead *hprt*$^+$ cells prior to PCR analysis. The procedure that was eventually developed involves the exposure of cells to a hypotonic shock followed by digestion with micrococcal nuclease (Grant et al. 1998). After 2 weeks of growth in complete selection medium in 96-well plates, the contents of "positive" wells, containing viable *hprt*$^-$ T cell clones, were transferred to individual sterile 0.6 ml thin-wall microfuge tubes for PCR analysis. To optimize the recovery of the low number of T cells in each clone, 2 × 10^4 TK6-D1 cells were added as a "carrier". Since TK6-D1 cells lack the *hprt* and flanking regions under study, they did not contribute to PCR background. Tubes were centrifuged for 5 min at 1,500 rpm (150 x g) and the supernatant was carefully removed and discarded. To the cell pellets, 20 µl of hypotonic

shock buffer (40 mM NaCl, 1 mM $CaCl_2$, 5 mM Hepes, pH 7.4) was added and samples were carefully mixed by gently tapping the sides of the tube. After 1 min, tonicity was restored with 4.4 µl of 0.5 M NaCl. Samples were incubated with 0.1 U/ml micrococcal nuclease at 37°C for 20 min. Nuclease digestion was terminated by chelating the remaining Ca^{2+} with 1.2 mM EGTA. The tubes were centrifuged at 1,500 rpm for 3 min and the supernatant was removed. Intact (i.e., *hprt*$^-$) T cells remaining in the tubes were lysed according to a modification of the protocol of Lui et al. (Liu et al. 1992). Cell pellets were lysed in 5 µl of lysis buffer (0.68% Tween 20, 0.68% NP-40, 76 mM KCl, 26.2 mM Tris-HCl, pH 8.3) and incubated with 100 µg/ml proteinase K for 30 min at 52°C. Proteinase K was heat-inactivated at 95°C for 20 min. Lysates were stored at 4°C until PCR analysis was carried out.

2.2.6 MP-PCR analysis of T cell clones

T cell clone lysates were divided into two 2.5 µl portions; one was used in a MP-PCR reaction to co-amplify *hprt* exons 2, 3 and 7/8 and a portion of the HF9 gene as an internal control (MP-PCR reaction #1). The remainder was used in a second MP-PCR reaction to amplify a portion of the HF9 gene along with two regions flanking the *hprt* gene; DXS79 that lies 400 kb from the 5' end of the *hprt* gene, and DXS86 that lies 800 kb from the 3' end (see Figure 2-2). The sequences of all primers and their positions in the genome are given in Appendix IV. Reactions were performed in thin-wall PCR tubes in a total of volume of 25

µl. MP-PCR reaction #1 contained 10% DMSO, 600 µM of each dNTP, 5 pmol of each *hprt* exon 2 and *hprt* exon 3 primer, 45 pmol of each *hprt* exon 7/8 primer, 3 pmol of each HF9 primer, 2.5 µl of cell lysate, 2 units AmpliTaq DNA polymerase and PCR buffer with a final concentration of 4 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 5 mM 2-mercaptoethanol, 6.8 µM EDTA and 65 mM Tris-HCl, pH 8.8. MP-PCR reaction #2 contained the reagents above, except that 400 µM of each dNTP was used and the following primer pairs and concentrations were employed; 20 pmol of each DXS79 and DXS86 primer and 1 pmol of each HF9 primer. Positive control reactions were run during each analysis using both 100 ng of TK6 genomic DNA and 100 ng of patient total WBC DNA. When patient WBC DNA was not available, 2.5 µl of non-mutant (*hprt* $^+$) T cell clone lysate was used as a positive control. A negative control, containing no DNA, was also included using 2.5 µl of sterile H_2O. One drop of DC 200/200 silicone oil was added to each tube and samples were heated to 94°C for 4 min in an MJ Research Programmable Thermal Controller. MP-PCR reaction #1 samples were cycled 35 times through a 30 sec 94°C denaturing step, a 2 min 54°C annealing step and a 2 min 68°C extension step. Samples were held for an additional 5 min at 68°C during the final round of amplification. MP-PCR reaction #2 samples were subjected to similar amplification conditions except that the annealing temperature was 58°C and an elongation temperature of 72°C was used. PCR products were separated on a 2% NuSieve+1.5% agarose gel. Gels were stained with 1 µg/ml ethidium bromide and visualized

under UV light.

2.2.7 Interpretation of data and statistical methods

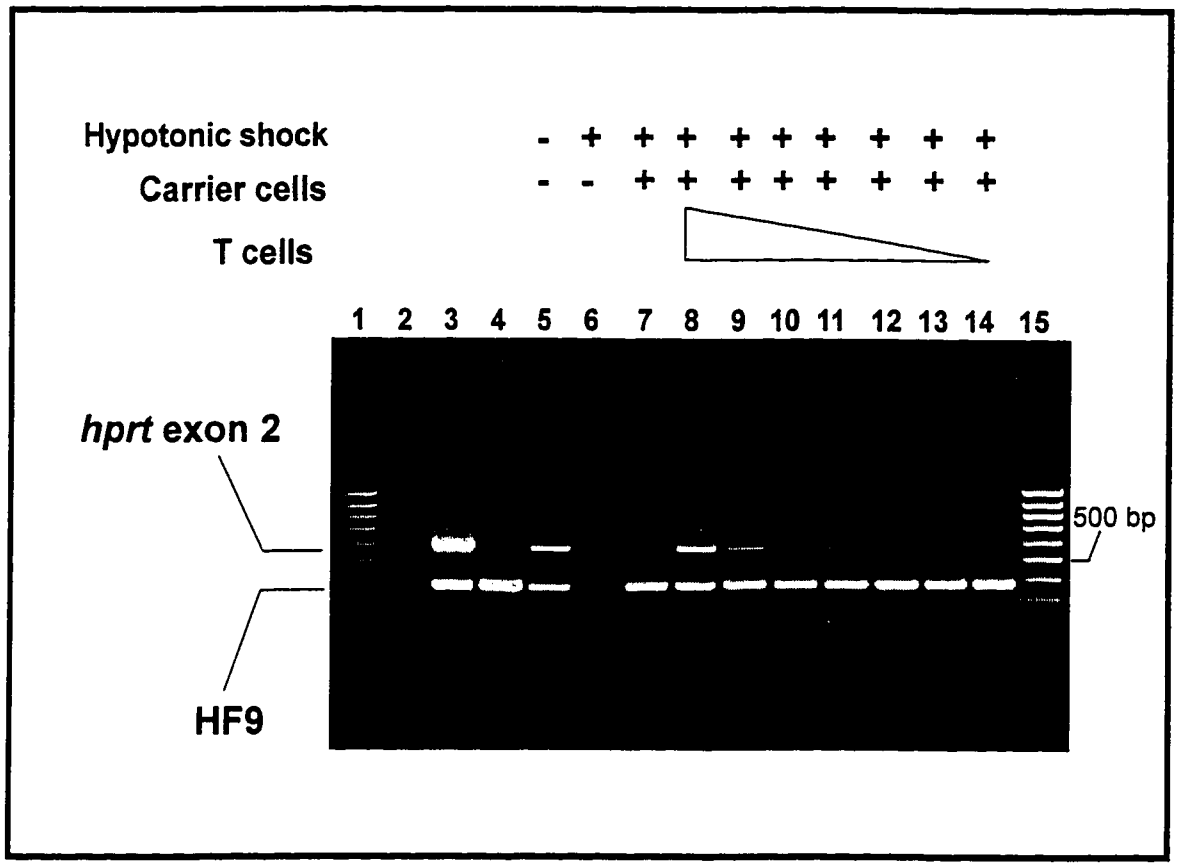
LSD mutations were identified as *hprt*$^-$ T cell clone lysates in which one or more PCR bands failed to amplify while the HF9 internal control was amplified. Differences between RA and OA T cell frequency of large-scale *hprt* deletion mutations were analysed using one-way analysis of variance (ANOVA).

2.3 Results

2.3.1 Hypotonic shock/micrococcal nuclease treatment

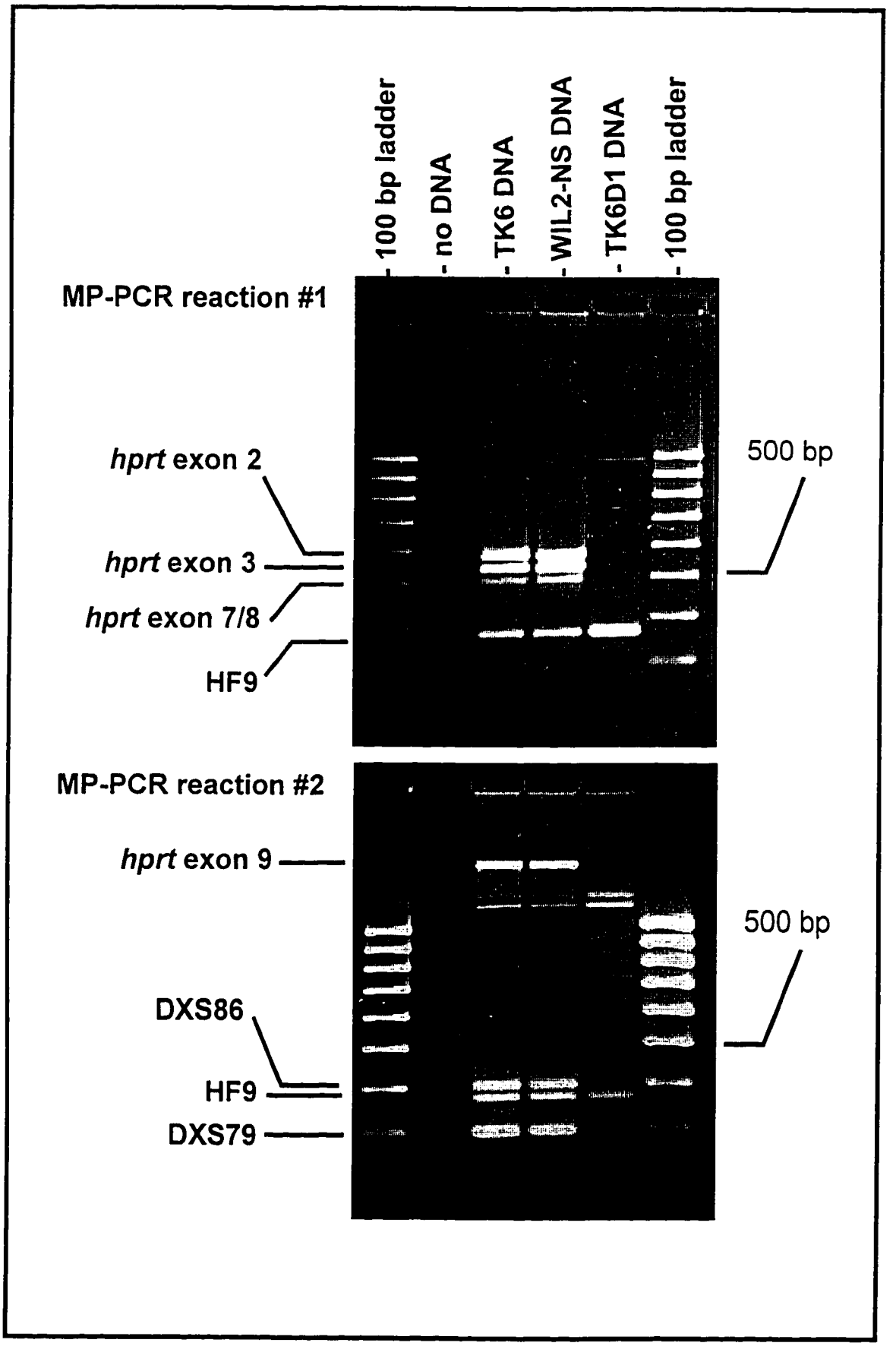
The process of cloning rare *hprt*$^-$ mutant T cells from a large population of non-mutant *hprt*$^+$ cells can give rise to clones consisting of fewer than 200 cells growing in the presence of large numbers of *hprt*$^+$ cells killed by 6-TG. DNA from these dead cells remained intact even after 2-4 weeks in 6-TG, as demonstrated by the production of a significant PCR signal when dead and dying *hprt*$^+$ PBMCs from "negative" wells were subjected to PCR (Figure 2-5, lane 5). Therefore, a method was developed to separate "live" cells from "dead" cells by means of hypotonic shock. By ethidium bromide staining it was evident that dead and dying *hprt*$^+$ cells were lysed or became permeable during this treatment while live *hprt*$^-$ T cells remained intact (data not shown). Subsequent treatment with micrococcal nuclease permitted the digestion of *hprt*$^+$ DNA and RNA prior to PCR analysis.

Figure 2-5. Hypotonic shock treatment of dead and dying *hprt*$^+$ PBMCs after 2 weeks in 6-TG. Lanes 2, 3 and 4 contain PCR products generated without DNA template, using 200 ng of TK6 genomic DNA or 200 ng TK6-D1 DNA, respectively. Lanes 5 and 6 contain PCR products generated from negative wells without carrier before and after hypotonic shock treatment, respectively. Lane 7 contains PCR products from a negative well that was first spiked with carrier and then exposed to hypotonic shock. Lanes 8-14 contain PCR products from a series of negative wells with carrier to which $5x10^3$, $1x10^3$, 500, 200, 100, 50 or 20 viable T cells were added prior to hypotonic shock/micrococcal nuclease treatment. Lanes 1 and 15 contain 250 ng of 100 base-pair ladder marker.


Hypotonic shock
- + + + + + + + + +
Carrier cells
- - + + + + + + + +
T cells
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
hprt exon 2
HF9
500 bp

After only 2 weeks of growth *ex vivo*, T cell clones consisted of a very small number of live cells existing in the presence of a large number of dead cells. To assess the efficacy of the hypotonic shock treatment, a series of "negative" selection wells containing dead and dying *hprt* $^+$ PBMCs were used to amplify *hprt* exon 2 and HF9 fragments before and after hypotonic shock/micrococcal nuclease treatment. The PCR signal associated with negative wells was eliminated after hypotonic shock/micrococcal nuclease treatment (Figure 2-5, lane 6). However, the number of cells from negative wells was small and it was possible that the material was simply lost from the walls of the tube during manipulations. To ensure that the small amount of material from the negative wells was in fact eliminated by the hypotonic treatment, negative wells were first "spiked" with carrier cells (2×10^4 TK6-D1 cells) to increase the efficiency of cell recovery. TK6-D1 cells were chosen as carrier since, lacking *hprt* exons 2, 3, 7/8 and 9 along with the flanking regions DXS79 and DXS86, they do not represent a source of PCR background signal (Figure 2-6). While the HF9 signal from the carrier cells could be detected, the *hprt* exon 2 signal associated with killed cells was again abolished by hypotonic shock /micrococcal nuclease treatment (Figure 2-5, lane 7). The efficiency of live T cell recovery subsequent to hypotonic shock/micrococcal nuclease treatment was then assessed. To determine the minimum number of cells that could be recovered to generate a PCR signal subsequent to the hypotonic shock treatment, a series of negative wells with carrier cells were spiked with decreasing numbers of live T

Figure 2-6. MP-PCR amplification of the *hprt* gene and flanking regions. 100 ng of TK6, WIL2-NS and TK6-D1 DNA was used to amplify *hprt* exons 2, 3, 7/8, 9 and flanking regions DXS79 and DXS86. A portion of the HF9 gene, distal to the *hprt* gene on the X chromosome, was co-amplified within each reaction as a positive control. While TK6 and WIL2-NS cells are positive for all regions examined, TK6-D1 cell are negative for all but the HF9 distal marker. A negative control subjected to identical PCR conditions but without DNA template was included as a negative control. Outside lanes contain 250 ng of 100 bp ladder marker.


100 bp ladder
no DNA
TK6 DNA
WIL2-NS DNA
TK6D1 DNA
100 bp ladder
MP-PCR reaction #1
hprt exon 2
hprt exon 3
hprt exon 7/8
HF9
500 bp
MP-PCR reaction #2
hprt exon 9
DXS86
HF9
DXS79
500 bp

cells, subjected to hypotonic shock/micrococcal nuclease treatment and analysed by PCR. Although the PCR signal associated with carrier cells remained constant, the *hprt* exon 2 band decreased in intensity as the number of live T cells was reduced (Figure 2-5, lanes 8-14). A PCR band corresponding to *hprt* exon 2 could be detected when as few as 20 live T cells were used (Figure 2-5, lane 14).

2.3.2 RA and OA patients

T cell clones mutated at the *hprt* locus were isolated from the peripheral blood of 16 RA patients and 6 OA patients. Their average age, disease duration and medication use at the time of analysis are given in Table 2-1.

Table 2-1. Age, disease duration and medication use of RA and OA patients. Age and duration of disease are given as the mean in years ± the standard deviation. Medication use is reported as drugs that were being taken at the time of *hprt*$^-$ T cell clone isolation. Several RA patients were being treated with more than one drug at the time that PB was taken.

	RA (n=16)	OA (n=6)
Age (years)	58.3 ± 8.1	59.0 ± 12.4
Disease duration (years)	10.7 ± 8.0	26.3 ± 17.3
Current medication (number of patients)		
methotrexate	10	0
cyclosporine	2	0
prednisone	9	1
myochrysine	1	0
NSAIDS	7	1

2.3.3 *Hprt* deletion mutations in *hprt*$^-$ T cell clones isolated from arthritis patients

A total of 54 *hprt*$^-$ T cell clones from 16 RA patients and 19 *hprt*$^-$ T cell clones from 6 OA patients were analysed for deletion mutations by MP-PCR. LSD mutant clones were identified as those that failed to amplify one or more PCR fragments while still amplifying the HF9 internal control. When two or more PCR bands failed to amplify, the intervening DNA sequence was assumed to have been deleted, although sequencing data will be needed to confirm this absolutely. The distance between primer pairs represents the minimum deletion size, since the exact ends of the LSD mutations were not mapped. Three RA patients produced a total of 5 clones with *hprt* LSD mutations whereas 1 OA patient produced 1 clone with a large *hprt* mutation. The clearest example of LSD mutations detected in *hprt*$^-$ T cell clones from an RA patient is shown in Figure 2-7. RA and OA patients whose *hprt*$^-$ T cell clones showed evidence of deletion mutations are identified as Patients A through D. The spectrum of *hprt* regions deleted in T cell clones from these patients is shown in Figure 2-8. Patient A produced 2 clones lacking *hprt* exons 2, 3 and 7/8, representing LSD mutations of at least 26 Kb, in addition to another clone missing *hprt* exons 2 and 3, a deletion of > 2.6 Kb. RA patient B had 1 *hprt*$^-$ clone lacking *hprt* exon 2 (> 572 bp deletion) while Patient C produced 1 clone missing *hprt* exon 3 (> 535 bp deletion). Patient D was the only OA patient to produce a LSD mutant T cell clone and this lacked *hprt* exon 2.

Figure 2-7. Large-scale *hprt* deletion mutations in *hprt*$^-$ T cell clones from a patient with RA. Lanes 3-7 represent positive controls and contain PCR products amplified using 100 ng TK6 DNA, 100 ng WBC DNA and cell lysates from 3 non-mutant T cell clones from one RA patient, respectively. Lanes 8-13 contain PCR products amplified using cell lysates from 6 *hprt*$^-$ (mutant) T cell clones isolated from the same patient. Two clones lack *hprt* exons 2, 3 and 7/8 (lanes 9 and 12) whereas another clone was negative for *hprt* exons 2 and 3 (lane 13). Lanes 1 and 14 contain 250 ng of 100 bp ladder marker.

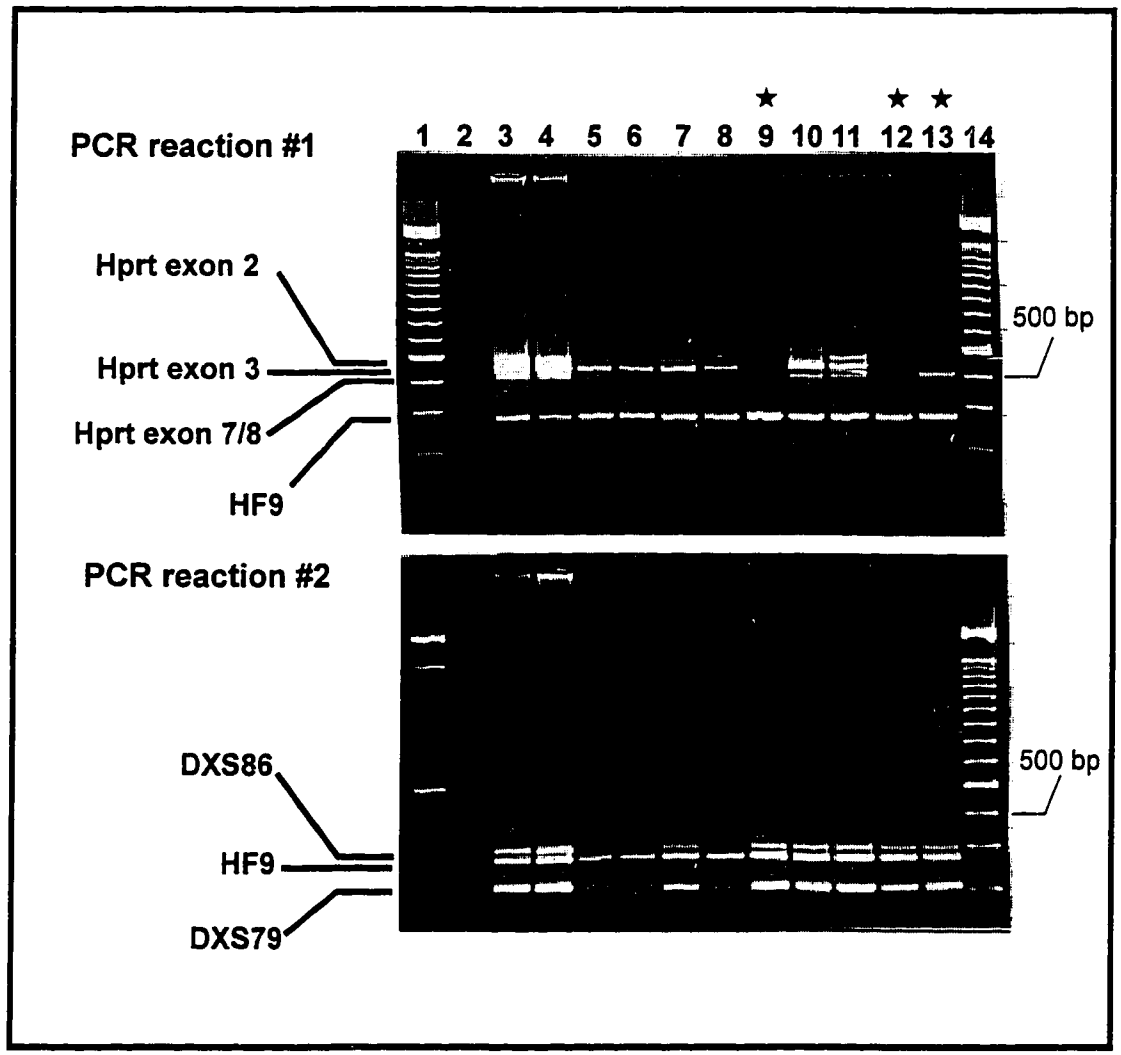
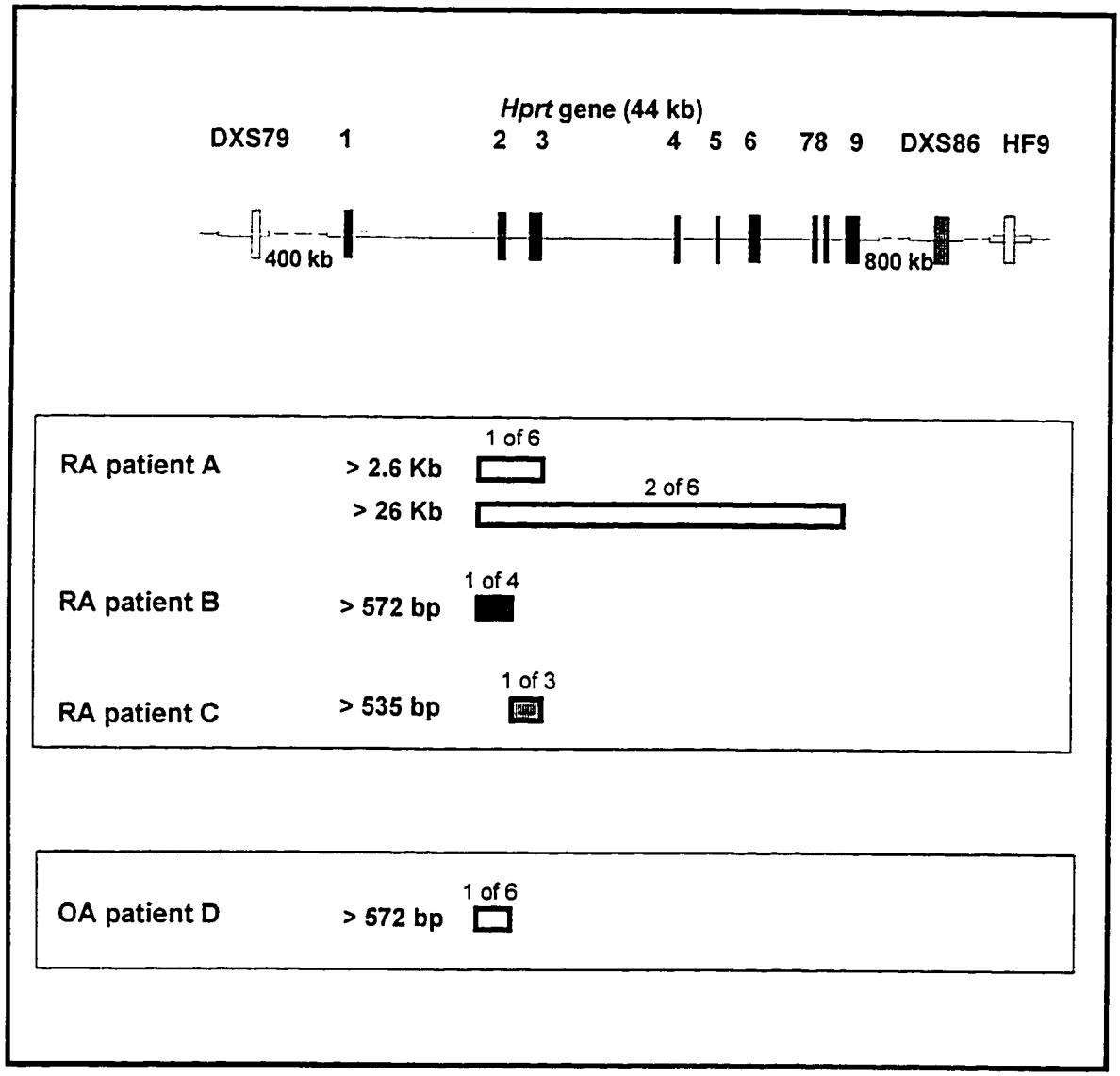

PCR reaction #1
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Hprt exon 2
Hprt exon 3
Hprt exon 7/8
HF9
500 bp
PCR reaction #2
DXS86
HF9
DXS79
500 bp

Figure 2-8. Summary of *hprt* LSD mutations found by MP-PCR in *hprt*$^-$ T cell clones from RA and OA patients. *Hprt*$^-$ clones were isolated from the peripheral blood of RA and OA patients and screened for LSD mutations by MP-PCR. Bars represent the minimum size of *hprt* LSD mutations, since the ends of the mutations were not mapped precisely. LSD mutants were identified as clones that failed to amplify one or more PCR fragments. If two or more PCR bands did not amplify, the intervening DNA sequence is assumed to have been deleted. Numbers correspond to the number of clones with LSD mutations out of the total number of *hprt*$^-$ T cell clones analyzed from that patient. The *hprt* gene and flanking regions are not drawn to scale.


Hprt gene (44 kb)
DXS79
1
2
3
4
5
6
78
9
DXS86
HF9
400 kb
800 kb
RA patient A
> 2.6 Kb
1 of 6
> 26 Kb
2 of 6
RA patient B
> 572 bp
1 of 4
RA patient C
> 535 bp
1 of 3
OA patient D
> 572 bp
1 of 6

Of the RA patients that produced *hprt*$^-$ T cell clones with LSD mutations, Patient A was 54 years of age with active RA, Patient B was 71 years of age with moderately active disease and Patient C was 64 years of age with stable disease status. The duration of RA for Patients A, B and C was 4, 13 and 12 years, respectively. Patient A and C were taking methotrexate at the time of study, while Patient B was not. Patient D, an individual with OA that produced 1 *hprt*$^-$ T cell clone with a LSD mutation, was 50 years of age and was not taking medication. Although the exact duration of disease was not available, OA had been long-standing and was secondary to a congenital displasia of the hip.

2.4 Discussion

2.4.1 Development of the hypotonic shock/micrococcal nuclease protocol

To detect deletion mutations by PCR, the mutant cells examined must represent a single clone and must not be contaminated with non-mutant DNA. Since deletion mutations are defined by the absence of PCR bands, non-mutant DNA could produce a signal that would obscure the presence of a mutation. After 2 weeks of growth in 6-TG, *hprt*$^-$ T cells were successfully isolated from the peripheral blood of RA and OA patients, but represented a mixture of viable *hprt*$^-$ T cells growing in the presence of a large number of dead and dying *hprt*$^+$ PBMCs. PCR analysis revealed that DNA from cells killed by 6-TG remained sufficiently intact to be detectable by PCR. Other investigators that examine genomic alterations in *hprt*$^-$ T cells have dealt with this problem of PCR

background signal in several ways. Many studies expand T cell clones beyond 2 weeks in culture prior to analysis, thereby "diluting" out cells that contribute to PCR background (Vrieling et al. 1992; Kusunoki et al. 1995) and also allowing the use of other techniques that require larger cell numbers, such as Southern blotting (Nicklas et al. 1987; Steingrimsdottir et al. 1993). Another study simply disregarded any *hprt*$^-$ T cell clones that amplified faint and strong PCR bands together, suggesting that the faint bands resulted from amplification of DNA from the feeder cells used, which contained a nearly intact *hprt* gene (Fuscoe et al. 1992). These practices have several disadvantages. T cells have a limited life-span *ex vivo* and in our experience, not every T cell clone remains viable beyond 2 weeks in culture. A bias is therefore introduced in studies that only examine T cell clones that survive for longer periods of time. In addition, the expansion of T cell clones beyond 2 weeks is time and labour-intensive. Therefore, we developed a methodology that allowed the early PCR analysis of *hprt*$^-$ T cell clones while eliminating DNA from cells that contribute to PCR background.

The treatment developed distinguished between viable cells and those killed by 6-TG on the basis of hypotonic shock. Dead and dying *hprt*$^+$ cells were lysed or became permeable while live *hprt*$^-$ cells withstood the hypotonic environment. After tonicity was restored, micrococcal nuclease, a Ca^{2+}-dependent exonuclease/endonuclease, was added to digest the DNA and RNA associated with lysed and permeabilized cells. Digestion could easily be terminated by the addition of EGTA, a Ca^{2+}-specific chelator. Viable *hprt*$^-$ T cells

were then lysed and the *hprt* gene was analysed by PCR for deletion mutations. Since the number of *hprt*$^-$ T cells within each clone was small (< 200 cells), a source of carrier material was needed to ensure the recovery of cells during isolation. TK6-D1 cells were chosen since PCR analysis revealed that they lacked the *hprt* exons and flanking regions under study. This 6-TG-resistant cell line was isolated following exposure to ionizing radiation (Wilkinson, 1997). Although the precise ends of the TK6-D1 mutation have not been identified, MP-PCR analysis suggests that this cell line possess a large *hprt* deletion (>1.2 Mb) that is consistent with mutations caused by radiation (Nicklas et al. 1991). This cell line was also used as a source of irradiated "feeder" cells during T cell cloning and therefore precluded additional PCR background. Sufficient numbers of carrier cells were used to form a visible pellet during the centrifugation steps of the hypotonic shock/micrococcal nuclease procedure; this allowed the detection of a PCR signal following treatment when as few as 20 live T cells were used. Once the hypotonic shock/micrococcal nuclease method was developed, it was subsequently used to generate *hprt*$^-$ T cell DNA from RA and OA patients for PCR analysis.

Although this method was used to analyse the *hprt* gene in T cell clones, the hypotonic shock/micrococcal nuclease treatment may have broader applications. Other selection systems to which this methodology could be applied include 8-azaadenine isolation of aprt$^-$ mutants (Pongsaensook et al. 1997) and the trifluorothymidine selection of tk$^-$ mutants (Spencer et al. 1994).

2.4.2 *Hprt* LSD mutations in T cell clones from arthritis patients

LSD mutations (affecting 1 or more *hprt* exons) were detected in *hprt*$^-$ T cell clones in both RA and OA patients, although the total number of patients analysed was too small for statistical inferences to be drawn. Since RA affects males and females at a ratio of approximately 1:3 (Grossman and Brahn, 1997), during the time of this project only 16 male RA patients and 6 male OA patients were analysed. Of the 54 *hprt*$^-$ RA T cell clones examined, 5 (9.3%) of the clones from 3 individuals had LSD mutations whereas 1 (5.3%) of the 19 *hprt*$^-$ OA T cell clones examined had a similar mutation. The remaining *hprt*$^-$ clones may have arisen from small mutations that were not detectable by PCR or mutations that fell within the *hprt* regions not studied (*hprt* exons 1, 4, 5 and 6). The LSD mutations observed were not due to an artifact of PCR or a reflection of the limited amount of DNA available, since all clones were positive for the HF9 distal marker and the flanking regions DXS79 and DXS86. Although the TK6-D1 cells used as carrier also amplify the HF9 PCR fragment, these cells are negative for the flanking regions. Therefore, there was sufficient DNA from all clones with and without *hprt* LSD mutations to be detected by PCR.

The majority of primers used in MP-PCR analysis of T cell lysates annealed to sequences within introns, and patient allelic variation represented a possible source of false LSD mutations. Therefore, total WBC DNA from each patient was included in MP-PCR analysis to ensure that failure of amplification did not result from patient allelic variation. WBC DNA from every patient

amplified all *hprt* and flanking regions under study, indicating that *hprt*$^-$ T cell deletion mutations did not result from the failure of primers to anneal to intronic sequences.

Of the 3 RA patients that generated *hprt*$^-$ T cell clones with deletion mutations, Patient A produced several clones with deletion of all *hprt* exons examined. Six *hprt*$^-$ T cell clones from this patient were examined; 2 lacked *hprt* exons 2, 3 and 7/8 while a third lacked *hprt* exons 2 and 3. Although the exact ends of these mutations were not mapped, these represent deletions of > 26 Kb and > 2.6 Kb, respectively. The possibility exists that the 2 clones with deletions > 26 Kb may have identical mutations and are siblings. Although a theoretical possibility, this seems unlikely since they were cloned from the peripheral blood. T cell receptor (TCR) expression is often used to distinguish between T cell clones; clones having identical TCRs are considered sibs whereas clones expressing different TCRs are not clonally related. The variable region of TCR β chain mRNA is usually amplified by RT-PCR and sequenced. However, many variable region families exist (at least 24 to date) and clones are screened for each separately. This technique is complex, requiring large numbers of T cells since multiple RT-PCR reactions are needed, and was therefore beyond the scope of this project. However, in studies of healthy subjects, 88-89 % of the *hprt*$^-$ T cell clones examined were not clonally related (Nicklas et al. 1987; Hou et al. 1993). The phenomenon of clonal expansion of RA T cells has been previously addressed. T cell clones from SF and synovial tissue have been

analysed for specific TCR usage, with the hope that the subset(s) of T cells that may participate in RA initiation and/or progression could be identified. Although there is some evidence that certain TCRs are over represented in RA joints, results are not consistent from patient to patient (Broker et al. 1993; Sakkas et al. 1994; Wagner et al. 1998). Therefore, there is evidence that clonal expansion can occur in the inflamed joints of RA patients but further studies are needed to determine what percentage of T cells with mutations at the *hprt* locus arise due to clonal expansion versus independent mutagenic events.

DNA damage, and therefore *hprt* mutations, can potentially be caused by drugs used in the treatment of RA. One such drug is methotrexate, an inhibitor of dihydrofolate reductase (DHFR) that interferes with DNA synthesis by depleting cells of thymidylate and purine nucleotides. *In vitro*, methotrexate has been found to increase DNA strand breaks in human HL-60 cells (Mietz et al. 1992). However, although cancers of the hematopoietic system occur more frequently in RA patients than in individuals without RA, in many studies these malignancies are not found more frequently in RA patients receiving methotrexate compared to RA patients that are not treated with this drug (Hoffmeister, 1983; Williams et al. 1996; Bologna et al. 1997). In addition, although 2 of the 4 patients with LSD mutations were being treated with methotrexate, 2 were not. Therefore, factors other than medication are likely to be responsible for the observed *hprt* mutations.

T cells from OA patients were also screened for *hprt* LSD mutations for

comparison with RA patients. *Hprt*$^-$ T cell clones from normal individuals were not initially examined for several reasons. An earlier study from our lab shows that the *hprt* MF of normal individuals is low (1 in 1 × 10^6 cells) and therefore obtaining clones mutated at this locus requires plating very large numbers of cells (Cannons et al. 1998). OA is a joint disease often associated with wear of the joint over time, and therefore the age of OA patients tends to be higher than that of RA patients. However, this age difference did not reach statistical significance in our study (58.3 ± 8.1 years (RA) vs 59.0 ± 12.4 (OA)). Therefore, OA *hprt*$^-$ T cells, presumably exposed to lower levels of NO, were also screened for deletion mutations and 1 LSD mutant was isolated. Few conclusions can be drawn because of the small number of patients examined thus far.

The occurrence of large-scale mutations in *hprt*$^-$ T cells from normal individuals must be established to determine conclusively if these mutational events arise more frequently in RA. Studies of *hprt*$^-$ T cell clones from healthy males have been conducted in other laboratories to characterize the spectrum of mutations that occur spontaneously. Four studies have previously examined peripheral T cell clones from healthy males for deletion of 1 or more *hprt* exons by MP-PCR. Fuscoe et al. (Fuscoe et al. 1992) screened a total of 218 *hprt*$^-$ clones from 6 normal males by MP-PCR using K-ras as a positive internal control. Of the 218 *hprt*$^-$ T cell clones, 16 (7%) arose due to a total gene deletion whereas 1 clone had a partial gene deletion, retaining *hprt* exons 1-4. Österholm et al. (Osterholm et al. 1995) studied a total of 156 *hprt*$^-$ T cell clones

from 14 non-smoking males (age 36.1 ± 8.9 years) by MP-PCR using the NAT2 gene as an internal PCR control. Five (3.2%) of the clones showed deletions of 1 or more *hprt* exons; 1 clone lacked the entire *hprt* gene, 1 was negative for part of exon 2, 1 was missing part of exon 3, another lacked all of exon 3 and 1 was negative for part of exon 4. In the study of Burkhart and Jones (Burkhart-Schultz and Jones, 1997), 27 (12%) of 217 *hprt*$^-$ T cell clones examined were missing 1 or more *hprt* exons and more deletions occurred in *hprt* exon 2 than in any other exon. Huttner et al. (Huttner and Holzapfel, 1996) studied 41 male blood donors for *hprt* mutations and found deletion mutations of 1 or more exons in 6% of all clones examined. From these studies it would appear that the percentage of LSD mutations that occur in normal individuals ranges from 3.2% to 12%.

Of the *hprt*$^-$ T cell clones from RA and OA patients examined during the course of this project, 9.3% and 5.3% arose due to the deletion of 1 or more *hprt* exons, respectively. Both of these numbers fall within the percentage of *hprt* LSD mutations that arise in healthy individuals, and do not differ significantly from each other. However, since not all regions of the *hprt* gene were examined by MP-PCR, the possibility exists that LSD mutations affecting *hprt* exons 1, 4, 5, 6 or 9 occurred in some T cell clones and were simply not detected. Alternatively, many *hprt*$^-$ T cells may have arisen due to mutagenic events not seen by MP-PCR, such as base-pair changes or small deletions. Among the *hprt*$^-$ T cell clones that had LSD mutations, all 6 were missing *hprt* exon 2 and/or 3. Other studies have noted that this region is often deleted (Osterholm et al.

1995; Burkhart-Schultz and Jones, 1997), suggesting that a mutational "hotspot" exists in this area. In addition, 2 *hprt*$^-$ T cell clones from an RA patient studied here lacked the entire *hprt* gene but not the flanking regions DXS79 and DXS86, consistent with mutations that are tolerated at this locus (Fuscoe et al. 1992; Morris et al. 1993).

Not every mutagen that causes an increase in *hprt* MF produces a distinct pattern of mutations. For example, although the *hprt* MF of peripheral T cells was shown to be elevated significantly by 36% in smokers when compared to non-smoking controls, no difference in the pattern of *hprt* mutations was found in these 2 populations (Vrieling et al. 1992). In contrast, exposure to ionizing radiation produces both an increase in peripheral T cell *hprt* MF and twice the expected number of *hprt* LSD mutations (Da Cruz and Glickman, 1997).

2.4.3 Summary and future work

Hprt$^-$ T cell clones from RA and OA patients were screened for LSD mutations by MP-PCR following isolation from *hprt*$^+$ cells by means of 6-TG selection and a newly-developed hypotonic shock/micrococcal nuclease treatment. Of 54 *hprt*$^-$ RA T cell clones and 19 *hprt*$^-$ OA T cell clones examined, 5 and 1 had deletions of 1 or more *hprt* exons, respectively. The percentages of RA and OA T cells with deletion mutations (9.3% and 5.3%) were not statistically different and both fell within the range of *hprt* LSD mutations found in healthy individuals. *Hprt* mutations detected included loss of *hprt* exons

2, 3 and 7/8, as well as deletions of *hprt* exon 2 or 3 or both; all are mutations previously reported in the literature. The issue of *hprt*$^-$ T cell clonallity was not addressed in this study. T cells from the synovium were not examined since they did not generally survive 2 weeks in culture and therefore did not yield a sufficient number of cells for study. In the future however, it would be of interest to screen these clones for LSD mutations by MP-PCR. Since evidence suggests that a mutagenic environment exists within the inflamed joints of RA patients, it is possible that LSD mutations may be more prevalent in synovial T cells than in peripheral T cells.

Current work is being conducted to screen a greater number of *hprt*$^-$ T cell clones from RA and OA patients and from normal age and sex-matched controls. This will give us information regarding the nature of the mutagenic events that occur in RA and may have led to the increase in T cell *hprt* MF observed in an earlier study. In addition to analysing the *hprt* gene at the genomic level, RT-PCR amplification of *hprt* mRNA and sequencing of the resulting cDNA would allow the characterization of small mutations not detectable by PCR. A new method of analysing TCR mRNA using consensus primers is being developed. This may allow the amplification of *hprt*$^-$ T cell clone TCR mRNA in one RT-PCR reaction rather than many. Information regarding the relationships between *hprt*$^-$ T cell clones will provide information on clonal expansion within the joints of RA patients and the number of *hprt*$^-$ T cells that arise from distinct mutational events.

Chapter 3: *Hprt* mutation frequency and deletion mutations in human cell lines exposed to NO-donating drugs

3.1 Introduction

3.1.1 Nitric oxide

Nitric oxide (NO) is a reactive, short-lived free radical capable of diffusing across cell membranes and is produced by many cell types. This molecule is generated from L-arginine and molecular oxygen by nitric oxide synthase (NOS). Neuronal NOS (nNOS) is expressed constitutively by cells of the nervous system and is involved in neuronal signalling, while endothelial NOS (eNOS) is expressed by the endothelium of the blood vessels and contributes to the maintenance of vascular tone. Both of these enzymes are Ca^{2+}-dependent and produce low concentrations (pmolar) of NO. The inducible form of NOS (iNOS) is expressed by immune cells such as macrophages and neutrophils and is responsible for many of the antimicrobial effects of these cells. iNOS is a Ca^{2+}-independent enzyme and is capable of producing large quantities of NO (μmolar) relative to the constitutively expressed NOS isoforms. Although NO is beneficial with respect to normal physiological responses and host defence, it is often produced in large amounts in inflammatory diseases such as rheumatoid arthritis (RA) and is thought to contribute to pathogenesis (McInnes et al. 1996; Wigand et al. 1997).

3.1.2 Nitric oxide and genetic damage

Previous studies have shown that NO can cause genetic damage *in vitro*. For example, NO was shown to be mutagenic when bacteria were incubated with aqueous NO or donors of NO such as nitroglycerin and spermine-NO (Wink et al. 1991). Analysis of the *his*G46 gene in these bacteria indicated that the majority of mutations arose due to DNA deaminations. In another study, incubation of plasmid DNA containing the *supF* gene with saturated aqueous NO produced a mutation rate that was 44-fold greater than spontaneously occurring mutations in human Ad293 cells (Routledge et al. 1993). The majority of point mutations analysed were C to T transitions. NO is thought to cause these transition mutations by deaminating cytosine to uracil that, when excised, is replaced with adenine (Wink et al. 1991). Exposure to aqueous NO was also found to increase the mutation frequency (MF) of the hypoxanthine phosphoribosyltransferase (*hprt*) gene in TK6 cells and to increase the number of DNA strand breaks (Nguyen et al. 1992). Although NO has been shown to induce genetic damage in the form of DNA deaminations, its ability to induce changes that exceed small mutational events has not been addressed to date. Observations within our lab, however, suggested that NO may be capable of producing large-scale deletion (LSD) mutations at the *hprt* locus in a mouse tumour cell line (Sandhu and Birnboim, 1997). LSD mutations are defined as mutations that result in the deletion of several hundred to several thousand base-pairs (see Chapter 1, section 1.3).

3.1.3 Nitric oxide, inflammation and genetic damage

Evidence suggests that NO is capable of inducing genetic damage *in vivo*. RA is an autoimmune disease characterized by chronic inflammation of the synovial joints and is associated with elevated serum and synovial fluid (SF) levels of nitrite, a more stable product of NO formation (Ueki et al. 1996). Levels of 3-nitrotyrosine are high in the serum and SF of RA patients but not in healthy controls (Kaur and Halliwell, 1994), indicating that NO-mediated oxidative damage occurs in the joints of RA patients. DNA single-strand breaks are found more frequently in mononuclear cells from RA patients than in mononuclear cells from controls (Bhusate et al. 1992). In macrophages stimulated to produce large quantities of NO, the MF of the *hprt* marker gene is elevated 15-fold above background, indicating that NO is capable of causing genetic damage (Zhuang et al. 1998). The *hprt* MF is also elevated in many inflammatory diseases that are associated with high levels of NO, including RA (Cannons et al. 1998), multiple sclerosis (Sriram, 1994) and systemic lupus erythematosus (Dawisha et al. 1994; Theocharis et al. 1995). Many inflammatory disorders associated with bacterial or parasitic infection and are also characterized by high levels of local NO production. In many cases, the risk of developing cancer at the site of inflammation is elevated, suggesting that NO can cause carcinogenic gene alterations (Ohshima and Bartsch, 1994). For example, *Helicobacter pylori* infection of the stomach induces a large production of NO (Tsuji et al. 1996) and causes gastric ulcers. These individuals are more likely to develop stomach

cancer than individuals without gastric ulcers (Kuipers, 1997). Individuals with chronic inflammatory diseases of the bowel such as Crohn's disease and ulcerative colitis, have an increased risk of developing colorectal cancer at the site of inflammation (Choi and Zelig, 1994). Chronic Hepatitis B infection of the liver is associated with a 200-fold increased risk of developing hepatocellular carcinoma (Milich et al. 1994).

3.1.4 Cell lines and NO-donating drugs

To address the question of whether NO is capable of inducing mutations of significant size, two human cell lines were exposed to NO-donating drugs *in vitro*. The two cell lines chosen to determine the mutagenicity of NO were TK6 and WIL2-NS. These two B lymphoblastoid cell lines were isolated from the spleen of a male patient and are karyotypically normal. While the TK6 cell line has been shown to express normal p53 protein, WIL2-NS cells over express mutant p53 protein (Zhen et al. 1995). When treated with radiation and other mutagens, WIL2-NS cells undergo apoptosis more slowly than TK6 cells (Greenwood et al. 1998).

Two NO-donating drugs were chosen for this study; nitroglycerin (NG) and sodium nitroprusside (SNP). NG is employed in the treatment of angina pectoris and SNP is used to reduce blood pressure in cases of hypertensive emergency. Both drugs require metabolism to donate NO, although the mechanisms through which this is accomplished are not fully elucidated.

3.1.5 Hypothesis

The NO donated by NG and SNP in cultures of TK6 and WIL2-NS cells is capable of causing genetic damage and is therefore expected to increase the *hprt* MF of these cells. The DNA damage induced by NO and resulting reactive oxygen and nitrogen species may produce characteristic mutations in the *hprt* gene, including LSD mutations.

3.1.6 Specific objectives

a) To determine if NO is capable of inducing DNA mutations *in vitro*, by assessing the *hprt* mutation frequency (MF) of TK6 and WIL2-NS cells treated with the NO-donating drugs NG and SNP.

b) To characterize the mutational events that occur at the *hprt* locus in drug-treated cells by analysing *hprt*$^-$ clones for LSD mutations by multiplex-PCR (MP-PCR).

Significance

This study is novel in that it represents the first attempt to determine if NO is capable of causing genetic damage that exceeds small mutational events such as DNA deaminations. Such mutations may form the basis of a mutational "fingerprint" that can be assigned to NO and allow the assessment of its genotoxic role in inflammatory diseases such as RA. Such information will also

contribute to the understanding of the mutational role of NO in the development of cancer.

3.2 Materials and methods

3.2.1 Metabolism of NG and SNP by TK6 and WIL2-NS cells

The ability of TK6 (ATCC CRL 8015) and WIL2-NS (ATCC CRL 8155) cells to metabolize NG or SNP and release NO was determined by measuring nitrite accumulation in drug-treated cultures with the colorimetric Griess reaction (Appendix V). For all experiments involving NO-donating drugs, fresh NG was obtained within 24 hours of treatment and filtered through a 0.2 µm membrane. Similarly, stock 50 mM SNP was prepared in RPMI 1640 medium at the time of treatment and filter-sterilized. Both NG and SNP are light sensitive and therefore were handled in a darkened laminar flow hood. Duplicate 1 ml cultures containing no cells or $2.5x10^5$ cells/ml ± 400 µM NG or 0.5 mM SNP were set up in 12-well plates for each time point (0, 4, 8 and 24 hours). Since phenol red interferes with the colorimetric Griess reaction, all samples were maintained in RPMI 1640 phenol red-free medium supplemented with 10% Fetal Calf Serum (FCS) and 2 mM L-glutamine at 37°C and 5% CO_2. At each time point, samples were transferred to 1.5 ml microfuge tubes and centrifuged at 1,500 rpm (150 x g) for 5 min. Supernatants were collected and analysed for nitrite formation by means of the Griess reaction. Equal volumes of Griess Reagent A (0.1% N-(1-napthyl) ethylene diamine dihydrochloride in ddH_2O) and Griess Reagent B (1%

p-aminobenzenesulfanilamide in 5% phosphoric acid) were combined and 800 µl of this solution was added to sterile 1.5 ml microfuge tubes. To the tubes, 200 µl of supernatant was added and samples were left in the dark at room temperature for 10 min. Sample absorbance at λ=543 nm was measured using a Perkin Elmer Lambda 4 UV Vis spectrophotometer. Nitrite concentrations were determined with reference to a standard curve generated using freshly prepared sodium nitrite.

3.2.2 Determining the cytotoxic effects of NG and SNP on TK6 and WIL2-NS cells

The cytotoxic effects of NG and SNP on TK6 and WIL2-NS cells were assessed by measuring the plating efficiency (PE) of cultures with and without drug treatment. Cells were plated in 10 cm tissue culture dishes containing $5x10^5$ cells/ml in 10 ml RPMI 1640 medium with 10% FCS, 2 mM L-glutamine and either no drug or increasing concentrations of NG or SNP. Cultures were incubated at 37°C and 5% CO_2 for 24 hours. Drug treatment was terminated by transferring the cultures to sterile 15 ml tubes, centrifuging for 5 min at 700 rpm (70 x g) and removing the supernatant. Cell pellets were washed with 5 ml phosphate buffered saline (PBS, 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4) and centrifuged again. PBS was removed, the cell pellet was brought up in RPMI 1640 and viable cell numbers were determined using trypan blue exclusion. Cells were plated in 200 µl RPMI 1640 medium containing 10%

FCS and 2 mM L-glutamine in triplicate 96-well plates at a density of either 0.25, 0.5, 1 or 2 cells per well. Plates were incubated at 37°C for 10 days and then examined by light microscopy for growth. The PE of each culture was determined, based on a Poisson distribution of clonable cells (Albertini et al. 1982), using the following formula:

PE = [-ln (fraction of negative wells) / number of cells per well] x 100

The average PE of control cultures was assigned a value of 100 and the relative PE of each treatment group was calculated as a percentage of the control value.

2.2.3 Treatment of TK6 and WIL2-NS cells with NG or SNP

To ensure that spontaneous *hprt* mutations were eliminated prior to all experiments, stock TK6 or WIL2-NS cells were thawed each time and placed immediately into medium selective for *hprt*$^+$ cells. Cultures were grown for 7 days in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine and supplemented with $1x10^{-5}$ M hypoxanthine, $1x10^{-6}$ M aminopterin and $1x10^{-5}$ M thymidine (HAT). Aminopterin, a folate analogue, selects for *hprt*$^+$ cells by poisoning the cell's *de novo* purine synthesis. Cells therefore rely on the *hprt*-dependent purine salvage pathway to obtain purine nucleotides for DNA synthesis; *hprt*$^+$ cells survive in HAT by converting hypoxanthine to its purine nucleotide while *hprt*$^-$ cells are incapable of producing purine nucleotides and fail to replicate. Because direct removal of cells from HAT had previously been shown to be toxic to cells due to the action of residual aminopterin, cultures were

removed from HAT-supplemented medium and grown for an additional 48 hours in medium supplemented with $1x10^{-5}$ M hypoxanthine and $1x10^{-5}$ M thymidine (HT) alone. Cells were then removed from HT-supplemented medium and washed once with PBS. Cells were counted by hemocytometer using trypan blue exclusion as a measure of viability, and plated in triplicate 10 cm tissue culture dishes containing $5x10^5$ viable cells/ml in 10 ml of medium supplemented with filter-sterilized NG or SNP. Since SNP metabolism releases cyanide (CN), in one experiment, cultures were exposed to 4 mM NaCN as a control. To determine the spontaneous *hprt* MF during each experiment, another set of triplicate cultures was maintained without drug. A positive control was included in each experiment and consisted of irradiated cultures. WIL2-NS cells were exposed to 400 rads using the X-ray source at the Ottawa Regional Cancer Centre, Cancer Research Group. TK6 cells have previously been shown to be sensitive to killing by radiation (Schwartz et al. 1995) and therefore were exposed to only 70 rads. After 24 hours of drug exposure, or 24 hours after irradiation, cells were removed from their treatment dishes, washed twice with PBS and replated in medium without drug. Cells were maintained as individual cultures for 8 days to allow the expression of the *hprt*$^-$ phenotype and were subcultured by dilution at a ratio of 1:5, approximately every 2 days. At the end of this period, cultures were challenged with 6-TG to select for *hprt*$^-$ cells.

3.2.4 Photo-degradation (NO-exhaustion) of NG and SNP

To determine if *hprt* mutations were induced by the NO released from NG and SNP, in one experiment these drugs were NO-depleted by exposure to light before being used to treat WIL2-NS cultures. Stock 10 mM SNP prepared in PBS, and stock 5 mg/ml NG, were transferred to clear 15 ml screw-cap Sarstedt tubes. Both drugs were exposed to room fluorescent light at room temperature for 7 days.

To confirm that these light-treated drugs no longer produced high levels of NO when metabolized, nitrite levels were measured by the Griess reaction in WIL2-NS cultures treated with 800 μM NG or 2 mM SNP before and after Photo-degradation. The *hprt* MF of WIL2-NS cells exposed to these NO-depleted drugs for 24 hours was then determined as described below.

3.2.5 Detection of *hprt*$^-$ TK6 and WIL2-NS cells

Hprt$^-$ cells were isolated 8 days after NO-donating drug or radiation treatment by selecting for *hprt*$^-$ cells in RPMI 1640 medium supplemented with 50 μM 6-TG. TK6 *hprt*$^-$ clones were isolated by limiting dilution in 96-well plates. WIL2-NS cells though, tended to aggregate giving rise to the possibility that *hprt*$^-$ mutants were being killed through metabolic cooperation. This situation is of concern when cells are in direct contact with one another; toxic 6-TG metabolites produced by *hprt*$^+$ cells can diffuse through cell membranes to kill off nearby *hprt*$^-$ cells. *Hprt*$^-$ WIL2-NS cells were therefore cloned in semi-solid

methocellulose (MethoCult)/RPMI 1640 medium containing 50 µM 6-TG.

3.2.5.1 Isolation of hprt⁻ TK6 clones by limiting dilution

Viable cells were counted by hemocytometer 8 days post-treatment using trypan blue exclusion, and plated at $1x10^4$ viable cells per well in triplicate 96-well plates in a total of 100 µl RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 50 µM 6-TG. To determine the PE of cultures at the time of 6-TG challenge, cells from each individual culture were plated at 0.5 cells per well in RPMI 1640 medium without 6-TG, in triplicate 96-well plates.

After 14 days, selection and PE plates were examined by light microscopy for growth. *Hprt⁻* clones from 6-TG selection plates were selected, transferred to 10 cm dishes and maintained in 10 ml of 50 µM 6-TG-supplemented medium until cell numbers were sufficient for DNA extraction (approximately 1 week). At this time, $1x10^6$ cells were removed and stored at -100°C in RPMI 1640 medium containing 20% FCS and 10% DMSO. DNA was extracted from the remaining cells according to the methodology described in Appendix II. In some cases, clones were further expanded and RNA was isolated according to the protocol described in Appendix V.

3.2.5.2 Isolation of hprt⁻ WIL2-NS clones in methocellulose

Viable cells were counted by trypan blue exclusion 8 days post-treatment and plated in triplicate wells at $1x10^6$ viable cells per well in 6-well plates. Each

well contained 2 ml of semi-solid medium with a final concentration of 0.6% methocellulose, 10% FCS, 2 mM L-glutamine and 50 μM 6-TG in RPMI 1640. To determine PE, 200 cells from each culture were plated in triplicate wells using 12-well plates. Each well contained 1 ml of semi-solid medium described above but without 6-TG. After 11 days, the number of colonies per well was determined for both selection and PE plates. *Hprt*$^-$ WIL2-NS colonies were isolated from selection plates and maintained in RPMI 1640 medium supplemented with 50 μM 6-TG until the time of DNA extraction. $1x10^6$ cells from each clone were also stored at -100°C as described above.

3.2.6 Determining TK6 and WIL2-NS *hprt* mutation frequencies

The *hprt* mutation frequency (MF) of TK6 cultures was determined from 96-well selection plates using the following formula:

MF = [-ln (fraction of negative wells) / number of cells per well]

The plating efficiency (PE) of TK6 cultures was determined by examination of 96-well PE plates and calculated using the formula described in section 3.2.2. The *hprt* MF of WIL2-NS cultures was obtained by scoring the number of *hprt*$^-$ colonies in 6-TG-supplemented methocellulose per 1×10^6 cells originally plated. The PE of WIL2-NS cultures was determined by counting the number of colonies per well in PE plates and calculated as the number of colonies per 200 cells originally plated.

The average PE of each individual culture was determined along with the

average *hprt* MF per $1x10^6$ cells plated. This MF value was adjusted for differences in PE between treatment groups by dividing the MF by the PE for each culture. This value is the corrected MF (cMF).

2.2.7 MP-PCR analysis of TK6 and WIL2-NS *hprt*$^-$ clones

After DNA was extracted from *hprt*$^-$ clones, DNA was quantified using the DABA method (Appendix III). From each clone, 100 ng of DNA was used in one MP-PCR reaction to amplify *hprt* exons 2 and 3 (MP-PCR reaction #1, see Section 2.2.6). Another 100 ng of DNA was used to amplify *hprt* exon 9 and 2 regions flanking the *hprt* gene, DXS79 and DXS86 (MP-PCR reaction #2). MP-PCR reaction #2 was performed as described as in Section 2.2.6 with the exception that *hprt* exon 9 was amplified instead of *hprt* exon 7/8. Within both MP-PCR reactions, a portion of the HF9 gene was co-amplified and served as a positive internal control. MP-PCR primer sequences are given in Appendix IV. PCR products were separated on a 2% NuSieve+1.5% agarose gel. Gels were stained with 1 µg/ml ethidium bromide and visualized under UV light.

2.2.8 Reverse-transcriptase-PCR analysis of TK6 mRNA

To detect *hprt* mutations that were too small to observe by MP-PCR, *hprt* mRNA from TK6 *hprt*$^-$ clones was examined by a reverse-transcriptase-PCR (RT-PCR) procedure. It was expected that, in some cases, *hprt* mRNA would not be detected. To ensure that such a lack of amplification was not due to

technical problems, first strand synthesis was primed with an oligo dT primer to make cDNA for the amplification of both *hprt* mRNA and a portion of thymidylate synthase (TS) mRNA as an internal control. Total RNA was isolated from TK6 *hprt*$^-$ clones, quantified according to the protocol described in Appendix VI, and 3 µg was used to synthesize cDNA. In sterile 0.6 ml PCR tubes, total RNA was combined with 30 pmol oligo $dT_{12\text{-}18}$ primer; sterile ddH_2O treated with diethyl procarbonate (DEPC) was added to a total volume of 10 µl. After 1 drop of silicone oil was added, tubes were incubated at 65°C for 5 min and then placed on ice. To the samples, 4 µl of 5x first strand buffer (375 mM KCl, 0.1 mM CDTA, 250 mM Tris-HCl, pH 8.3) and 2 µl of 10 mM dNTPs was added. Samples were incubated at 50°C for 1- 2 min, after which 1 µl of 60 mM $MgCl_2$, 2 µl of 0.1 M dithiolthrietol and 1 µl of SuperScript reverse transcriptase (RT from GibcoBRL) was added. Negative controls were included to demonstrate the absence of chromosomal DNA, and consisted of 3 µg total wild-type TK6 RNA, treated as above but without RT. cDNA was synthesized by incubating samples at 50°C for 60 min. From this, 2 µl was used as a template for the PCR amplification of *hprt* and TS cDNA. Primer sequences are given in Appendix IV. PCR reactions were performed in sterile 0.6 ml PCR tubes in a total volume of 25 µl and consisted of 2 µl cDNA, 10% DMSO, 400 µM of each dNTP, 10 pmol of each *hprt* mRNA primer, 5 pmol of each TS primer, 2 units AmpliTaq DNA polymerase and PCR buffer with a final concentration of 4 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 5 mM 2-mercaptoethanol, 6.8 µM EDTA and 65 mM Tris-HCl, pH 8.8.

One drop of silicone oil was added and the tubes were placed in an MJ Research Programmable Thermal Controller. The following amplification conditions were employed: 4 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 56°C and 1 min at 72°C. After the final round of amplification, samples were held for an additional 5 min at 72°C. RT-PCR products were separated on a 2% agarose gel that was stained with 1 µg/ml ethidium bromide and visualized under UV light.

3.2.9 Interpretation of data and statistical methods

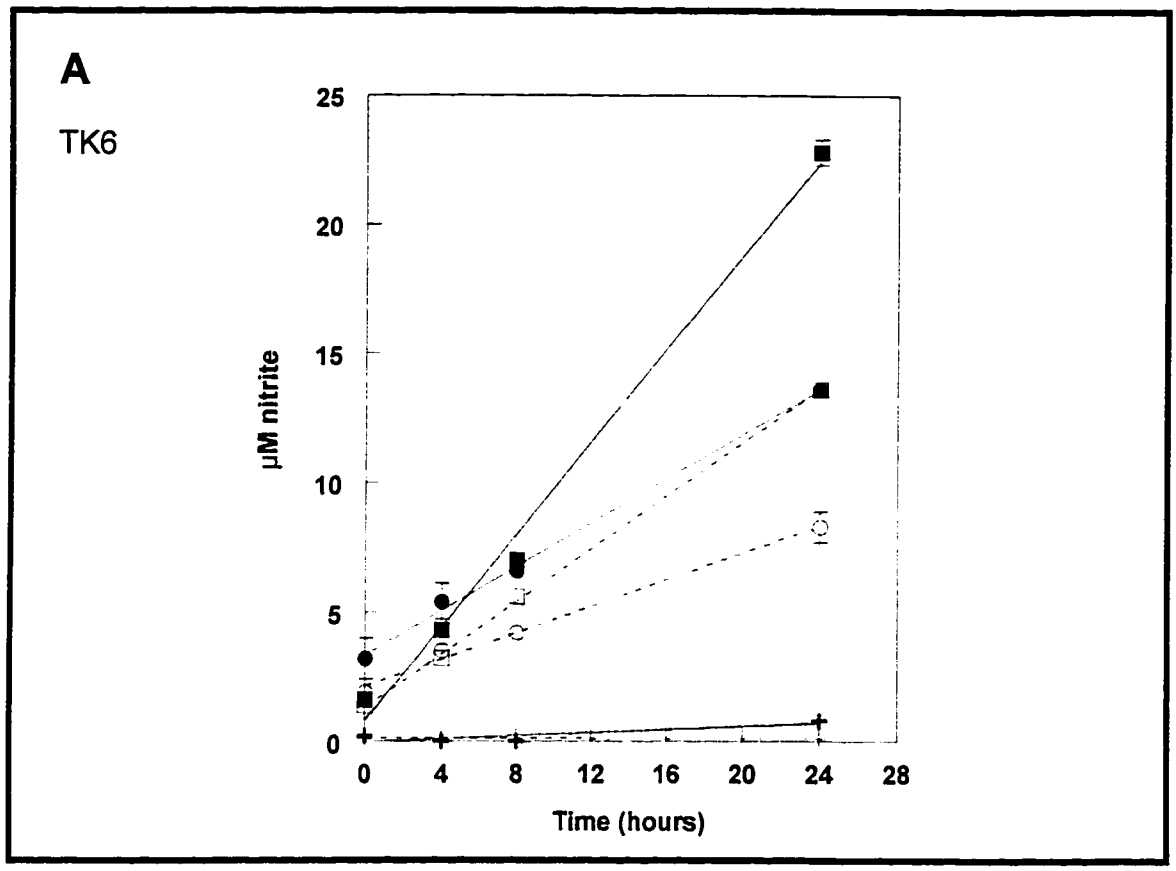
Statistical differences between the *hprt* MF of spontaneous and drug-treated cultures were determined using one-way analysis of variance (ANOVA) Dunnett's post-test. Differences in the number *hprt* LSD mutations occurring in spontaneous versus drug-treated cultures were also analysed by one-way ANOVA.

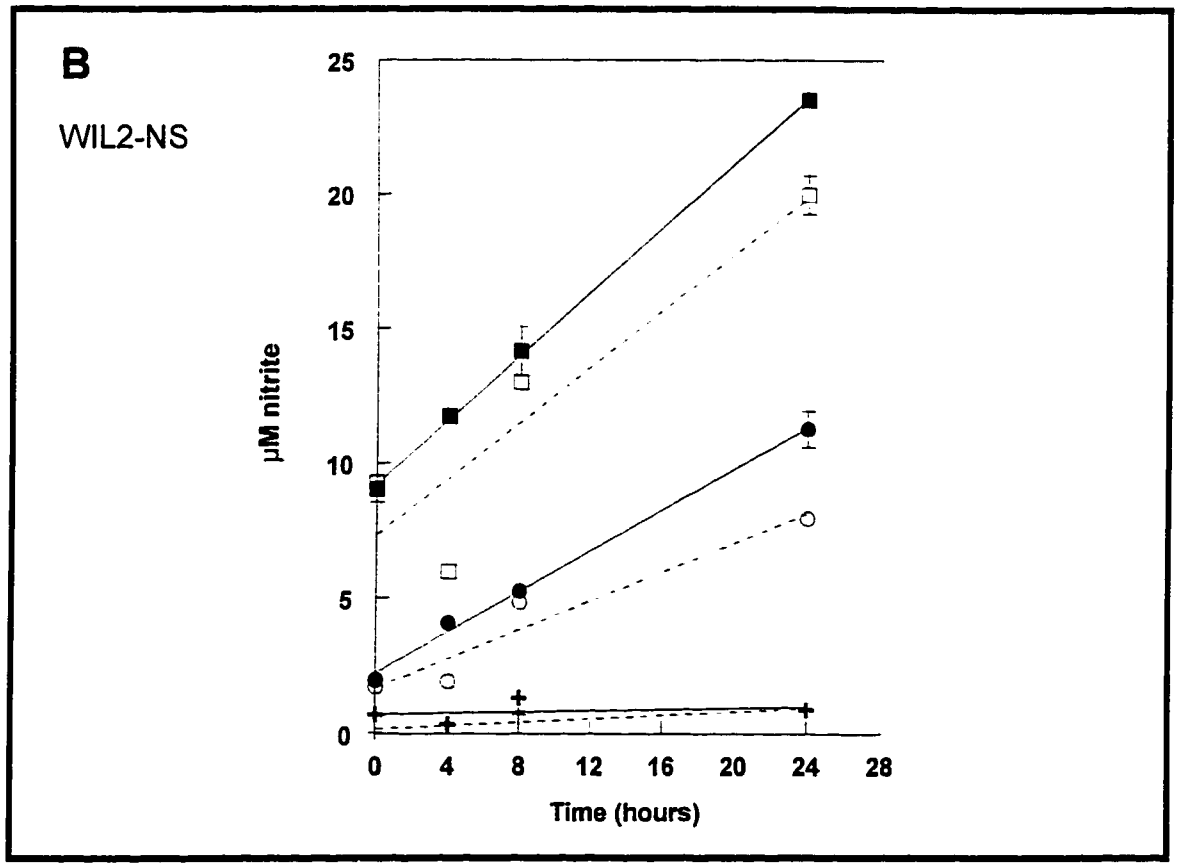
3.3 Results

3.3.1 Metabolism of NG and SNP by TK6 and WIL2-NS cells

Both NG and SNP caused an increase in nitrite concentrations in TK6 and WIL2-NS cultures over 24 hours, indicating that these cells were capable of metabolizing the two drugs with the release of NO (Figure 3-1). Both NG and SNP also produced a rise in nitrite levels in the absence of cells (open symbols), indicating that the spontaneous release of NO contributed significantly to the

Figure 3-1. Supernatant nitrite levels of TK6 and WIL2-NS cultures treated with 0.5 mM SNP or 400 μM NG. Supernatant nitrite levels of TK6 cultures (A) and WIL2-NS cultures (B) were assessed using the Griess reaction. Cultures with 2.5×10^5 cells per ml (closed symbols) or without cells (open symbols) were incubated for various time points with 0.5 mM SNP (circles) or with 400 μM NG (squares) in phenol red free RPMI 1640 medium. Cultures without any drug treatment (pluses) were included as negative controls. Data points represent the mean of duplicate samples ± SEM. Error bars, when not shown, appear within the symbol.


A
TK6
µM nitrite
25
20
15
10
5
0
0
4
8
12
16
20
24
28
Time (hours)

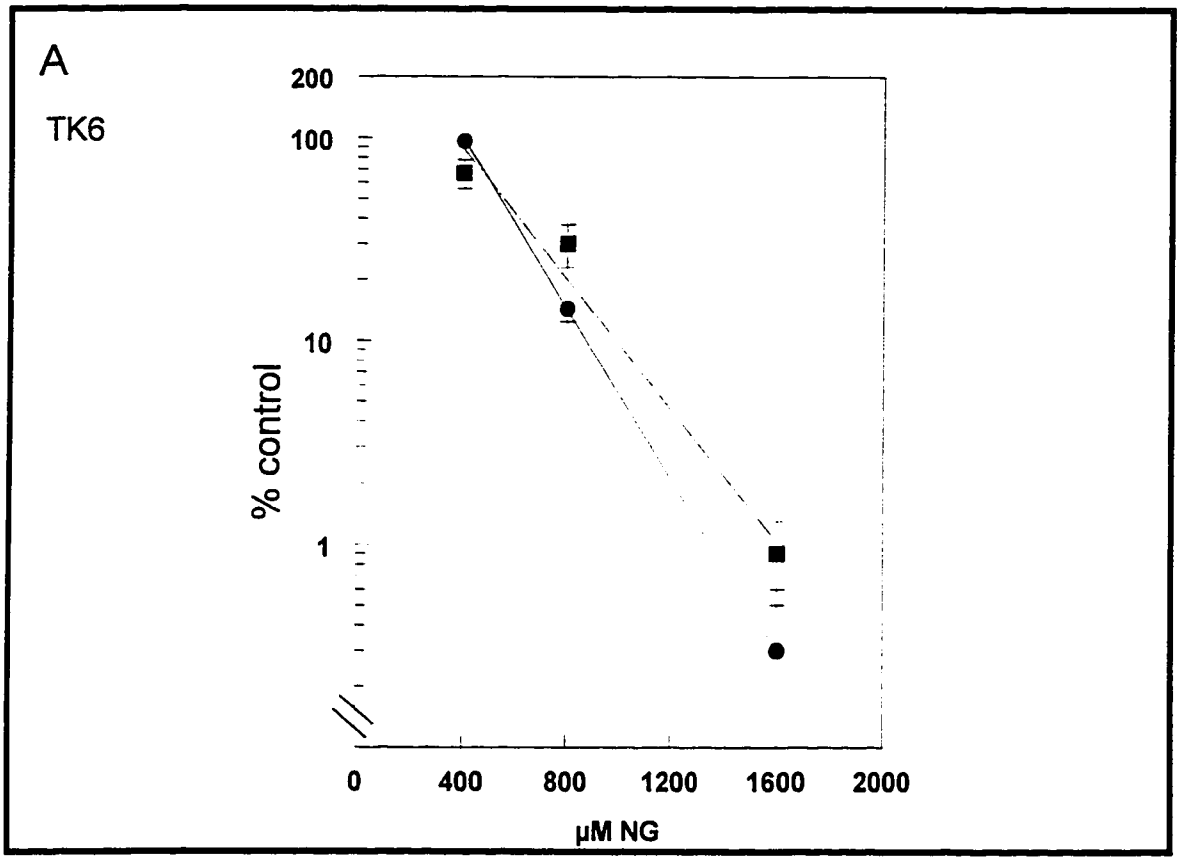

B
WIL2-NS
µM nitrite
25
20
15
10
5
0
0
4
8
12
16
20
24
28
Time (hours)

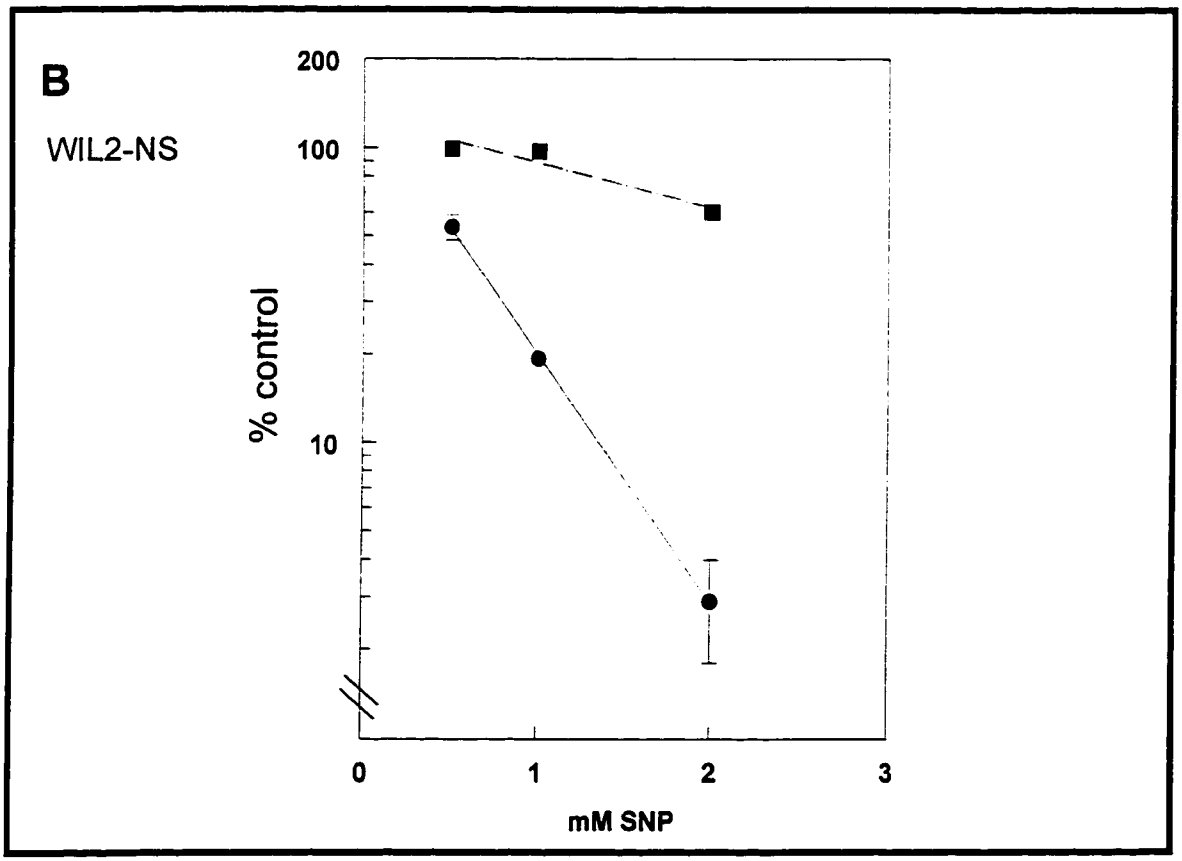
rise in nitrite levels. However, after 24 hours, nitrite concentrations were found to be significantly higher (2-tailed t-test) in drug-treated cultures that included cells (closed symbols) than in those without cells. After 24 hours, nitrite concentrations of TK6 and WIL2-NS cultures treated with 400 μM NG were similar (22.0 ± 0.5 μM and 23.5 ± 0.3 μM , respectively) as were TK6 and WIL2-NS cultures treated with 0.5 mM SNP (13.6 ± 0.3 μM and 11.3 ± 0.7 μM , respectively).

3.3.2 The cytotoxic effect of NG and SNP on TK6 and WIL2-NS cells

As excessive killing of TK6 and WIL2-NS cells could potentially obscure *hprt* MF increases caused by NG and SNP, the effect of these drugs on cell viability was first examined. The PE of both TK6 and WIL2-NS cultures decreased in a similar manner with increasing NG concentrations (Figure 3-2, panel A). At a concentration of 400 μM NG, the PE of TK6 and WIL2-NS cultures was 96.7 ± 9.6 % and 67.0 ± 11.0 % of control values, respectively. At a concentration of 800 μM NG, TK6 and WIL2-NS PE was reduced to 14.4 ± 2.0 % and 30.1 ± 7.2 % of control values, respectively. Higher NG concentrations decreased PE values to lower than 1 % of controls. Therefore, NG concentrations of 400 μM and 800 μM were chosen to determine the effect of this NO-donating drug on the *hprt* MF of TK6 and WIL2-NS cells, respectively. Although the cytotoxic effect of NG was similar in both TK6 and WIL2-NS cultures, the PE of these two cell lines treated with SNP was remarkably different

Figure 3-2. The cytotoxic effects of NG and SNP on TK6 and WIL2-NS cells. TK6 (●) and WIL2-NS (■) cultures (5×10^5 cells/ml) were treated with increasing concentrations of NG (A) or SNP (B) for 24 hours. The cytotoxic effects of NG and SNP were determined by comparing the plating efficiency (PE) of drug-treated cultures to that of controls. Values are expressed as percent of control PE and represent the mean of three replicates ± SEM. Error bars, when not shown, appear within the symbol.


A
TK6
200
100
10
1
% control
0
400
800
1200
1600
2000
µM NG


B
WIL2-NS
200
100
10
% control
0
1
2
3
mM SNP

(Figure 3-2, panel B). Whereas the PE of TK6 cultures was reduced to 53.6 ± 5.2 % of controls at 0.5 mM SNP and to 2.9 ± 1.1 % of controls at 2 mM SNP, the PE of WIL2-NS cultures was not reduced as dramatically. PE values remained close to control values at 0.5 mM and 1 mM SNP (99.2 ± 0.8 % and 97.2 ± 1.7 %, respectively) and decreased slightly to 60.3 ± 4.8 % of control values at 2 mM SNP. Therefore TK6 cultures were treated with 0.5 mM SNP during NO-donating drug mutational studies while WIL2-NS cells were treated with 1 mM SNP or higher. Although cyanide toxicity was not examined in detail, surprisingly, overt cell killing was not evident by gross observation of WIL2-NS cells treated with 4 mM NaCN.

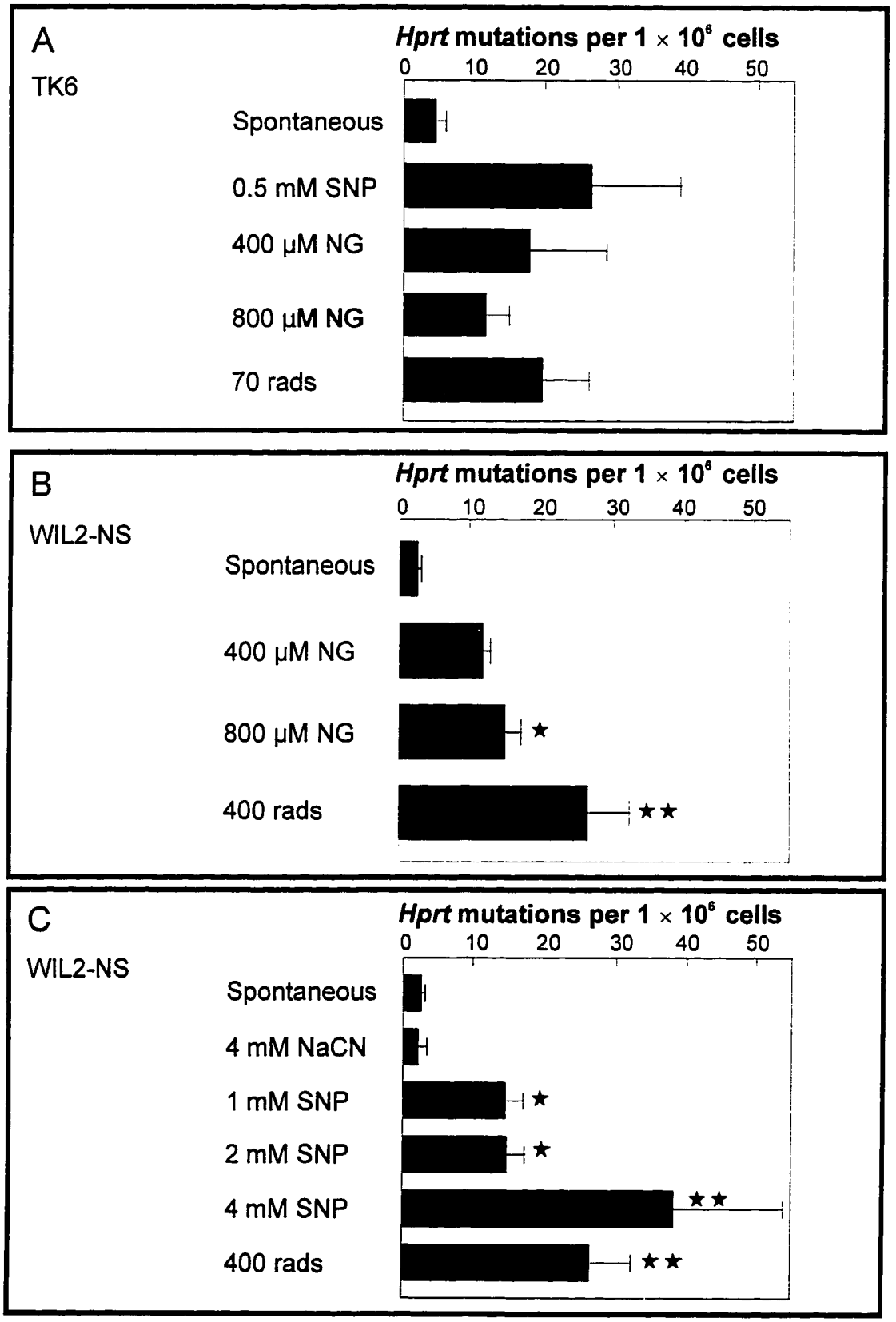
3.3.3 The *hprt* MF of TK6 and WIL2-NS cells treated with NG and SNP

To determine if the NO-donating drugs NG and SNP increase the *hprt* MF of human cells, TK6 and WIL2-NS cultures were treated with either NG or SNP for 24 hours. The "spontaneous" *hprt* MF was determined during each experiment in cultures that had received no drug treatment. Cultures exposed to radiation and then maintained for the same time period as drug-treated cultures were also included in each mutational assay as positive controls. All *hprt* MFs are expressed as the number of 6-TG-resistant (*hprt*$^-$) clones per 1 × 10^6 cells plated and are corrected for differences in PE (cMF). All values represent the mean of cMFs from at least three independent treatments ± the standard error of the mean.

The spontaneous *hprt* cMF of TK6 cells was 4.6 ± 1.4 *hprt*$^-$ mutants per 1 × 10^6 cells plated (Figure 3-3, panel A). Although the *hprt* cMF of drug-treated and irradiated TK6 cultures tended to be higher than that of spontaneous cultures (26.4 ± 12.4, 17.7 ± 10.6, 11.2 ± 3.3 and 19.7 ± 6.4 *hprt*$^-$ mutants per 1 × 10^6 cells for cultures treated with 400 μM NG, 800 μM NG, 0.5 mM SNP and 70 rads, respectively), these increases did not reach statistical significance ($p>0.05$) when analysed by one-way ANOVA. The PE values varied greatly within each treatment group, contributing to the large scatter of cMFs. For example, the PE of cultures treated with no drug, 400 μM NG, 0.5 mM SNP and 70 rads ranged from 11% to 77%, 2% to 77%, 29% to 48% and from 20% to 53%, respectively. Because of the inconsistent *hprt* cMFs observed in TK6 cells, WIL2-NS cells, a B lymphoblastoid cell line isolated from the same male patient as TK6, were employed to further study the effect of NG and SNP on *hprt* MF further.

The spontaneous *hprt* cMF of WIL2-NS cells was 2.6 ± 0.5 *hprt*$^-$ mutants per 1 × 10^6 cells plated (Figure 3-3, panel B), while the *hprt* cMF of cultures treated with 400 μM NG and 800 μM was 11.8 ± 1.1 and 15.0 ± 2.3 *hprt*$^-$ mutants per 1 × 10^6 cells, respectively. The mean *hprt* cMF of WIL2-NS cells treated with 800 μM NG was significantly higher than the cMF of spontaneous controls ($p<0.05$). The *hprt* cMF of cultures treated with 400 rads as a positive control was 26.5 ± 5.8 *hprt*$^-$ mutants per 1 × 10^6 cells, significantly higher than the spontaneous *hprt* cMF ($p<0.01$). The PE of drug-treated and untreated

Figure 3-3. The *hprt* cMF of TK6 and WIL2-NS cells treated with NO-donating drugs. TK6 (A) and WIL2-NS (B and C) cultures (5×10^5 cells/ml) were treated with NG or SNP for 24 hours or irradiated. WIL2-NS cells were also exposed to 4 mM NaCN for 24 hours as an SNP negative control. Cultures were maintained for 8 days following drug removal and the *hprt* MF was calculated by determining the number of 6-TG-resistant mutants per 1×10^6 cells. Cultures maintained for 8 days but treated with no drug were included in each experiment as negative controls and represent the spontaneous MF. Values represent the mean MF corrected for PE (cMF) from at least 3 independent treatments ± SEM. Values that were found to be significantly higher than the overall spontaneous cMF by one-way ANOVA are marked with an asterix (★$p<0.05$, ★★$p<0.01$).


A
TK6
Hprt mutations per 1 × 10⁶ cells
0 10 20 30 40 50
Spontaneous
0.5 mM SNP
400 µM NG
800 µM NG
70 rads
B
WIL2-NS
Hprt mutations per 1 × 10⁶ cells
0 10 20 30 40 50
Spontaneous
400 µM NG
800 µM NG
400 rads
C
WIL2-NS
Hprt mutations per 1 × 10⁶ cells
0 10 20 30 40 50
Spontaneous
4 mM NaCN
1 mM SNP
2 mM SNP
4 mM SNP
400 rads

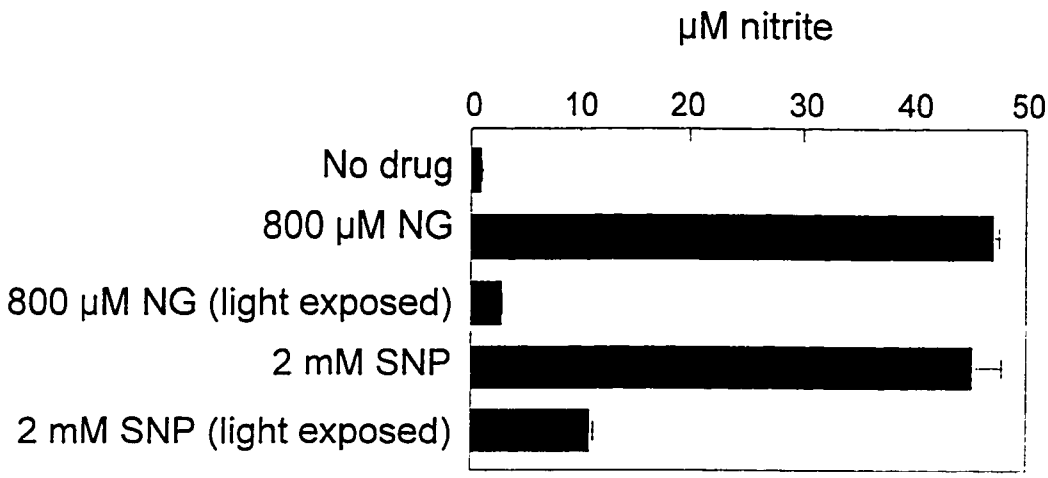
cultures did not vary greatly and ranged from 60% to 82% for all experiments.

The *hprt* cMF of WIL2-NS cultures treated with SNP was found to be elevated when compared to controls (Figure 3-3, panel C). Although the *hprt* cMF did not increase in a dose-dependent manner (14.6 ± 2.6, 14.8 ± 2.6 and 38.2 ± 15.7 *hprt*$^-$ mutants per 1 × 10^6 cells for cultures treated with 1 mM, 2 mM and 4 mM SNP, respectively), the *hprt* cMF of SNP-treated cultures was significantly higher than spontaneous controls ($p<0.05$). The metabolism of SNP releases both NO and cyanide (Feelisch and Stamler, 1996). However, the mutagenic effect of SNP was not due to cyanide since the *hprt* cMF of WIL2-NS cells treated with 4 mM NaCN was not significantly different from the spontaneous *hprt* cMF (Figure 3-3, panel C).

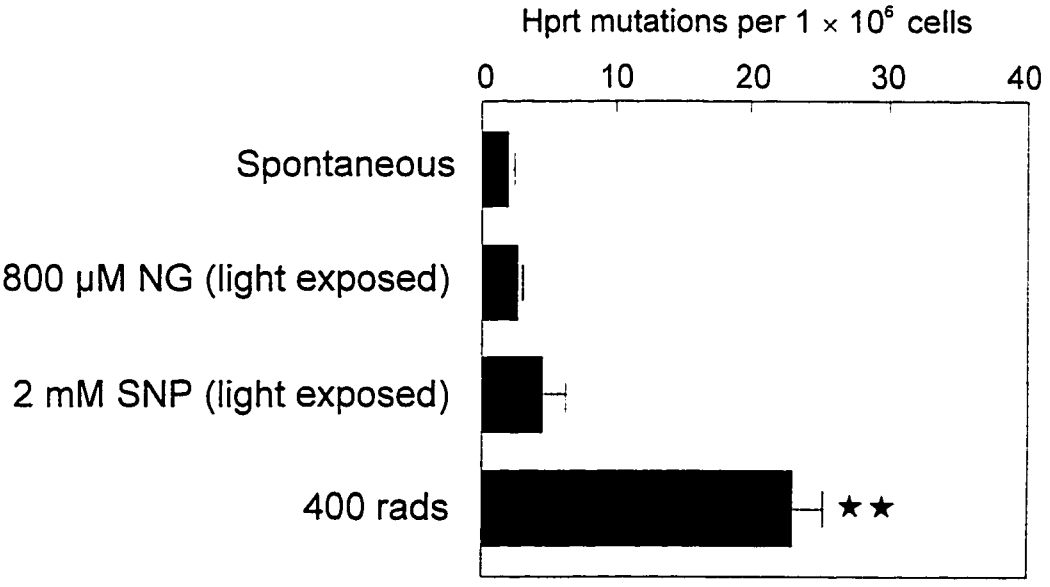
To determine if the observed increase in *hprt* cMF of WIL2-NS cultures treated with NO-donating drugs was indeed due to NO, stock NG and SNP were inactivated by exposure to light for 1 week. To ensure that inactivation was successful, supernatant nitrite levels were measured in WIL2-NS cultures treated with freshly prepared or light-exposed drugs by means of the Griess reaction (see section 3.2.1). After 24 hours of drug treatment, supernatant nitrite levels were found to be elevated in cultures exposed to 800 µM NG and 2 mM SNP prior to light inactivation (Figure 3-4, panel A). Culture supernatant nitrite levels were reduced by 94% and 76% after treatment with light-inactivated NG and SNP, respectively (Figure 3-4, panel A). When WIL2-NS cultures were treated with either 800 µM of light-exposed NG or 2 mM of light-exposed SNP and then

Figure 3-4. Supernatant nitrite levels and *hprt* cMF of WIL2-NS cultures treated with 800 μM NG or 2 mM SNP before and after drug inactivation. (A) Cells were plated at 2.5 × 10^5 cells/ml in phenol red free medium and treated with drug for 24 hours. Supernatant nitrite concentrations were determined using the Griess reaction. Values represent the mean of duplicate samples ± SEM. (B) WIL2-NS cultures (5 × 10^5 cells/ml) were treated for 24 hours with either freshly prepared or photoinactivated 800 μM NG or 2 mM. Cultures were maintained for 8 days following drug removal and the *hprt* MF was calculated by determining the number of 6-TG-resistant mutants per 1 × 10^6 cells. Cultures maintained for 8 days but treated with no drug were included as negative controls and represent the spontaneous MF. Cultures irradiated with 400 rads were included as positive controls. Values represent the mean corrected MF (cMF) from at least 3 independent treatments ± SEM. Values that were found to be significantly higher than the mean spontaneous cMF by one-way ANOVA are marked with an asterix (★★$p<0.01$).

A


μM nitrite
0
10
20
30
40
50
No drug
800 μM NG
800 μM NG (light exposed)
2 mM SNP
2 mM SNP (light exposed)

B


Hprt mutations per 1 × 10⁶ cells
0
10
20
30
40
Spontaneous
800 μM NG (light exposed)
2 mM SNP (light exposed)
400 rads
★★

subjected to an *hprt* mutational assay, the resulting *hprt* cMFs were1.9 ± 0.5 and 2.6 ± 0.4 *hprt*$^-$ mutants per 1 × 10^6 cells, respectively (Figure 3-4, panel B). Therefore, the *hprt* cMF of WIL2-NS cultures treated with 800 µM NG or 2 mM SNP was reduced by 82.7% and 69.9%, respectively, compared to cultures treated with active drugs (Figure 3-3, panels B and C). This decrease in *hprt* cMF was not due to failure of the *hprt* mutational assay as the *hprt* cMF of WIL2-NS cells exposed to 400 rads in the same experiment was significantly higher (22.9 ± 2.2 *hprt*$^-$ mutants per 1 × 10^6 cells) than the spontaneous *hprt* cMF ($p<0.01$) and similar to the radiation-induced *hprt* cMFs of other experiments (Figure 3-3, panels B and C).

3.3.4 MP-PCR analysis of *hprt*$^-$ TK6 and WIL2-NS clones

To determine the nature of the mutational events giving rise to the increase in *hprt* MF observed in drug-treated cultures, *hprt*$^-$ TK6 and WIL2-NS clones that arose spontaneously or after drug treatment were screened by MP-PCR for LSD mutations. 100 ng of DNA from *hprt*$^-$ clones was used to amplify *hprt* exons 2, 3, 9 and flanking regions DXS79 and DXS86.

A total of 5 spontaneous *hprt*$^-$ TK6 clones were analysed by MP-PCR, along with 26 *hprt*$^-$ clones isolated after treatment with 400 µM NG and 14 *hprt*$^-$ clones obtained after exposure to 0.5 mM SNP. *Hprt*$^-$ TK6 clones showed a high level of spontaneously occurring deletion mutations; 4 of the 5 spontaneous clones had large-scale *hprt* mutations. Of the 26 NG-treated *hprt*$^-$ TK6 clones,

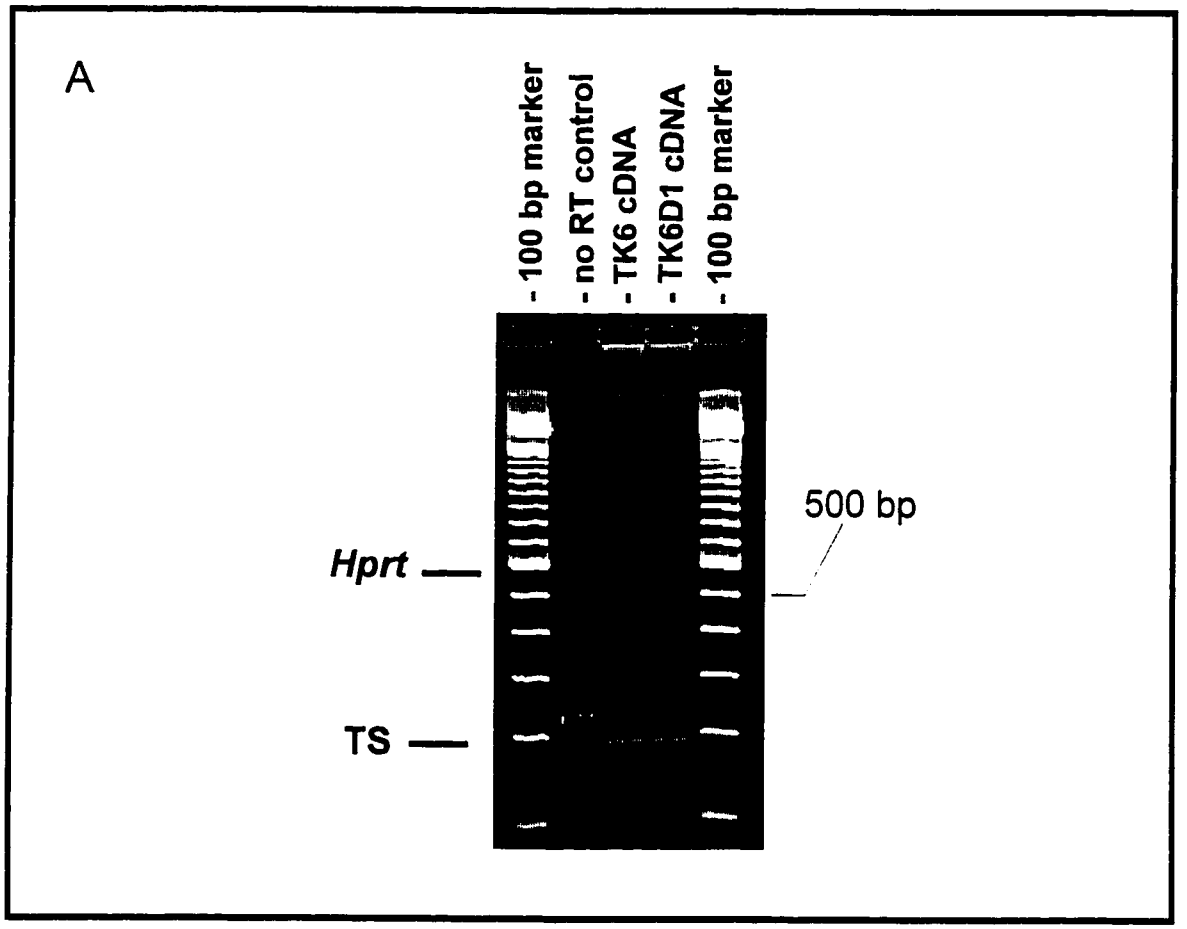
only 1 had a LSD mutation, affecting *hprt* exons 2, 3 and 9. All *hprt*$^-$ TK6 clones isolated following 0.5 mM SNP treatment had LSD mutations; 13 lacked *hprt* exon 2 and 1 was negative for *hprt* exon 2 and 3. A summary of *hprt* deletion mutations found in *hprt*$^-$ TK6 clones isolated before and after NO-donating drug treatment is given in Table 3-1.

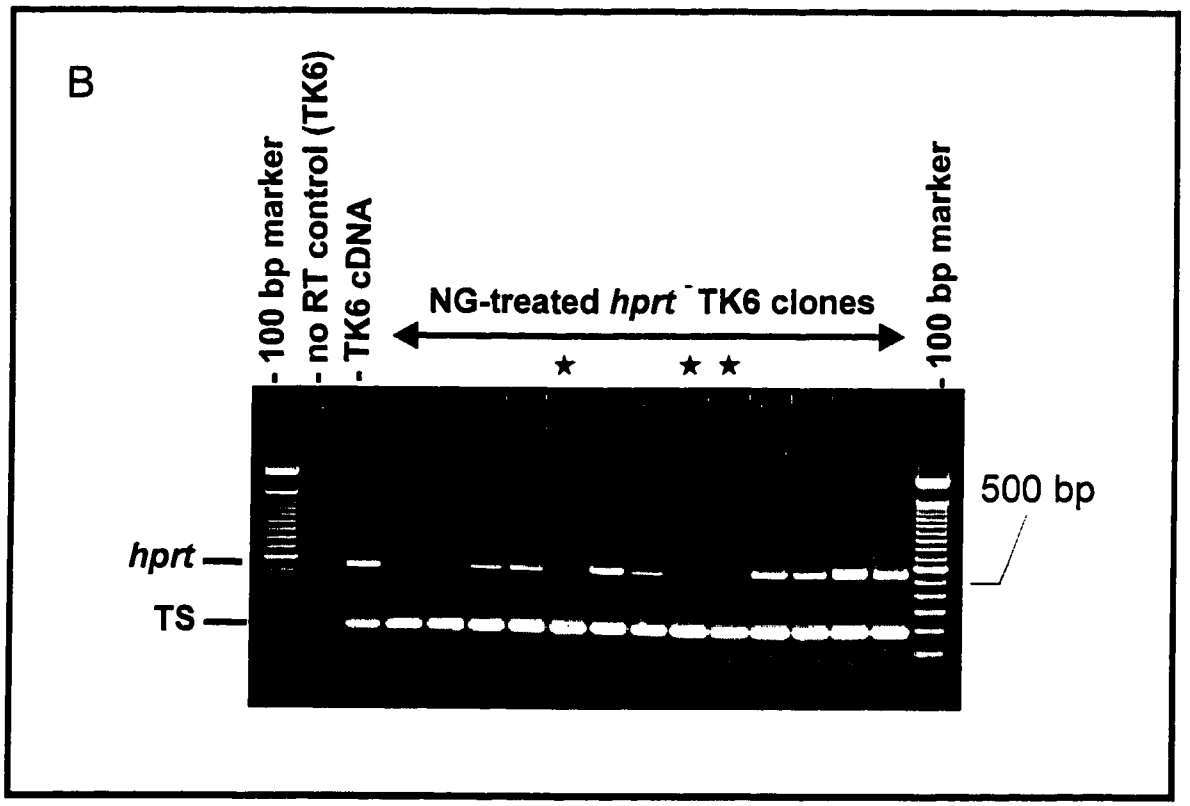
To determine if the *hprt* mutational events described above affected *hprt* mRNA transcription, total RNA was isolated from all *hprt*$^-$ TK6 clones and mRNA was amplified by RT-PCR. To ensure that the RT-PCR assay was functional even in cases where *hprt* mRNA was not detected, a portion of TS mRNA was co-amplified as an internal control (Figure 3-5, panel A). All *hprt*$^-$ TK6 clones that had *hprt* LSD mutations failed to yield detectable *hprt* mRNA, with the exception of 1 spontaneous *hprt*$^-$ clone that generated a faint *hprt* RT-PCR product but was shown to lack *hprt* exon 9 by MP-PCR. Of the 26 *hprt*$^-$ clones isolated after NG treatment and without detectable genomic *hprt* deletion mutations, 4 lacked detectable *hprt* mRNA and presumably had small mutations that were not detected by MP-PCR. RT-PCR products from several *hprt*$^-$ clones isolated following NG treatment are shown in Figure 3-5, panel B. All 14 of theSNP-treated *hprt*$^-$ TK6 clones lacked *hprt* exon 2 or *hprt* exons 2 and 3, and none of these produced detectable levels of *hprt* mRNA (data not shown). Because of the high rate of spontaneous LSD mutations found in TK6 cells, further studies into the mutagenic effects of NG and SNP were conducted with WIL2-NS cells.

Table 3-1. Summary of *hprt* deletion mutations found by MP-PCR in TK6 *hprt*$^-$ clones. *Hprt*$^-$ clones arising spontaneously or following treatment with 400 μM NG or 0.5 mM SNP were isolated and screened for *hprt* deletion mutations by MP-PCR. Clones that failed to amplify one or more *hprt* exons or flanking regions but not the HF9 internal control were considered deletion mutants. The precise ends of deletion mutations were not mapped.

Treatment group	Number of TK6 *hprt*⁻ clones analysed	Number of *hprt* deletion mutations	Description of *hprt* deletion mutations	Number of *hprt*⁻ clones with described deletion mutation
Spontaneous	5	4	*hprt* exon 9	1
			hprt exons 2, 3 and 9	3
400 µM NG	26	1	*hprt* exons 2, 3 and 9	1
0.5 mM SNP	14	14	*hprt* exon 2	13
			hprt exons 2 and 3	1

Figure 3-5. RT-PCR products of RNA from TK6 cells, TK6-D1 cells and *hprt*$^-$ TK6 clones isolated after NG treatment. (A) Total RNA (3 µg) from TK6 and TK6-D1 cells was used to synthesize cDNA using an oligo dT primer. PCR products were amplified from 2 µl of cDNA using primers specific for a portion of the *hprt* and TS cDNA. A negative control was included and consisted of 3 µg of TK6 RNA subjected to identical RT-PCR conditions but without reverse transcriptase (RT). Outside lanes contain 250 ng of 100 bp ladder marker. (B) *Hprt* and TS products were amplified using 3 µg of total RNA extracted from 6-TG-resistant TK6 clones isolated after treatment with 400 µM NG. Although the TS fragment is amplified consistently, some clones (★) lack detectable *hprt* mRNA. Negative and positive controls were included and consist of 3 µg TK6 RNA subjected to amplification without RT (no RT control) and 3 µg of TK6 RNA amplified with RT, respectively. Outside lanes contain 250 ng of 100 bp ladder marker.


A
- 100 bp marker
- no RT control
- TK6 cDNA
- TK6D1 cDNA
- 100 bp marker
500 bp
Hprt
TS


B
- 100 bp marker
- no RT control (TK6)
- TK6 cDNA
NG-treated hprt⁻ TK6 clones
- 100 bp marker
500 bp
hprt
TS

A total of 17 spontaneously arising *hprt*$^-$ WIL2-NS clones were analysed by MP-PCR, along with 20 *hprt*$^-$ clones isolated after exposure to 800 μM NG and 16 *hprt*$^-$ clones obtained after treatment with 1 mM SNP. The high frequency of spontaneous *hprt* LSD mutations that occurred in TK6 cells was not observed in WIL2-NS clones. Of the 17 spontaneous *hprt*$^-$ WIL2-NS clones, only 2 showed deletion mutations and all lacked *hprt* exon 9. However, larger deletion mutations were detected in *hprt*$^-$ WIL2-NS clones isolated after NG treatment (Figure 3-6) and following SNP treatment (Figure 3-7). Of the 20 *hprt*$^-$ WIL2-NS clones isolated after NG treatment, 8 showed no detectable LSD mutations, 3 lacked *hprt* exon 9, 2 were negative for *hprt* exon 2, 2 were missing *hprt* exons 2 and 3, 4 were negative for *hprt* exons 2, 3, and 9 and 1 lacked *hprt* exons 2, 3, 9 and flanking region DXS86. Of the 16 *hprt*$^-$ WIL2-NS clones isolated following SNP treatment, 3 had no mutations detectable by MP-PCR, 7 lacked *hprt* exon 9, 2 were negative for *hprt* exons 2 and 3, 2 were missing *hprt* exons 2, 3, 9 and flanking region DXS86 and 2 more were negative for *hprt* exons 2, 3, 9 and both flanking regions DXS86 and DXS79. A summary of *hprt* LSD mutations observed by MP-PCR in spontaneous and drug-treated WIL2-NS clones is given in Figure 3-8. When analysed by one-way ANOVA, drug- treated *hprt*$^-$ WIL2-NS clones had significantly more LSD mutations than did spontaneously arising *hprt*$^-$ clones ($p<0.01$).

Figure 3-6. *Hprt*$^-$ WIL2-NS LSD mutants isolated after NG treatment. 100 ng of DNA from wild-type WIL2-NS cells, or from *hprt*$^-$ WIL2-NS clones isolated after treatment with 800 μM NG, was used to amplify portions of the *hprt* gene and flanking regions. Wild-type WIL2-NS DNA was positive for all regions examined while 7 of 8 *hprt*$^-$ clones showed deletion mutations (★) involving *hprt* exon 9 (3 of 8), *hprt* exons 2 and 3 (2 of 8) or *hprt* exons 2, 3 and 9 (2 of 8). All *hprt*$^-$ clones were positive for the HF9 internal control. Outside lanes contain 250 ng 100 bp ladder marker.

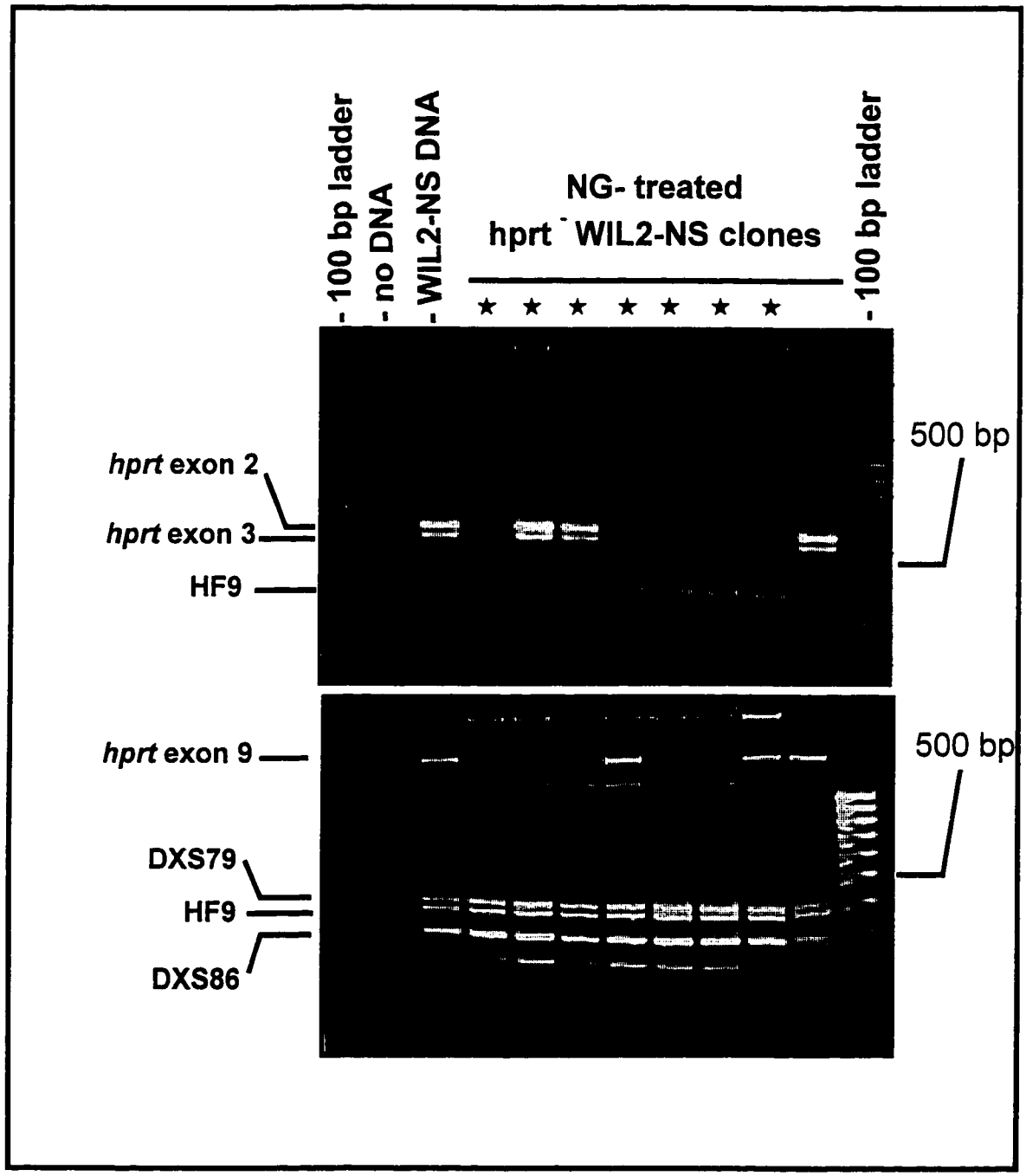

- 100 bp ladder
- no DNA
- WIL2-NS DNA
NG- treated
hprt - WIL2-NS clones
- 100 bp ladder
★ ★ ★ ★ ★ ★ ★
hprt exon 2
hprt exon 3
HF9
500 bp
hprt exon 9
DXS79
HF9
DXS86
500 bp

Figure 3-7. *Hprt*$^-$ WIL2-NS LSD mutants isolated after SNP treatment. 100 ng of DNA from wild-type WIL2-NS cells or from *hprt*$^-$ WIL2-NS clones isolated after treatment with 1 mM SNP was used to amplify portions of the *hprt* gene and flanking regions. Wild-type WIL2-NS cells were positive for all regions examined while 5 of 8 *hprt*$^-$ clones showed deletion mutations (★) involving *hprt* exons 2 and 3 (2 of 8), *hprt* exon 9 (1 of 8) or *hprt* exons 2, 3, 9 and flanking regions DXS79 and DXS86 (2 of 8). All *hprt*$^-$ clones were positive for the HF9 internal control. Outside lanes contain 250 ng of 100 bp ladder marker.

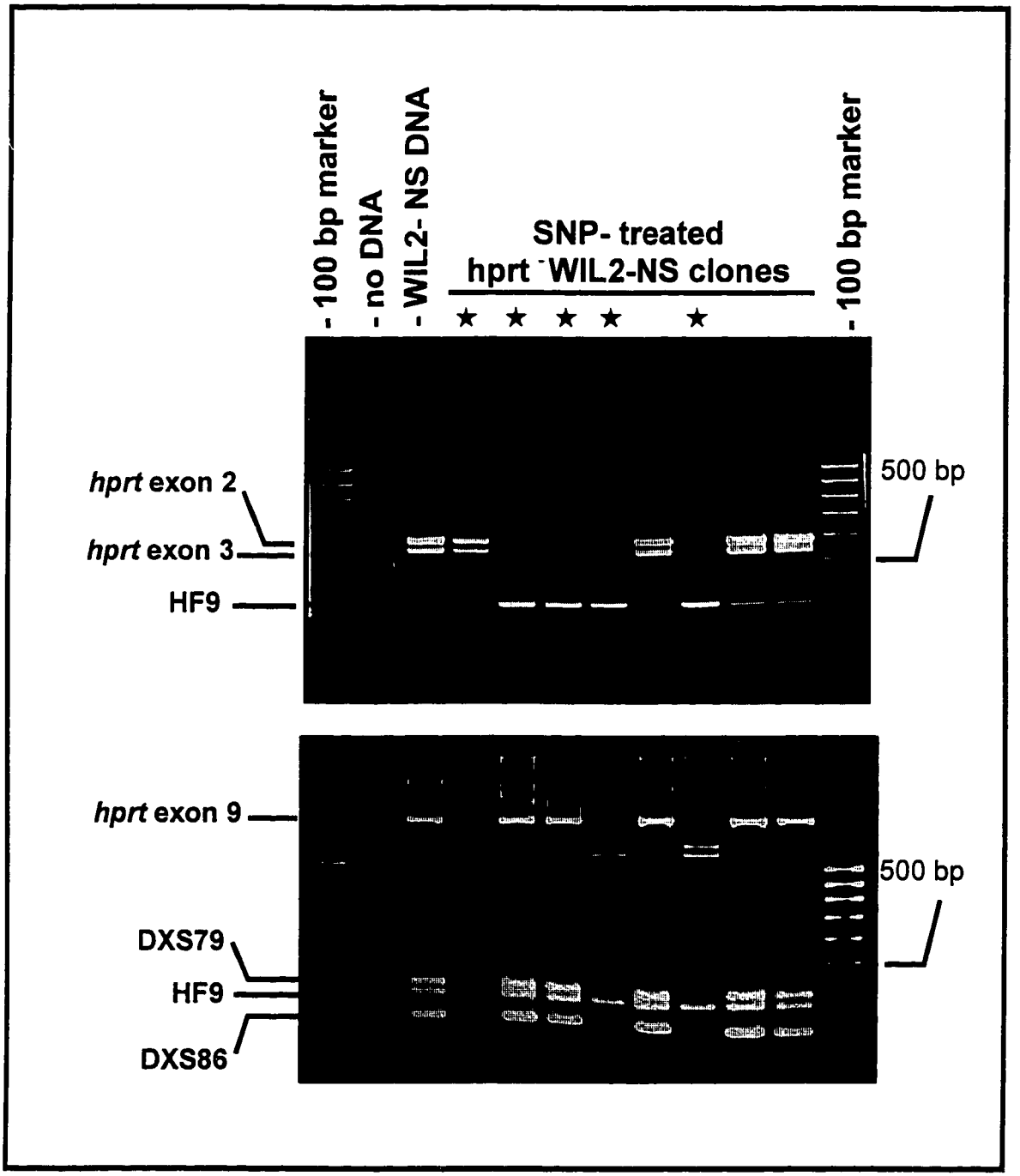
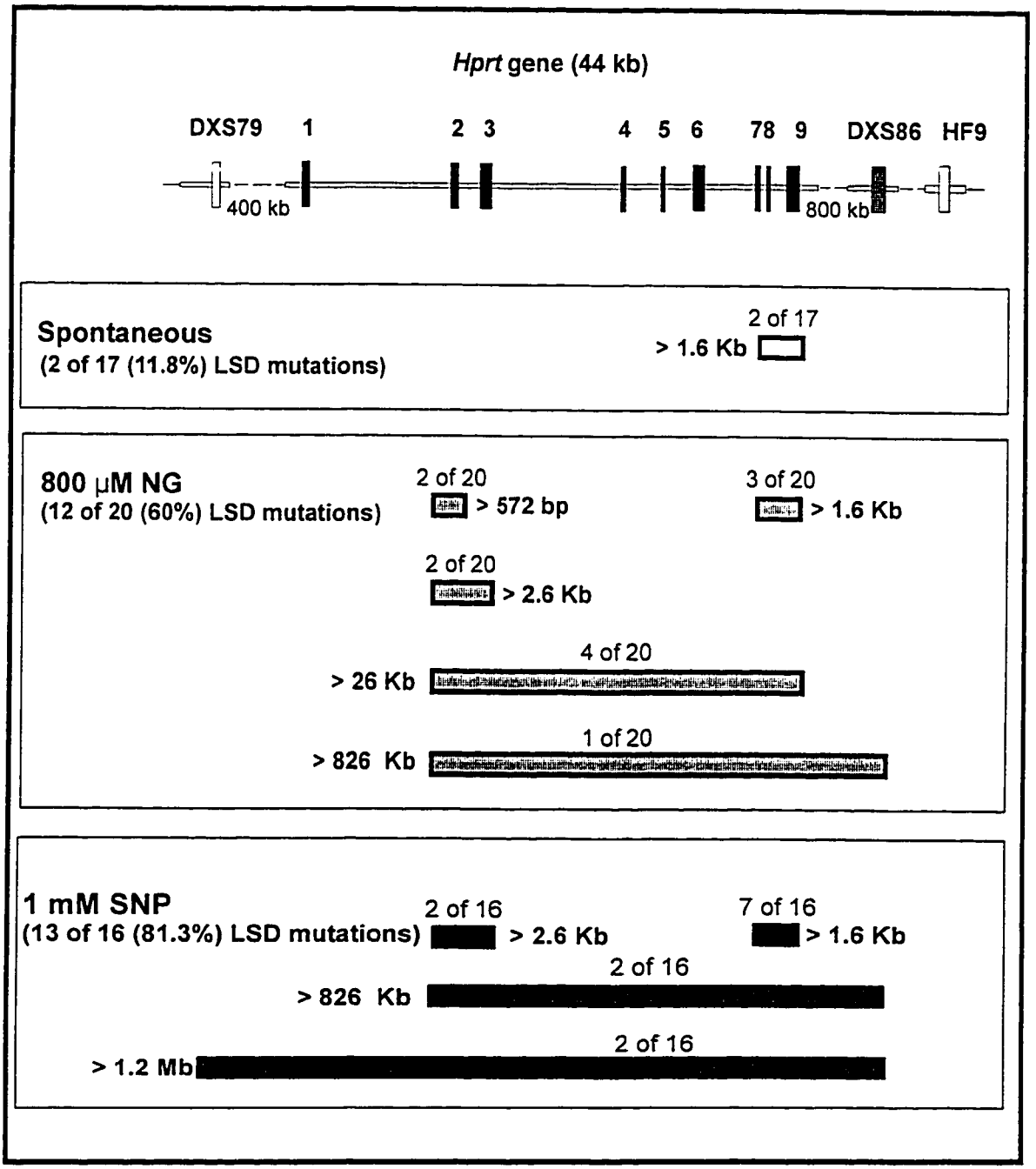

- 100 bp marker
- no DNA
- WIL2- NS DNA
SNP- treated
hprt - WIL2-NS clones
- 100 bp marker
hprt exon 2
hprt exon 3
HF9
500 bp
hprt exon 9
DXS79
HF9
DXS86
500 bp

Figure 3-8. Summary of *hprt* LSD mutations found by MP-PCR in *hprt*$^-$ WIL2-NS clones following treatment with NO-donating drugs. *Hprt*$^-$ WIL2-NS clones were isolated from either untreated cultures (spontaneous) or cultures treated with 800 μM NG or 1 mM SNP, and then screened for LSD mutations by MP-PCR. Bars represent the minimum size of *hprt* deletions, since the ends of the LSD mutations were not mapped precisely. LSD mutants were identified as clones that failed to amplify one or more PCR fragments while amplifying the HF9 internal control. If two or more PCR bands did not amplify, the intervening DNA sequence is assumed to have been deleted. Numbers correspond to the number of clones with LSD mutations out of the total number clones analyzed. The *hprt* gene and flanking regions are not drawn to scale.


Hprt gene (44 kb)
DXS79
1
2 3
4 5 6
78 9
DXS86
HF9
400 kb
800 kb
Spontaneous
(2 of 17 (11.8%) LSD mutations)
2 of 17
> 1.6 Kb
800 µM NG
(12 of 20 (60%) LSD mutations)
2 of 20
> 572 bp
3 of 20
> 1.6 Kb
2 of 20
> 2.6 Kb
4 of 20
> 26 Kb
1 of 20
> 826 Kb
1 mM SNP
(13 of 16 (81.3%) LSD mutations)
2 of 16
> 2.6 Kb
7 of 16
> 1.6 Kb
2 of 16
> 826 Kb
2 of 16
> 1.2 Mb

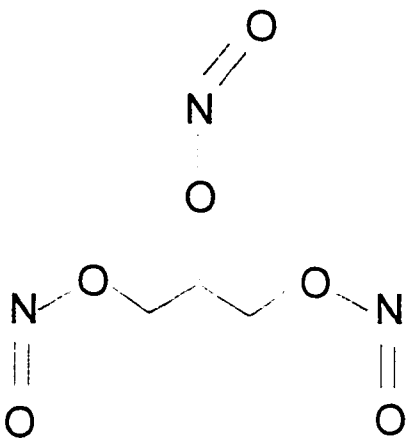
3.4 Discussion

3.4.1 TK6 and WIL2-NS metabolism of NG and SNP

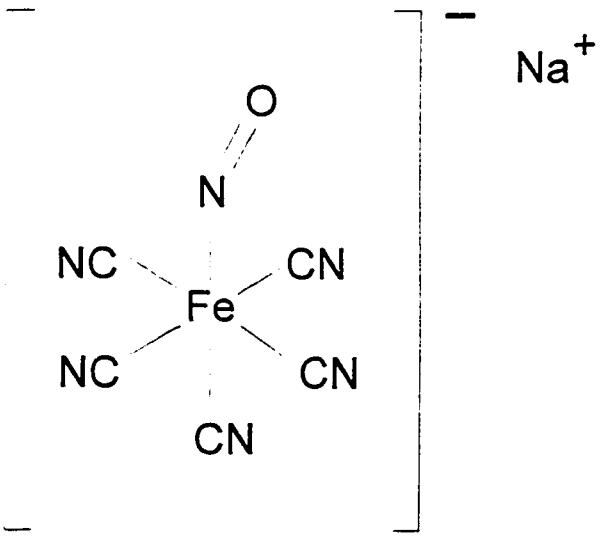
Prior to *hprt* mutational studies involving NG and SNP, the ability of TK6 and WIL2-NS cells to metabolize these drugs was assessed by determining the nitrite levels in culture supernatants. Both TK6 and WIL2-NS cells were capable of metabolizing NG and SNP as determined by the Griess reaction. The mechanisms through which NG and SNP are metabolized to release NO are not fully understood. NG is an organic nitrate (Figure 3-9) used as a vasorelaxant in the treatment of angina. Release of NO from this compound has been detected in both intact cells and subcellular fractions and, although more than one pathway has been implicated in the metabolism of NG, NO release is thought to be dependent in part on interactions with thiols (Feelisch and Stamler, 1996). SNP is a transition metal nitrosyl used to reduce blood pressure in cases of hypertensive emergency (Figure 3-9). SNP undergoes a one-electron reduction, either enzymatically, non-enzymatically or upon exposure to light, that liberates both CN and NO (Feelisch and Stamler, 1996). In our experiments, both NG and SNP were found to release NO spontaneously.

Although TK6 and WIL2-NS cells responded in the same manner to the cytotoxic effects of NG, WIL2-NS cells were found to be significantly more resistant to killing by SNP. Furthermore, in WIL2-NS cells cultured with 4 mM NaCN, overt cytotoxicity was not observed when cells were examined by light microscopy. WIL2-NS is a cell line, derived from the same patient as TK6, that is

Figure 3-9. Structure of the NO-donating drugs NG and SNP.



Nitroglycerin



Sodium nitroprusside

mutated at codon 237 of the p53 tumour suppressor gene (Zhen et al. 1995). These cells over express mutant p53 protein while TK6 cells express normal amounts of wild-type p53. Although the WIL2-NS mutant form of p53 is capable of binding to DNA (Carrier et al. 1996), WIL2-NS are more resistant to radiation-induced apoptosis than are TK6 cells (Amundson et al. 1993; Schwartz et al. 1995; Zhen et al. 1995). For this reason, WIL2-NS cells may be more likely to survive exposure to SNP than TK6 cells that readily undergo apoptosis. Metabolism of NG, however, may produce cytotoxic species that act independently of p53-mediated apoptosis, and therefore kill both TK6 and WIL2-NS cells with equal efficiency.

3.4.2 *Hprt* MF of TK6 and WIL2-NS cells exposed to NG or SNP

The ability of NG and SNP to induce mutations was assessed by measuring the *hprt* MF of drug-treated TK6 and WIL2-NS cells. Although in TK6 experiments, the *hprt* cMF tended to be higher in drug-treated and irradiated cultures than in non-drug-treated cultures, these increases did not reach statistical significance. There was a large scatter in TK6 PE between treatment dishes and the corresponding *hprt* cMF of these cultures. Others have also noted a lack of dose response in TK6 cells that may result from a rapid apoptotic response (Wilkinson, 1997). When TK6 and WIL2-NS cells are exposed to radiation, TK6 cells undergo apoptosis within 24 hours whereas the p53 mutant WIL2-NS cell line demonstrates delayed apoptosis (>40 hours) and the number

of cells undergoing apoptosis is much lower than in TK6 cultures (Schwartz et al. 1995). For this reason presumably, fewer radiation-induced chromosomal aberrations are detected in TK6 cells than in WIL2-NS cells (Schwartz and Jordan, 1997); mutant p53 status allows WIL2-NS cells with large mutations to survive in culture whereas mutated TK6 cells become apoptotic and are lost from the population. However, if apoptosis is inhibited in TK6 cells by the addition of phorbol 12-myristate 13-acetate (PMA), the number and magnitude of radiation-induced chromosomal aberrations in TK6 and WIL2-NS cultures is similar (Schwartz and Jordan, 1997). TK6 cells have also been reported to undergo apoptosis earlier than WIL2-NS cells when treated with agents other than radiation, such as ethyl nitrosourea, an alkylating agent and etoposide, a topoisomerase II inhibitor (Greenwood et al. 1998). Therefore TK6 cells may also be sensitive to NG and SNP treatment; a robust apoptotic response may have led a sporadic representation of *hprt*$^-$ TK6 cells upon 6-TG challenge. For this reason, WIL2-NS cells were chosen to continue *hprt* mutational analysis.

The PE of drug-treated and untreated WIL2-NS cultures was similar and therefore *hprt* MFs were not artificially inflated when corrected for PE (cMF). The *hprt* cMF of WIL2-NS cells treated with 800 μM NG or 1 mM SNP was significantly higher than the spontaneous cMF. Although the *hprt* cMF of WIL2-NS cells increased with increasing drug concentrations, a linear dose-response was not observed for NG or SNP. This suggests that cultures reached a saturation point with respect to drug metabolism, beyond which the addition of

drug only contributed to spontaneous NO release. Alternatively, larger numbers of cells may have been killed at higher drug concentrations and therefore fewer *hprt*$^-$ cells were detected upon 6-TG challenge than actually arose due to drug treatment. The increase in *hprt* cMF of drug-treated WIL2-NS cultures was largely attributed to NO, as photo-degradation of these drugs resulted in a large reduction in cMF. In the case of SNP, exposure of WIL2-NS cells to photo-depleted drugs did not completely return the *hprt* cMF to spontaneous control values. It has recently been suggested that even after SNP has lost the capacity to donate NO, it may still retain pro-oxidative effects due to the presence of an iron moiety. *In vitro*, NO-depleted SNP was still able to generate hydroxyl radicals and cause lipid peroxidation in brain homogenates, effects that were abrogated by the addition of deferoxamine, an iron chelator (Rauhala et al. 1998). Therefore, following exposure to light, SNP may still retain a portion of its genotoxic character and produce a slight increase in WIL2-NS *hprt* cMF when compared to controls.

Release of NO from SNP also liberates cyanide (Feelisch and Stamler, 1996). Cyanide is a weak mutagen that has been shown to cause an increase in DNA strand breaks *in vitro* (Bhattacharya and Lakshmana Rao, 1997). Therefore, the genotoxic potential of 4 mM NaCN was determined in WIL2-NS cells using an *hprt* mutational assay. No significant increase in *hprt* cMF was found in cyanide-treated cultures versus controls. This suggests that the rise of *hprt* cMF in WIL2-NS cells treated with SNP was largely due to the actions of NO

rather than cyanide. The fact that no excessive cell killing was noted in WIL2-NS cells treated with 4 mM NaCN may also be due to the mutant p53 status of this cell line.

3.4.3 *Hprt* deletion mutations in TK6 and WIL2-NS cells exposed to NG or SNP

LSD mutations were detected at the *hprt* locus in TK6 and WIL2-NS *hprt*$^-$ clones following NG and SNP treatment, as well as in untreated cultures. In TK6 experiments the possibility that *hprt*$^-$ clones might be sibs was not addressed, since they were grown in bulk culture prior to being cloned in 96-well plates. Therefore, some TK6 *hprt*$^-$ clones may not have occurred as a result of independent mutational events. In WIL2-NS experiments however, the issue of clonal expansion was addressed; only WIL2-NS *hprt*$^-$ clones that were isolated from separate treatment plates or that had a unique pattern of MP-PCR deletion mutations were considered to have arisen from independent mutational events. In TK6 *hprt*$^-$ clones that arose spontaneously, a high background of LSD mutations was observed. In spontaneous *hprt*$^-$ clones isolated from two separate experiments, 4 out of 5 (80%) had large *hprt* deletions; 1 lacked *hprt* exon 9 (a deletion of > 1.6 Kb) whereas 3 others were negative for all *hprt* exons studied (> 26 Kb deletion). A high background rate of TK6 *hprt* deletions has been reported previously. For example, in one study of spontaneously arising *hprt*$^-$ TK6 clones, 45% of the clones had deletions detectable by MP-PCR (Giver

et al. 1993). In several other studies, the number of *hprt* LSD mutations induced by radiation was not significantly different than in spontaneously arising *hprt*$^-$ TK6 clones (Nelson et al. 1994; Phillips et al. 1997). Although the reason for this high frequency of LSD mutations in these cells is not known, template-directed misalignment (slippage) during replication has been suggested as a mechanism for these spontaneous mutations (Lichtenauer-Kaligis et al. 1993). Since we hoped to observe a difference in *hprt* deletion mutations between spontaneous and NO-donating drug-treated cultures, TK6 cells with their high rate of spontaneous deletions were not appropriate for use in this study. We therefore examined by MP-PCR the *hprt* deletion mutations arising in WIL2-NS cultures. The high rate of spontaneously arising LSD mutations seen in TK6 cells was not observed in WIL2-NS cells. In *hprt*$^-$ spontaneous WIL2-NS clones, only 2 out of 17 (11.8%) clones had *hprt* deletions detectable by MP-PCR, and both of these mutations affected *hprt* exon 9, representing deletions of > 1.6 Kb. *Hprt*$^-$ WIL2-NS clones isolated after treatment with NG or SNP, however, showed evidence of *hprt* LSD mutations in 60% and 81.3% of the clones examined, respectively. The size of these mutations tended to be much larger than those found in spontaneously arising *hprt*$^-$ clones. LSD mutations in drug-treated *hprt*$^-$ clones ranged from the size seen in spontaneously arising clones (~1.6 Kb) to greater than 1.2 Mb and included the *hprt* flanking regions DXS79 and DXS86. This evidence suggests that NO released from NG and SNP is capable of directly or indirectly inducing DNA double-strand breaks that, when ligated together, result

in large deletions of genomic material. A recent report has found evidence that NO may be capable of inducing genetic damage that exceeds small base-pair changes. Zhuang et al. noted that in murine macrophages induced to produce large quantities of NO, *hprt* cDNA mutations involving the deletion of several exons occurred, but were not detected in macrophages that produced little or no NO (Zhuang et al. 1998). Whether or not these mRNA changes resulted from genomic LSD mutations was not addressed in this study.

Mutated p53 status has been associated with genomic instability in many types of cancer. Could the altered p53 expression in WIL2-NS cells contribute to the formation of *hprt* mutations observed in this study? If this were the case, large *hprt* deletions might have been expected to be detected more readily in the spontaneous population. In addition, it has been shown that mutant p53 status alone is insufficient to produce genetic mutations; cells must first complete the cell cycle in the presence of mutagens for DNA damage to occur (Paulson et al. 1998). It has also been shown that WIL2-NS do not have a reduced capacity to repair DNA breaks (Bill et al. 1997). Therefore the *hprt* mutations observed were most likely due to the genotoxic effects of NO and related species. The fact that larger *hprt* deletion mutations were observed in WIL2-NS cells than in TK6 cells likely reflects the ability of WIL2-NS cells to survive such mutations due to their altered p53 status.

What products of NO formation could be responsible for the increase in genetic damage observed in cells exposed to NO-donors? NO is a short-lived

free radical capable of reacting with oxygen species to form other reactive compounds that, in turn, cause cellular damage (see Chapter 2, Figure 2-1). Assessing the contribution of any one of these species to mutagenesis has been difficult, largely due to the speed with which NO reacts with other compounds and the difficulty of dissecting the resulting pathways. However, the nitroxyl radical (NO^-), also thought to be formed by NOS, has recently been shown to be capable of inducing DNA double-strand breaks *in vitro* (Wink et al. 1998), a situation that may result in genomic deletion mutations. The hydroxyl radical ($OH^\cdot$), also formed in the breakdown of NO, can mediate DNA cleavage (Kang and Kim, 1997). Further study of the genotoxic products formed by NO is required to understand their contribution to mutagenesis.

3.4.4 Summary and future work

The NO-donating drugs NG and SNP were found to increase the *hprt* MF in WIL2-NS cells. This effect could be reduced by the Photo-degradation (NO-exhaustion) of these drugs prior to the *hprt* MF assay and was not likely to be due to the genotoxic effects of cyanide. LSD mutations, detected by MP-PCR, occurred more frequently in drug-treated *hprt*$^-$ WIL2-NS clones than in *hprt*$^-$ clones that arose spontaneously. LSD mutations were also larger in drug-treated cells compared to unexposed cells. A 1.6 Kb region was found to be deleted in several spontaneous *hprt*$^-$ WIL2-NS whereas LSD mutations in drug-treated clones ranged from >1.6 Kb to >1.2 Mb. This is strong evidence that NO

and its subsequent reactive species are capable of inducing significant genetic damage, information that had not previously been reported at the time of this study.

Chapter 4: General discussion and conclusions

We have developed a novel method of isolating *hprt*$^-$ T cell clones that permits PCR analysis of the *hprt* gene at an early stage of clonal growth. Any bias associated with a requirement for prolonged proliferative capacity is minimized. To examine the nature of the mutagenic events that occur in RA T cells, peripheral *hprt*$^-$ T cell clones from RA and OA patients were screened for LSD mutations by MP-PCR using this technique. Large *hprt* deletions were detected in both RA and OA T cells, although these fell within the range of LSD mutations reported to occur in *hprt*$^-$ T cells from normal individuals. If these frequencies of *hprt* LSD mutations remain constant when larger numbers of clones are examined, then the mutational events that lead to the reported increase in T cell *hprt* MF in RA patients will most likely be due to smaller mutations. The possibility also exists that LSD mutations occurred in these T cells in *hprt* regions not examined by MP-PCR.

MP-PCR analysis of the *hprt* gene required male T cell clones and therefore large numbers of patients were not available for analysis during the time course of this project. However, if *hprt* cDNA rather than genomic DNA is sequenced for mutations, females may be included and the constraint of analysing only male patients is removed. Others have successfully amplified and sequenced *hprt* cDNA from *hprt*$^-$ T cell clones to identify mutations within exons (Podlutsky et al. 1998). In the future, this strategy might be employed to include female RA patients in the study, and to infer changes at the genomic

level based on *hprt* mRNA mutations.

During the course of this study, only peripheral *hprt*$^-$ T cell clones have been examined for LSD mutations, due to technical difficulties in growing synovial *hprt*$^-$ T cell clones. It may therefore by of interest to examine synovial *hprt*$^-$ T cell clones from RA and OA patients in the future for *hprt* LSD mutations. Since the *hprt* MF of RA T cells was previously shown to be elevated in the synovium when compared to peripheral RA T cells (Cannons et al. 1998), it is possible that the induction of LSD mutations is also more prevalent in T cells from the joint than in peripheral blood T cells.

To determine if NO is capable of inducing genetic damage that produces LSD mutations, TK6 and WIL2-NS cell lines were exposed to the NO-donating drugs NG and SNP. Although results were inconsistent in TK6 cultures, the *hprt* MF and the frequency of large-scale (1.6 Kb to 1.2 Mb) mutations detected by MP-PCR was clearly increased in WIL2-NS cultures exposed to NG and SNP compared to controls. This indicates that NO and its related species are capable of producing large-scale genetic damage. This may have implications for the treatment of diseases in which NO levels are elevated. Since NO is elevated in many inflammatory diseases, more detailed knowledge of the genotoxic capabilities of NO may lead to a greater understanding of the relationship that exists between inflammation and the development of cancer.

T lymphocytes were not used in the study of the genotoxic potential of NG and SNP for several reasons. Elimination of *hprt*$^-$ cells by culture in HAT-

supplemented medium followed by drug exposure, *hprt*$^-$ phenotype expression and 6-TG selection require approximately 3-4 weeks of culture. Due to the limited life-span of T cells, and since the response of these cells to NG and SNP was not known, it was decided that *in vitro* experiments using T lymphocytes exposed to NO-donating drugs would not be practical. Therefore, two immortalized human B lymphoblastoid cell lines were exposed to NG and SNP, two NO-donating drugs previously found to be mutagenic in our lab. Other donors of NO exist, including DEA/NO, which do not require metabolism and release NO spontaneously. However, experiments in our lab and in other laboratories have failed to detect an increase in *hprt* mutations using this compound (Felley-Bosco et al. 1995; Donovan et al. 1997). The half-life of DEA/NO is less than 2 min and it is possible that the level of NO produced by DEA/NO over such a short period of time is insufficient to cause DNA mutations. Alternatively, this burst of NO may be capable of killing a great number of cells, leading to an underrepresentation of *hprt*$^-$ mutants. NG and SNP, drugs that require metabolism and thought to release NO slowly over time, were therefore chosen to more closely simulate the inflammatory conditions of the RA joint.

This study is novel in that it represents the first attempt to determine if large-scale deletion mutations occur within the mutagenic environment of the inflamed joint in RA. Although LSD *hprt* mutagenic events did not predominate in RA T cells in our series of patients, the number of clones analysed was too small from which to draw conclusions. More mutants from normal controls need

to be examined, in addition to *hprt*$^-$ synovial T cell clones from RA and OA patients. *In vitro* evidence gathered here suggests that NO is capable of inducing LSD mutations. Since genetic damage may contribute to the pathogenesis of RA, this study provides additional evidence that targeting NO formation may have therapeutic benefits in this disease.

References

Akiyama, M., Kyoizumi, S., Hirai, Y., Kusunoki, Y., Iwamoto, K.S. and Nakamura, N. (1995) Mutation frequency in human blood cells increases with age. Mutat. Res. DNAging Genet. Instability Aging 338, 141.

Albertini, R.J., Castle, K.L. and Borcherding, W.R. (1982) T-cell cloning to detect the mutant 6-thioguanine-resistant lymphocytes present in human peripheral blood. Proc. Natl. Acad. Sci. USA. 79, 6617.

Albertini, R.J., O'Neill, J.P., Nicklas, J.A., Heintz, N.H. and Kelleher, P.C. (1985) Alterations of the hprt gene in human in vivo-derived 6-thioguanine-resistant T lymphocytes. Nature 316, 369.

Amin, A.R. and Abramson, S.B. (1998) The role of nitric oxide in articular cartilage breakdown in osteoarthritis. Curr. Opin. Rheumatol. 10, 263.

Ammenheuser, M.M., Hastings, D.A., Whorton, E.B., Jr. and Ward, J.B., Jr. (1997) Frequencies of hprt mutant lymphocytes in smokers, non-smokers, and former smokers. Environ. Mol. Mutagen. 30, 131.

Amundson, S.A., Xia, F., Wolfson, K. and Liber, H.L. (1993) Different cytotoxic and mutagenic responses induced by X-rays in two human lymphoblastoid cell lines derived from a single donor. Mutat. Res. 286, 233.

Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H. and Luthra, H.S. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 31, 315.

Aupperle, K.R., Boyle, D.L., Hendrix, M., Seftor, E.A., Zvaifler, N.J., Barbosa, M. and Firestein, G.S. (1998) Regulation of synoviocyte proliferation, apoptosis, and invasion by the p53 tumor suppressor gene. Am. J. Pathol. 152, 1091.

Bhattacharya, R. and Lakshmana Rao, P.V. (1997) Cyanide induced DNA fragmentation in mammalian cell cultures. Toxicology 123, 207.

Bhusate, L.L., Herbert, K.E., Scott, D.L. and Perrett, D. (1992) Increased DNA strand breaks in mononuclear cells from patients with rheumatoid arthritis. Ann. Rheum. Dis. 51, 8.

Bill, C.A., Yu, Y., Miselis, N.R., Little, J.B. and Nickoloff, J.A. (1997) A role for

p53 in DNA end rejoining by human cell extracts. Mutat. Res. 385, 21.

Bologna, C., Picot, M.C., Jorgensen, C., Viu, P., Verdier, R. and Sany, J. (1997) Study of eight cases of cancer in 426 rheumatoid arthritis patients treated with methotrexate. Ann. Rheum. Dis. 56, 97.

Broker, B.M., Korthauer, U., Heppt, P., Weseloh, G., de la Camp, R., Kroczek, R.A. and Emmrich, F. (1993) Biased T cell receptor V gene usage in rheumatoid arthritis. Oligoclonal expansion of T cells expressing V alpha 2 genes in synovial fluid but not in peripheral blood. Arthritis Rheum. 36, 1234.

Burkhart-Schultz, K.J., Thompson, C.L. and Jones, I.M. (1996) Spectrum of somatic mutation at the hypoxanthine phosphoribosyltransferase (*hprt*) gene of healthy people. Carcinogenesis 17, 1871.

Burkhart-Schultz, K.J. and Jones, I.M. (1997) Deletion and insertion in vivo somatic mutations in the hypoxanthine phosphoribosyltransferase (hprt) gene of human T-lymphocytes [published erratum appears in Environ Mol Mutagen 1998;31(2):195]. Environ. Mol. Mutagen. 30, 371.

Cannons, J., Goldstein, R., Karsh, J. and Birnboim, H.C. (1998) Hprt- mutant T cells in the peripheral blood and synovial tissue of patients with rheumatoid arthritis. Arthritis Rheum. 41, 1772.

Carrier, F., Bae, I., Smith, M.L., Ayers, D.M. and Fornace, A.J., Jr. (1996) Characterization of the GADD45 response to ionizing radiation in WI-L2- NS cells, a p53 mutant cell line. Mutat. Res. 352, 79.

Choi, P.M. and Zelig, M.P. (1994) Similarity of colorectal cancer in Crohn's disease and ulcerative colitis: implications for carcinogenesis and prevention. Gut 35, 950.

Cibere, J., Sibley, J. and Haga, M. (1997) Rheumatoid arthritis and the risk of malignancy. Arthritis Rheum. 40, 1580.

Clancy, R.M., Amin, A.R. and Abramson, S.B. (1998) The role of nitric oxide in inflammation and immunity. Arthritis Rheum. 41, 1141.

Connor, J.R., Manning, P.T., Settle, S.L., Moore, W.M., Jerome, G.M., Webber, R.K., Tjoeng, F.S. and Currie, M.G. (1995) Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. Eur. J. Pharmacol. 273, 15.

Da Cruz, A.D. and Glickman, B.W. (1997) Nature of mutation in the human hprt gene following in vivo exposure to ionizing radiation of cesium-137. Environ. Mol. Mutagen. 30, 385.

Dawisha, S.M., Gmelig-Meyling, F. and Steinberg, A.D. (1994) Assessment of clinical parameters associated with increased frequency of mutant T cells in patients with systemic lupus erythematosus. Arthritis Rheum. 37, 270.

de Mello, S.B., Novaes, G.S., Laurindo, I.M., Muscara, M.N., Maciel, F.M. and Cossermelli, W. (1997) Nitric oxide synthase inhibitor influences prostaglandin and interleukin-1 production in experimental arthritic joints. Inflamm. Res. 46, 72.

Donovan, P.J., Smith, G.T., Lawlor, T.E., Cifone, M.A., Murli, H. and Keefer, L.K. (1997) Quantification of diazeniumdiolate mutagenicity in four different in vitro assays. Nitric. Oxide. 1, 158.

Feelisch, M. and Stamler, J.S. (1996) Donors of nitrogen oxides. In: M. Feelisch and J.S. Stamler (Eds.), *Methods in* Nitric Oxide Research. John Wiley & Sons, Inc. New York, p. 71.

Felley-Bosco, E., Mirkovitch, J., Ambs, S., Macé, K., Pfeifer, A., Keefer, L.K. and Harris, C.C. (1995) Nitric oxide and ethylnitrosourea: Relative mutagenicity in the *p53* tumor suppressor and hypoxanthine-phosphoribosyltransferase genes. Carcinogenesis 16, 2069.

Firestein, G.S., Echeverri, F., Yeo, M., Zvaifler, N.J. and Green, D.R. (1997) Somatic mutations in the p53 tumor suppressor gene in rheumatoid arthritis synovium. Proc. Natl. Acad. Sci. USA. 94, 10895.

Fuscoe, J.C., Zimmerman, L.J., Harrington-Brock, K. and Moore, M.M. (1992) Large deletions are tolerated at the hprt locus of in vivo derived human T-lymphocytes. Mutat. Res. 283, 255.

Fuscoe, J.C., Nelsen, A.J. and Pilia, G. (1994) Detection of deletion mutations extending beyond the HPRT gene by multiplex PCR analysis. Somat. Cell Mol. Genet. 20, 39.

Giver, C.R., Nelson, S.L. and Grosovsky, A.J. (1993) Spectrum of spontaneous HPRT$^-$ mutations in TK6 human lymphoblasts. Environ. Mol. Mutagen. 22, 138.

Grabowski, P.S., Wright, P.K., Van 't Hof, R.J., Helfrich, M.H., Ohshima, H. and Ralston, S.H. (1997) Immunolocalization of inducible nitric oxide synthase in synovium and cartilage in rheumatoid arthritis and osteoarthritis. Br. J.

Rheumatol. 36, 651.

Grant, D.D., Goldstein, R., Karsh, J. and Birnboim, H.C. (1998) Elimination of non-viable 6-thioguanine-sensitive T cells from viable T cells prior to PCR analysis. J. Immunol. Methods (in press).

Greenwood, S.K., Armstrong, M.J., Hill, R.B., Bradt, C.I., Johnson, T.E., Hilliard, C.A. and Galloway, S.M. (1998) Fewer chromosome aberrations and earlier apoptosis induced by DNA synthesis inhibitors, a topoisomerase II inhibitor or alkylating agents in human cells with normal compared with mutant p53. Mutat. Res. 401, 39.

Grossman, J.M. and Brahn, E. (1997) Rheumatoid arthritis: current clinical and research directions. J. Womens. Health 6, 627.

Hoffmeister, R.T. (1983) Methotrexate therapy in rheumatoid arthritis: 15 years experience. Am. J. Med. 75, 69.

Hou, S.M., Steen, A.M., Falt, S. and Andersson, B. (1993) Molecular spectrum of background mutation at the hprt locus in human T-lymphocytes. Mutagenesis 8, 43.

Hukkanen, M., Hughes, F.J., Buttery, L.D., Gross, S.S., Evans, T.J., Seddon, S.X., Riveros-Moreno V, Macintyre, I., Polak, J.M., Seddon, S. and Riveros-Moreno, V. (1995) Cytokine-stimulated expression of inducible nitric oxide synthase by mouse, rat, and human osteoblast-like cells and its functional role in osteoblast metabolic activity. Endocrinology 136, 5445.

Huttner, E. and Holzapfel, B. (1996) HPRT mutant frequencies and detection of large deletions by multiplex- PCR in human lymphocytes of vinyl chloride exposed and non-exposed populations. Toxicol. Lett. 88, 175.

Jendro, M.C., Ganten, T., Matteson, E.L., Weyand, C.M. and Goronzy, J.J. (1995) Emergence of oligoclonal T cell populations following therapeutic T cell depletion in rheumatoid arthritis. Arthritis Rheum. 38, 1242.

Kang, J.H. and Kim, S.M. (1997) DNA cleavage by hydroxyl radicals generated in the Cu,Zn-superoxide dismutase and hydrogen peroxide system. Mol. Cells 7, 777.

Kaur, H. and Halliwell, B. (1994) Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. FEBS Lett. 350, 9.

Kuipers, E.J. (1997) Helicobacter pylori and the risk and management of associated diseases: gastritis, ulcer disease, atrophic gastritis and gastric cancer. Aliment. Pharmacol. Ther. 11 Suppl 1:71-88, 71.

Kusunoki, Y., Kodama, Y., Hirai, Y., Kyoizumi, S., Nakamura, N. and Akiyama, M. (1995) Cytogenetic and immunologic identification of clonal expansion of stem cells into T and B lymphocytes in one Atomic-bomb survivor. Blood 86, 2106.

Laakso, M., Mutru, O., Isomaki, H. and Koota, K. (1986) Cancer mortality in patients with rheumatoid arthritis. J. Rheumatol. 13, 522.

Lichtenauer-Kaligis, E.G., Thijssen, J., Den Dulk, H., Van de Putte, P., Tasseron-de Jong, J.G. and Giphart-Gassler, M. (1993) Genome wide spontaneous mutation in human cells determined by the spectrum of mutations in hprt cDNA genes. Mutagenesis 8, 207.

Liu, A.H., Creadon, G. and Wysocki, L.J. (1992) Sequencing heavy- and light-chain variable genes of single B-hybridoma cells by total enzymatic amplification [published erratum appears in Proc Natl Acad Sci U S A 1992 Oct 15;89(20): 9974]. Proc. Natl. Acad. Sci. USA. 89, 7610.

Lo, Y.Y., Conquer, J.A., Grinstein, S. and Cruz, T.F. (1998) Interleukin-1 beta induction of c-fos and collagenase expression in articular chondrocytes: involvement of reactive oxygen species. J. Cell Biochem. 69, 19.

Maini, R.N. and Zvaifler, N.J. (1994) Rheumatoid arthritis and spondyloarthropathy. In: J.H. Klippel and P.A. Dieppe (Eds.), Rheumatology. Mosby, Times Mirror International Publishers Limited, Toronto.

McCartney-Francis, N., Allen, J.B., Mizel, D.E., Albina, J.E., Xie, Q.W., Nathan, C.F. and Wahl, S.M. (1993) Suppression of arthritis by an inhibitor of nitric oxide synthase. J. Exp. Med. 178, 749.

McInnes, I.B., Leung, B.P., Field, M., Wei, X.Q., Huang, F.P., Sturrock, R.D., Kinninmonth, A., Weidner, J., Mumford, R. and Liew, F.Y. (1996) Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. J. Exp. Med. 184, 1519.

Miesel, R., Kurpisz, M. and Kröger, H. (1996) Suppression of inflammatory arthritis by simultaneous inhibition of nitric oxide synthase and NADPH oxidase. Free Radic. Biol. Med. 20, 75.

Mietz, J.A., Unger, T., Huibregtse, J.M. and Howley, P.M. (1992) The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. EMBO J. 11, 5013.

Milich, D.R., Jones, J., Hughes, J. and Maruyama, T. (1994) Hepatitis B virus infection, the immune response and hepatocellular carcinoma. Ciba. Found. Symp. 187:113-29; discussi, 113.

Morris, T., Masson, W., Singleton, B. and Thacker, J. (1993) Analysis of large deletions in the *HPRT* gene of primary human fibroblasts using the polymerase chain reaction. Somat. Cell Mol. Genet. 19, 9.

Muller-Ladner, U., Kriegsmann, J., Gay, R.E. and Gay, S. (1995) Oncogenes in rheumatoid arthritis. Rheumatic Disease Clinics of North America 21, 675.

Murrell, G.A., Doland, M.M., Jang, D., Szabo, C., Warren, R.F. and Hannafin, J.A. (1996) Nitric oxide: an important articular free radical. J. Bone Joint Surg. Am. 78, 265.

Nelson, S.L., Giver, C.R. and Grosovsky, A.J. (1994) Spectrum of X-ray-induced mutations in the human *hprt* gene. Carcinogenesis 15, 495.

Nguyen, T., Brunson, D., Crespi, C.L., Penman, B.W., Wishnok, J.S. and Tannenbaum, S.R. (1992) DNA damage and mutation in human cells exposed to nitric oxide in vitro. Proc. Natl. Acad. Sci. USA 89, 3030.

Nicklas, J.A., Hunter, T.C., Sullivan, L.M., Berman, J.K., O'Neill, J.P. and Albertini, R.J. (1987) Molecular analyses of in vivo hprt mutations in human T-lymphocytes. I. Studies of low frequency 'spontaneous' mutants by Southern blots. Mutagenesis 2, 341.

Nicklas, J.A., O'Neill, J.P., Sullivan, L.M., Hunter, T.C., Allegretta, M., Chastenay, B.F., Libbus, B.L. and Albertini, R.J. (1988) Molecular analyses of in vivo hypoxanthine-guanine phosphoribosyltransferase mutations in human T-lymphocytes: II. Demonstration of a clonal amplification of hprt mutant T-lymphocytes in vivo. Environ. Mol. Mutagen. 12, 271.

Nicklas, J.A., Hunter, T.C., O'Neill, J.P. and Albertini, R.J. (1991) Fine structure mapping of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene region of the human X chromosome (Xq26). Am. J. Hum. Genet. 49, 267.

Ohshima, H. and Bartsch, H. (1994) Chronic infections and inflammatory processes as cancer risk factors: Possible role of nitric oxide in carcinogenesis.

Mutat. Res. Fundam. Mol. Mech. Mutagen. 305, 253.

Okamoto, T., Akaike, T., Nagano, T., Miyajima, S., Suga, M., Ando, M., Ichimori, K. and Maeda, H. (1997) Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide. Arch. Biochem. Biophys. 342, 261.

Osterholm, A.M., Falt, S., Lambert, B. and Hou, S.M. (1995) Classification of mutations at the human hprt-locus in T-lymphocytes of bus maintenance workers by multiplex-PCR and reverse transcriptase-PCR analysis. Carcinogenesis 16, 1909.

Paulson, T.G., Almasan, A., Brody, L.L. and Wahl, G.M. (1998) Gene amplification in a p53-deficient cell line requires cell cycle progression under conditions that generate DNA breakage. Mol. Cell Biol. 18, 3089.

Phillips, E.N., Gebow, D. and Liber, H.L. (1997) Spectra of X-ray-induced and spontaneous intragenic HPRT mutations in closely related human cells differentially expressing the p53 tumor suppressor gene. Radiat. Res. 147, 138.

Podlutsky, A., Osterholm, A.M., Hou, S.M., Hofmaier, A. and Lambert, B. (1998) Spectrum of point mutations in the coding region of the hypoxanthine- guanine phosphoribosyltransferase (hprt) gene in human T-lymphocytes in vivo. Carcinogenesis 19, 557.

Pongsaensook, P., Smith, L.E. and Grosovsky, A.J. (1997) Isolation of an APRT heterozygote from TK6 human lymphoblasts: predominance of multi-locus loss of heterozygosity among spontaneous APRT-mutants. Mutat. Res. 377, 27.

Radomski, M.W., Palmer, R.M. and Moncada, S. (1990) Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc. Natl. Acad. Sci. USA. 87, 10043.

Ralston, S.H. and Grabowski, P.S. (1996) Mechanisms of cytokine induced bone resorption - role of nitric oxide, cyclic guanosine monophosphate, and prostagladins. Bone 19, 29.

Rauhala, P., Khaldi, A., Mohanakumar, K.P. and Chiueh, C.C. (1998) Apparent role of hydroxyl radicals in oxidative brain injury induced by sodium nitroprusside [In Process Citation]. Free Radic. Biol. Med. 24, 1065.

Rep, M.H., van Oosten, B.W., Roos, M.T., Ader, H.J., Polman, C.H. and van Lier, R.A. (1997) Treatment with depleting CD4 monoclonal antibody results in a

preferential loss of circulating naive T cells but does not affect IFN- gamma secreting TH1 cells in humans. J. Clin. Invest. 99, 2225.

Routledge, M.N., Wink, D.A., Keefer, L.K. and Dipple, A. (1993) Mutations induced by saturated aqueous nitric oxide in the pSP189 *supF* gene in human Ad293 and *E. coli* MBM7070 cells. Carcinogenesis 14, 1251.

Sakkas, L.I., Chen, P.F. and Platsoucas, C.D. (1994) T-cell antigen receptors in rheumatoid arthritis. Immunol. Res. 13, 117.

Sakurai, H., Kohsaka, H., Liu, M.F., Higashiyama, H., Hirata, Y., Kanno, K., Saito, I. and Miyasaka, N. (1995) Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J. Clin. Invest. 96, 2357.

Sandhu, J.K. and Birnboim, H.C. (1997) Mutagenicity and cytotoxicity of reactive oxygen and nitrogen species in the MN-11 murine tumor cell line. Mutat. Res. 379, 241.

Schwartz, J.L., Jordan, R., Sedita, B.A., Swenningson, M.J., Banáth, J.P. and Olive, P.L. (1995) Different sensitivity to cell killing and chromosome mutation induction by gamma rays in two human lymphoblastoid cell lines derived from a single donor: Possible role of apoptosis. Mutagenesis 10, 227.

Schwartz, J.L. and Jordan, R. (1997) Selective elimination of human lymphoid cells with unstable chromosome abberations by p53-dependant apoptosis. Carcinogenesis 18, 201.

Sfikakis, P.P., Tesar, J., Theocharis, S., Klipple, G.L. and Tsokos, G.C. (1994) Increased frequency of in vivo *hprt* gene-mutated T cells in the peripheral blood of patients with systemic sclerosis. Ann. Rheum. Dis. 53, 122.

Skandalis, A., Da Cruz, A.D., Curry, J., Nohturfft, A., Curado, M.P. and Glickman, B.W. (1997) Molecular analysis of T-lymphocyte HPRT- mutations in individuals exposed to ionizing radiation in Goiania, Brazil. Environ. Mol. Mutagen. 29, 107.

Spencer, D.L., Hines, K.C. and Caspary, W.J. (1994) An in situ protocol for measuring the expression of chemically-induced mutations in mammalian cells. Mutat. Res. 312, 85.

Sriram, S. (1994) Longitudinal study of frequency of *HPRT* mutant T cells in patients with multiple sclerosis. Neurology 44, 311.

St Clair, E.W., Wilkinson, W.E., Lang, T., Sanders, L., Misukonis, M.A., Gilkeson, G.S., Pisetsky, D.S., Granger, D.L. and Weinberg, J.B. (1996) Increased expression of blood mononuclear cell nitric oxide synthase type 2 in rheumatoid arthritis patients. J. Exp. Med. 184, 1173.

St.Clair, E.W. (1998) Nitric oxide--friend or foe in arthritis? J. Rheumatol. 25, 1451.

Steingrimsdottir, H., Rowley, G., Waugh, A., Beare, D., Ceccherini, I., Cole, J. and Lehmann, A.R. (1993) Molecular analysis of mutations in the *hprt* gene in circulating lymphocytes from normal and DNA-repair-deficient donors. Mutat. Res. DNA Repair 294, 29.

Swann, P.F., Waters, T.R., Moulton, D.C., Xu, Y.Z., Zheng, Q.G., Edwards, M. and Mace, R. (1996) Role of postreplicative DNA mismatch repair in the cytotoxic action of thioguanine. Science 273, 1109.

Tamura, T., Nakanishi, T., Kimura, Y., Hattori, T., Sasaki, K., Norimatsu, H., Takahashi, K. and Takigawa, M. (1996) Nitric oxide mediates interleukin-1-induced matrix degradation and basic fibroblast growth factor release in cultured rabbit articular chondrocytes: A possible mechanism of pathological neovascularization in arthritis. Endocrinology 137, 3729.

Tanaka, S., Matsui, T., Murakami, T., Ishizuka, T., Sugiura, M., Kawashima, K. and Sugita, T. (1998) Immunological abnormality associated with the augmented nitric oxide synthesis in adjuvant-induced arthritis [In Process Citation]. Int. J. Immunopharmacol. 20, 71.

Theocharis, S., Sfikakis, P.P., Lipnick, R.N., Klipple, G.L., Steinberg, A.D. and Tsokos, G.C. (1995) Characterization of in vivo mutated T cell clones from patients with systemic lupus erythematosus. Clin. Immunol. Immunopathol. 74, 135.

Tsuji, S., Kawano, S., Tsujii, M., Takei, Y., Tanaka, M., Sawaoka, H., Nagano, K., Fusamoto, H. and Kamada, T. (1996) *Helicobacter pylori* extract stimulates inflammatory nitric oxide production. Cancer Lett. 108, 195.

Turner, D.R., Morley, A.A., Haliandros, M., Kutlaca, R. and Sanderson, B.J. (1985) In vivo somatic mutations in human lymphocytes frequently result from major gene alterations. Nature 315, 343.

Ueki, Y., Miyake, S., Tominaga, Y. and Eguchi, K. (1996) Increased nitric oxide levels in patients with rheumatoid arthritis. J. Rheumatol. 23, 230.

Vrieling, H., Thijssen, J.C., Rossi, A.M., van Dam, F.J., Natarajan, A.T., Tates, A.D. and van Zeeland, A.A. (1992) Enhanced hprt mutant frequency but no significant difference in mutation spectrum between a smoking and a non-smoking human population. Carcinogenesis 13, 1625.

Wagner, U.G., Koetz, K., Weyand, C.M. and Goronzy, J.J. (1998) Perturbation of the T cell repertoire in rheumatoid arthritis. Proc. Natl. Acad. Sci. USA. 95, 14447.

Wigand, R., Meyer, J., Busse, R. and Hecker, M. (1997) Increased serum NG-hydroxy-L-arginine in patients with rheumatoid arthritis and systemic lupus erythematosus as an index of an increased nitric oxide synthase activity. Ann. Rheum. Dis. 56, 330.

Wilkinson, D. (1997) Investigations into genomic instability of endogenous and transfected hprt genes in mouse and human tumour cells. University of Ottawa Thesis.

Williams, C.A., Bloch, D.A., Sibley, J., Haga, M., Wolfe, F., Raynauld, J., Singh, G., Hickey, A.R. and Fries, J.F. (1996) Lymphoma and leukemia in rheumatoid arthritis: are they associated with azathioprine, cyclophosphamide, or methotrexate? A matched case-control study in the Arthritis, Rheumatism and Aging Medical Information System (ARAMIS) population. J Clin Rheumatol 2, 64.

Wink, D.A., Kasprzak, K.S., Maragos, C.M., Elespuru, R.K., Misra, M., Dunams, T.M., Cebula, T.A., Koch, W.H., Andrews, A.W., Allen, J.S. and Keefer, L.K. (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science 254, 1001.

Wink, D.A., Feelisch, M., Fukuto, J., Chistodoulou, D., Jourd'heuil, D., Grisham, M.B., Vodovotz, Y., Cook, J.A., Krishna, M., DeGraff, W.G., Kim, S., Gamson, J. and Mitchell, J.B. (1998) The cytotoxicity of nitroxyl: possible implications for the pathophysiological role of NO. Arch. Biochem. Biophys. 351, 66.

Zhen, W., Denault, C.M., Loviscek, K., Walter, S., Geng, L. and Vaughan, A.T.M. (1995) The relative radiosensitivity of TK6 and WI-L2-NS lymphoblastoid cells derived from a common source is primarily determined by their p53 mutational status. Mutat. Res. Lett. 346, 85.

Zhuang, J.C., Lin, C., Lin, D. and Wogan, G.N. (1998) Mutagenesis associated with nitric oxide production in macrophages. Proc. Natl. Acad. Sci. USA. 95, 8286.

Appendix I

List of reagents and suppliers

Reagent name	Supplier
agarose	Boehringer Mannheim
p-aminobenzenesulfanilamide	Sigma
aminopterin	Sigma
ammonium sulfate	BDH
AmpliTaq polymerase	Perkin Elmer
CDTA (trans-1,2-diaminocyclohexane)	Sigma
chloroform	BDH
3,5 diaminobenzoic acid (DABA)	Aldrich
diethyl procarbonate (DEPC)	Kodak
EDTA (ethylene diaminetetraacetic acid)	BDH
ethidium bromide	Sigma
dithiolthrietol	Gibco BRL
EtOH	Commercial Alcohols Inc.
Fetal calf serum (FCS)	ImmunoCorp
Ficoll-Hypaque	ImmunoCorp
glutamine	Gibco BRL
Hybridoma Serum Free Medium	Gibco BRL
hypoxanthine	Sigma
Interleukin-2 (IL-2)	Sigma
isopropanol	BDH

Reagent name	Supplier
lithium chloride (LiCl)	BDH
magnesium chloride	Fisher Scientific
2-mercaptoethanol	BDH
MethoCult	StemCell Technologies Inc.
micrococcal nuclease	Sigma
microfuge tubes	Rose Scientific
N-(1-napthyl) ethylene diamine dihydrochloride	Sigma
nitroglycerin	Sabex
NP-40	BDH
NuSieve	FMC BioProducts
oligodT_{12-18} primer	Pharmacia
phenol	BDH
phosphoric acid	BDH
phytohemagglutinin (PHA)	Sigma
polyethylene tubes (15ml)	Sarstedt
potassium chloride (KCl)	BDH
potassium dihydrogen orthophosphate (KH_2PO_4)	BDH
Proteinase K	EM Industries
RPMI 1640 medium	Gibco BRL
RPMI 1640 phenol red free medium	Gibco BRL
silicone oil (DC 200/200)	BDH
sodium acetate	BDH

Reagent name	Supplier
sodium citrate	BDH
sodium dodecylsulfate (SDS)	BDH
sodium nitrite	Fisher Scientific
sodium nitroprusside	Aldrich
SuperScript reverse transcriptase	Gibco BRL
6-thioguanine (2-amino-6-mercaptopurine, 6-TG)	Sigma
tissue culture plates (96-well)	Costar
Tris-HCl (tris (hydroxymethyl) methylamine)	BDH
trypan blue	MCB
Tween 20	Sigma
urea	BDH

Appendix II

Isolation of DNA from peripheral white blood cells

Blood was centrifuged at 3,000 rpm (1,300 x g) for 10 min using a Sorvall refrigerated centrifuge and the buffy coat was transferred to a sterile 2 ml screw-cap microfuge tube. The buffy coat was brought up in 1 ml PBS and the red blood cells were lysed with 100 µl of S/P Lysing Reagent. The microfuge tube was centrifuged for 3 min at 13,000 rpm (14,000 x g) using a microcentrifuge. The remaining WBCs were washed three times with 1 ml PBS, then lysed in 750 µl of DNA extraction solution (1 M LiCl, 1 M urea, 50 mM TrisHCl, pH 8.0, 5 mM CDTA and 0.2% SDS) and incubated overnight at 45°C with 100 µg/ml proteinase K. 800 µl of phenol/chloroform (1 g phenol/ 1 ml chloroform) was added and the sample was shaken for 20 min. After 5 min of centrifugation, the aqueous phase was transferred to a new tube and the phenol/chloroform treatment was repeated. After a second centrifugation, the aqueous phase was transferred to a new tube and 1 ml chloroform was added. The sample was shaken briefly, centrifuged for another 5 min and the aqueous phase was removed to a new tube. To this, an equal volume of isopropanol was added and after several inversions the tube was left to stand at room temperature for 20 min. The precipitate was pelletted for 3 min at 13,000 rpm (14,000 x g) and the supernatant was removed. The pellet was washed with 500 µl of an alcohol/salt solution (70% EtOH, 30% [0.2 M LiCl, 10 mM TrisHCl, pH 7.5, 1 mM CDTA]) and vortexed. After 20 min at -20°C, the sample was centrifuged again for 2 min and

the supernatant was removed. The pellet was dried under vacuum and brought up in 200-500 µl of CT (1 mM CDTA, 10 mM TrisHCl, pH 7.5). DNA concentrations were determined using the DABA fluorometric method (Appendix III).

Appendix III

DABA method for the quantitation of DNA

All DNA samples were analysed in duplicate. 200 µl of DABA reagent (15% 3, 5-diaminobenzoic acid in ddH_2O) was placed in a sterile 1.5 microfuge tubes and to this 1 µl of DNA sample was added. Samples were incubated at 60°C for 30 min and then transferred to 16 x 100 mm glass tubes containing 2.5 ml of 1 N HCl. Fluorescence (Ex=410 nm, An=500 nm) was measured using a Perkin Elmer LS-5 Fluorescence-Spectrophotometer. The concentration of DNA was determined by comparing the average fluorescence value to a standard curve generated using standard, sonicated WBC DNA.

Appendix IV

MP-PCR and RT-PCR primers

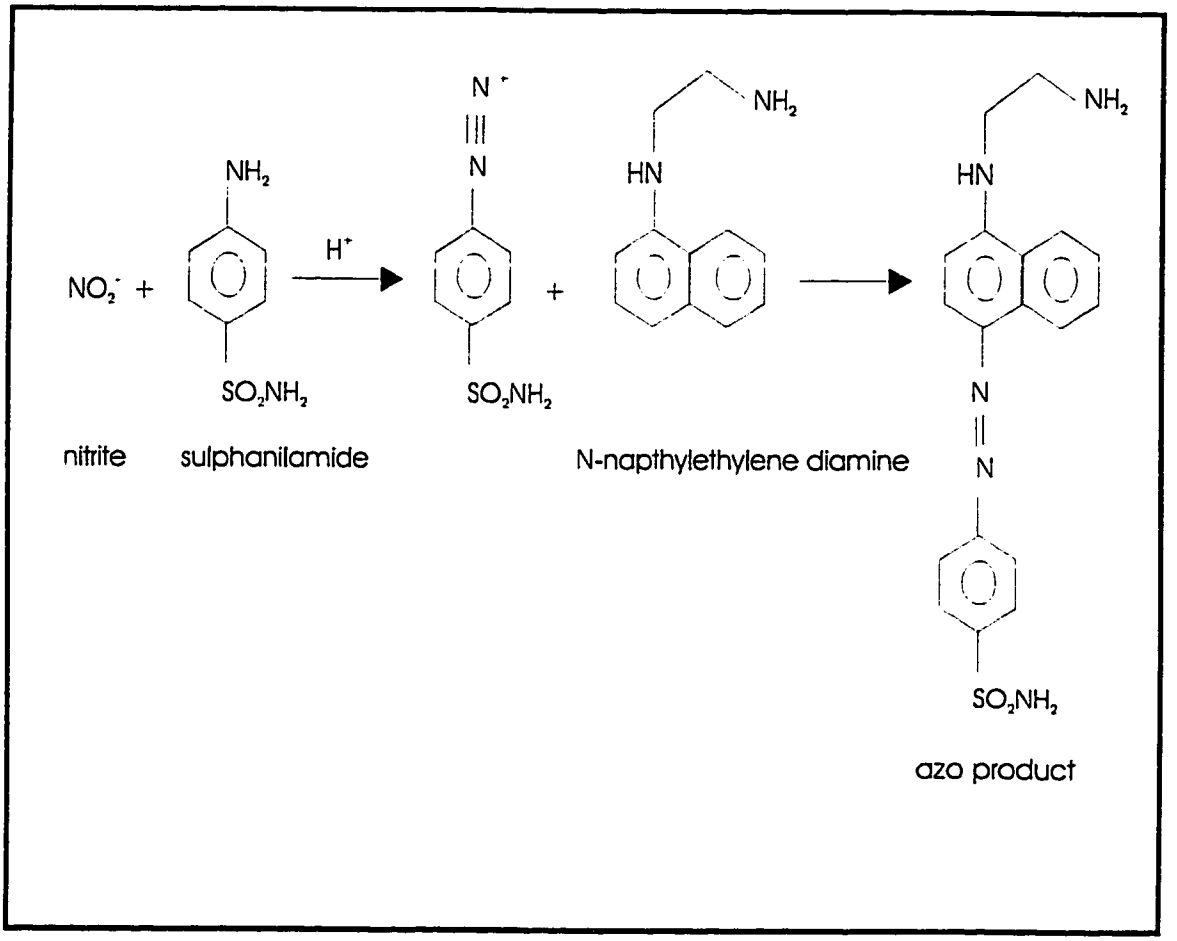
Primer name	Sequence (5' to 3')	Position in genomic DNA	Expected PCR product size (bp)
hprt exon 2 forward	TGGGATTACACGTGTGAACCAACC	14578	572
hprt exon 2 reverse	GACTCTGGCTAGAGTTCCTTCTTC	15126	
hprt exon 3 forward	GGGGGCTATAAATTCTTTGCTGAC	16676	535
hprt exon 3 reverse	GGGATTTCTTCAATTCGCCAATAC	17187	
hprt exon 7/8 forward	AAAGGACCCCACGAA	39827	492
hprt exon 7/8 reverse	TTCAACTATTTCAGCCAACA	40299	
hprt exon 9 forward	GGATATGCCCTTGACTATAA	40069	1653
hprt exon 9 reverse	CTATAGGCTCATAGTGCAAA	41702	
DXS79 forward	AGGAATAACTTGACATTTGA	33	302
DXS79 reverse	TGTTACATGGAAAAGAAG	317	
DXS86 forward	GCATTTAGGATTCCAATA	12	390
DXS86 reverse	ATAAGTGCATTCTTTCTGAT	382	
HF9 forward	AGAGCAAAAGCGAAATGTGA	33818	370
HF9 reverse	TCCCCCACTATCTCCTTGAC	34168	
hprt mRNA forward	GCCTCCTCCTCTGCTCCG	16	547

Primer name	Sequence (5' to 3')	Position in genomic DNA	Expected PCR product size (bp)
hprt mRNA reverse	CTTGACCATCTTTGGATTAT ACTGCCTGAC	533	
TS mRNA forward	CATCATGTGCGCTTGGAAT C	636	186
TS mRNA reverse	TGCGCAATCATGTACGTGA G	802	

Position in genomic DNA refers to the numbering of the gene in which the primer anneals.

Appendix V

Schematic of the Griess reaction



Appendix VI

Extraction of RNA from suspension cells

Cells were transferred to a 15 ml polyethylene tube and centrifuged at 4°C for 5 min at 700 rpm (70 x g). Medium was removed and the cell pellet was washed twice with 5 ml cold PBS. After the final removal of PBS, the cell pellet was vortexed briefly and 3-4 ml RNA extraction solution (RES, 0.5 M LiCl, 1 M urea, 5 mM CDTA, 1% SDS) was shot in. The sample was sonicated briefly to reduce viscosity and then digested at 50°C for 30 min with 100 µg/ml proteinase K. The tube was chilled on ice for 2 min and brought to room temperature. DNA and RNA were precipitated by the addition of 100 µl 4 M sodium acetate and 2 volumes cold 95% ethanol. After mixing, the sample was placed at -20°C for 20 min and then centrifuged at 10,000 rpm (9,000 x g) for 10 min at 0°C using a Sorvall high-speed refrigerated centrifuge. After the supernatant was discarded, the pellet was dissolved in 0.9 ml RES and digested a second time with 100 µg/ml proteinase K at 50°C for 30 min. The sample was transferred to a 1.5 ml microfuge tube to which 100 µl phenol/chloroform (1g phenol/1 ml chloroform) was added. The tube was vortexed several times over a 3 min period and centrifuged at 13,000 rpm (14,000 x g) for 5 min. The aqueous layer was transferred to another microfuge tube while the interface material was back extracted with 100 µl RES. After being vortexed for 10 sec and centrifuged for 2 min, the supernatant was combined with the first extract. 100 µl of chloroform was added to the tube, the sample was vortexed for 20 sec and centrifuged at

13,000 rpm for 2 min. The supernatant was transferred to a 2 ml microcentrifuge tube and RNA was precipitated with 3 µl 2 N acetic acid and 1 ml LiCl/ ethanol (3 volumes 5 M LiCl: 2 volumes 95% ethanol). The sample was mixed and placed on ice overnight. The tube was centrifuged at 13,000 rpm (14,000 x g) for 5 min and the supernatant was discarded. The pellet was dissolved in 0.4 ml CCS (1 mM sodium citrate, 1 mM CDTA, 0.1% SDS), transferred to a 1.5 ml microfuge tube and combined with 18 µl 4 M sodium acetate and 1 ml ethanol. The sample was mixed and placed at -70°C for 5 min, then at -20°C for 15 min. The tube was centrifuged at 13,000 rpm for 2 min and the supernatant was discarded. This ethanol precipitation was repeated once more. The final pellet was dissolved in 50 µl CCS. RNA concentration was determined by measuring sample absorbance at λ=260 nm.