TUMOUR PROMOTION IN BLADDER CANCER - THE INFLUENCE OF INFLAMMATION ON DNA DAMAGE AND PROTECTION FROM THIS DAMAGE BY CHROMOSOME 11

by

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B.Sc. (Kinesiology), Simon Fraser University, 1990

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in the School of Kinesiology

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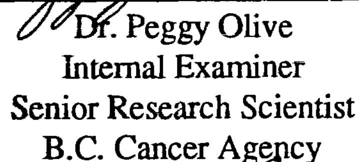

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ABSTRACT

Little information is available on the forces that drive tumour promotion and progression in humans. The knowledge we have comes mainly from animal studies. The goal of this thesis was to study the processes involved in human tumour promotion by focusing on one aspect that is receiving much current attention. A hallmark activity of tumour promoters is their ability to recruit inflammatory cells and to stimulate them to release reactive oxygen species (ROS). This thesis uses both *in vivo* and *in vitro* model systems to explore the role of inflammation in inducing genetic damage in human populations.

In vivo, we looked at the influence of inflammation on DNA damage in patients on indwelling urinary catheterization, a population characterized by repeated bacterial infection in the urinary bladder. Risk for urinary bladder cancer is elevated 20-fold among such individuals. Micronucleus (MN) frequencies of exfoliated urothelial cells (an index of DNA damage) and urinary white blood cell counts from patients at a long-term care facility in Vancouver were compared to non-catheterized controls. Although urine from the catheterized group had significantly larger numbers of white blood cells compared to controls ($p < 0.001$), no significant difference in MN frequencies in the 2 groups existed (mean MN frequencies, controls: 0.10 $\pm$ 0.03%; catheterized: 0.14 $\pm$ 0.03%, $p = 0.13$). We found, however, that a high intake of vitamins and non-steroidal anti-inflammatory drugs was occurring in the catheterized group; hence, the inflammatory reaction cannot be eliminated as a risk factor.

The remaining studies in this thesis explored the hypothesis that altered sensitivity of clones of cells to reactive oxygen species in a tissue could facilitate clonal expansion under conditions of chronic inflammation. Loci on chromosome 11 have previously been shown to play a role in altering the sensitivity of tumour

cells to X-irradiation. To test the influence of chromosome 11 on ROS-induced DNA damage *in vitro,* we used a model system consisting of three cell lines: 1) a primary bladder carcinoma cell line ('parent'); 2) a derivative of this cell line into which a normal chromosome 11 had been inserted by microcell fusion ('hybrid'); and 3) a 'revertant' cell line that had spontaneously lost the insert. These studies showed that the chromosome insert provided a significant protection against induction of MN by hydrogen peroxide or x-rays ($p < 0.0001$). Two mechanistic studies of the processes underlying this protection showed that: 1) catalase levels were significantly increased in the 'hybrid' cell line ($p < 0.001$), and 2) the fidelity and efficiency of DNA double strand break (dsb) rejoining does not differ in nuclear extracts from 'parent', 'hybrid' and 'revertant' cells, although the insertion of chromosome 11 did affect the types of reaction products that are produced. Although these studies suggest that altered cell sensitivity to ROS may play a role in carcinogenesis, further molecular studies are required to elucidate this role.

DEDICATION

To my parents, Stanley W. Hofseth and E. Jeannette Curtis for both their emotional and financial support. To my brother, Lance Hofseth, for being supportive in his own unique way. Finally, to all my closest friends who believed in me. I thank you.

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TABLE OF CONTENTS

APPROVAL	**ii**
ABSTRACT	**iii**
DEDICATION	**v**
ACKNOWLEDGMENTS	**vi**
TABLE OF CONTENTS	**vii**
LIST OF FIGURES	**xiv**
LIST OF TABLES	**xvi**
LIST OF ABBREVIATIONS	**xvii**
THESIS FORMAT	**xviii**

CHAPTER 1

INTRODUCTION 1

1.1 Tumour Promotion Models 2

1.2 *Schistosoma haematobium* and Bladder Cancer 5
- 1.2.1 Epidemiology
- 1.2.2 Etiology of schistosomiasis-associated bladder cancer

1.3 Indwelling Urinary Catheters and Bladder Cancer 9
- 1.3.1 Epidemiology
- 1.3.2 Complications associated with catheterization
- 1.3.3 Etiology of catheter-associated bladder cancers

1.4 Inflammation and Cancer 14
- 1.4.1 The respiratory burst
- 1.4.2 Reactive oxygen species (ROS)
- 1.4.3 Lipid peroxidation
- 1.4.4 The arachidonic acid cascade
  - 1.4.4.1 Direct DNA binding
  - 1.4.4.2 Carcinogen bioactivation
    - *Prostaglandin H synthase in bioactivation*
    - *Myeloperoxidase in bioactivation*

1.4.4.3 Free radical production during arachidonic acid metabolism
1.4.5 Nitric oxide and nitrosamines

1.5 Micronuclei (MN) as a Quantifiable Indicator of *In Vitro* and *In Vivo* DNA Damage 25

1.6 Chromosome 11 and Bladder Cancer 27

1.7 Loci on Chromosome 11 Associated with Sensitivity to ROS 30
1.7.1 Chromosome 11 and catalase activity
1.7.2 Chromosome 11 and DNA repair

1.8 References 35

CHAPTER 2
HYPOTHESES, OBJECTIVES AND APPROACHES 61

2.1 *In Vitro* Studies 62
2.1.1 Hypothesis 2
2.1.2 Objective and Approach
2.1.3 Hypothesis 3
2.1.4 Objective and Approach
2.1.5 Hypothesis 4
2.1.6 Objective and Approach

2.2 *In Vivo* Study 65
2.2.1 Hypothesis 1
2.2.2 Objective and Approach

2.3 References 66

CHAPTER 3

The influence of chromosome 11 and associated catalase levels on DNA damage induced by hydrogen peroxide 67

3.1 Abstract 68

3.2 Introduction 69

3.3 Materials and methods 71
- 3.3.1 Cells and culture conditions
- 3.3.2 Micronucleus (MN) assay
- 3.3.3 Catalase assay
- 3.3.4 Statistical analysis

3.4 Results and Discussion 74

3.5 References 79

CHAPTER 4

Rejoining of DNA double strand breaks after the introduction of chromosome 11 into a radiosensitive bladder carcinoma cell line 82

4.1 Abstract 83

4.2 Introduction 84

4.3 Materials and methods 87
- 4.3.1 Cells and culture conditions
- 4.3.2 Description of plasmid pUC18 DNA
- 4.3.3 Preparation of plasmid and nuclear extracts
- 4.3.4 Plasmid rejoining assay
- 4.3.5 Gel electrophoresis and Southern analysis
- 4.3.6 Quantification of DNA bands in Southern blots
- 4.3.7 Bacterial transformation and rejoin fidelity
- 4.3.8 Statistical analysis

4.4 Results 93
4.4.1 Rejoining of *Sal*I-induced dsb by 'parent' extracts
4.4.2 Effect of insertion of chromosome 11 into bladder carcinoma cell line
4.4.3 Rejoining of dsb induced by other restriction enzymes
4.4.4 Transformation frequencies and fidelity of rejoining of DNA dsb

4.5 Discussion 101

4.6 References 104

CHAPTER 5
Micronucleus frequencies in urothelial cells of catheterized patients with chronic bladder inflammation 108

5.1 Abstract 109

5.2 Introduction 110

5.3 Materials and methods 112
5.3.1 Populations
5.3.2 Sample collection
5.3.3 Sample processing
5.3.4 Sample analysis
5.3.4.1 Assessment of urinary white blood cells (WBC)
5.3.4.2 Assessment of MN frequencies
5.3.5 Effect of urinary catheters on release of ROS from human neutrophils
5.3.6 Statistical analysis

5.4 Results 117
5.4.1 Population profiles
5.4.2 Presence of inflammatory cells in urine from catheterized and control groups
5.4.3 MN frequencies in catheterized and control groups
5.4.4 Effect of a siliconized latex urinary catheter on human neutrophil activity

5.5 Discussion 124

5.6 References 128

CHAPTER 6
DISCUSSION 134

6.1 Review of the research 135

6.2 Future studies 137

6.3 Conclusions 138

6.4 References 140

APPENDICES 142
APPENDIX A
Methods development 143

1. *In vitro* studies 144
1.1 Evolution of the double strand break repair model 144
1.1.1 Preparation of nuclear extracts
1.1.2 Correction of plasmid heterogeneity
1.1.3 Optimizing transfection conditions
1.1.4 Conditions used for labeling DNA
2. *In vivo* study 150

3. References 152

APPENDIX B

Administrative forms required for the organization of the *in vivo* study outlined in chapter 5. 153

Proposal to staff at George Pearson Medical Centre requesting access to catheterized patients for urine collection 154

Ethical approval for collection of urine from catheterized patients 160

Formal consent regarding collection of urine from catheterized patients 162

Example of the informed consent form each catheterized patient signed before urine was collected 164

Example of one of the notices handed out to staff at George Pearson Centre during the study 166

Diagram of the catheter system used by patients at George Pearson Medical Centre 169

Example of the information sheet used when collecting information from the patient files at George Pearson Medical Centre 171

The questionnaire filled out by non-catheterized controls 175

APPENDIX C

Scoresheets 178

Scoresheet for MN determination in exfoliated urothelial cells from catheterized patients at George Pearson Medical Centre 179

Scoresheet for analysis of urine from catheterized patients at George Pearson Medical Centre 181

Scoresheet for MN determination in cultured bladder cells 183

LIST OF FIGURES

Figure 1.	Interrelationship between bladder pathology and cellular changes associated with carcinogenesis in schistosomal bladders	8
Figure 2.	Etiology of a bladder infection	13
Figure 3.	The respiratory burst	16
Figure 4.	The arachidonic acid cascade	21
Figure 5.	Micronucleus formation in epithelial tissues	26
Figure 6.	Strand exposure and repair model for deletion formation at the sites of short direct repeats	33
Figure 7.	The effect of H_2O_2 treatment on micronucleus formation in 'parent', 'hybrid' and 'revertant' cultures	75
Figure 8.	Catalase activity (± S.E.) in 'parent', 'hybrid' and 'revertant' cultures	76
Figure 9.	Micronucleus (MN) induction in 'parent', 'hybrid' and 'revertant' bladder carcinoma cells after exposure to xanthine/xanthine oxidase (X/XO), TPA-stimulated neutrophils, hydrogen peroxide (H_2O_2) and X-rays	86
Figure 10.	Plasmid pUC18 DNA	88
Figure 11.	Example of the bands that were examined after nuclear extracts were incubated with linearized pUC18 DNA	90
Figure 12.	Quantification of rejoined pUC18 DNA after incubation with nuclear extracts	91
Figure 13.	Autoradiogram showing rejoining of linearized (LIN) pUC18 DNA	93
Figure 14.	Autoradiogram comparing the distribution of rejoining by 'parent', 'hybrid', and 'revertant' nuclear extracts	95
Figure 15.	The effect of nuclear extracts from 'parent', 'hybrid', and 'revertant' bladder carcinoma cells on the total amount of rejoining of *Sal*I-, *Eco*RI-, and *Kpn*I-induced dsb	97
Figure 16.	The effect of nuclear extracts from 'parent', 'hybrid', and 'revertant' bladder carcinoma cells on the rejoining of linearized DNA into circular products or linear products. pUC18 DNA was linearized with either *Sal*I, *Eco*RI, and *Kpn*I restriction endonucleases	98

Figure 17. Frequency of micronucleated urothelial cells in samples from long-term urinary catheterized patients and non-catheterized controls 121
Figure 18. Results of transfection of DH5α bacteria with pUC18 DNA 147
Figure 19. A typical Southern blot showing dsb rejoining characteristics after 'parent', 'hybrid' and 'revertant' nuclear extracts were incubated with *SalI*-broken plasmid pUC18 149
Figure 20. Labeled diagram of products observed after urinary sediments from catheterized samples were centrifuged with Percoll 150

LIST OF TABLES

Table 1.	Inflammation and cancer	4
Table 2.	Carcinogenic pathways associated with urinary catheterization	14
Table 3.	Genetic alterations in human bladder cancer	28
Table 4.	Loci of chromosome 11 that play a role in DNA repair and other cellular processes	34
Table 5.	Transformation frequencies of *Eco*RI, *Sal*I, and *Kpn*I linearized pUC18 DNA after incubation with 'parent', 'hybrid', and 'revertant' nuclear extracts	99
Table 6.	Fidelity of rejoining of pUC18 by human nuclear extracts from 'parent', 'hybrid', and 'revertant' cells	100
Table 7.	General data for catheterized patients acquired from hospital records and questionnaires	117
Table 8.	Number of positive bacteria cultures for catheterized patients taken during 2 years prior to sample collection (December, 1990 - December, 1992)	118
Table 9.	Selected characteristics of catheterized patients and non-catheterized controls	119
Table 10.	Urinary white blood cells and MN frequencies in catheterized and control subjects	120
Table 11.	Density of bacteria in catheterized and control samples	120
Table 12.	Nitrite contamination in catheterized and control samples	120
Table 13.	Effects of segments of siliconized latex urinary catheters or eluates obtained from them on reactive oxygen species release from neutrophils	123
Table 14.	Determination of optimal Percoll concentration to use in collection of urothelial cells from patients on indwelling urinary catheterization	151

LIST OF ABBREVIATIONS

DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
MN	Micronuclei
H_2O_2	Hydrogen peroxide
S. haematobium	*Schistosoma haematobium*
NSAIDs	Non-steroidal anti-inflammatory drugs
DSB	Double strand break
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

THESIS FORMAT

This thesis is comprised of six chapters including a general introduction; the hypotheses, objectives and approaches; three papers, two of which are written for publication; and a general discussion and conclusion. An appendix is also included.

The introduction (chapter 1) provides background information for the three papers which follow. It covers the concept of tumour promotion in cancer, which presently is a poorly understood phenomenon. There is a special emphasis on the use of population groups such as patients with *Schistosoma haematobium* infections or patients on indwelling urinary catheterization to study promotion in humans. This chapter also summarizes current knowledge of the paths by which inflammation could act to increase cancer development. Finally, this chapter gives a review of the micronucleus assay as a biomarker in tumourigenesis and a brief background on the potential involvement of loci on chromosome 11 in reducing the sensitivity of bladder cells to inflammatory-related reactive oxygen species.

Chapter 2 covers four central hypotheses examined in this thesis and describes the objectives and approaches used to explore them.

Chapter 3 is the first of two chapters that explores the influence of chromosome 11 in the protection against ROS-induced DNA damage *in vitro.* The study outlined in this chapter found that the insertion of a normal human chromosome 11 protects bladder carcinoma cells from DNA damage induced by hydrogen peroxide. We also determined that the catalase activity was elevated in the bladder cells with an extra chromosome 11; however, the significance of this elevation has yet to be determined.

In chapter 4 we found that a cell line with an extra chromosome 11 has a reduced sensitivity to irradiation and hypothesized that repair loci on chromosome 11 might play a role in this phenomenon. We demonstrated that chromosome 11 does not influence the efficiency or the fidelity of double strand break rejoining. However the data suggest that insertion of this chromosome

may alter the relative amount of the different rejoined products. Whether this alteration plays a role in the 'parent' cell's radiosensitivity has yet to be determined.

In chapter 5, we tested the hypothesis that DNA damage is induced in the urothelium during inflammation by products of activated inflammatory cells. Individuals on long-term indwelling urinary catheterization were used as a study population. Although this group had chronic inflammation in the bladder there was no indication that DNA damage was elevated. Although the reasons for this observation are yet to be determined, possible explanations included the pathophysiology of the inflammatory reaction and the influence of vitamins, non-steroidal anti-inflammatory drugs and the catheter itself in the protection against inflammatory cell - mediated DNA damage.

Chapter 6 discusses the significance of this research project. It also discusses some unresolved issues, provides insight into prospective studies that would be useful in solving some of these issues and presents a general conclusion.

Finally, an appendix is included which covers methods development (appendix A), administrative activities required for the completion of the population study in chapter 5 (appendix B), and, examples of the scoresheets used in data collection (appendix C).

CHAPTER 1

INTRODUCTION

1.1 Tumour Promotion Models

The multistage concept of carcinogenesis was first developed from studies on skin carcinogenesis in mice (Berenblum and Shubik, 1947). This model includes three stages of carcinogenesis: initiation, promotion and progression. Initiation involves irreversible genetic damage to central control genes (protooncogenes and tumour suppressor genes). Promotion is usually a reversible process with a long latency period in which an altered phenotype of the initiated cell becomes expressed (Farber, 1981; Kinzel *et al.*, 1986). It involves gene activation and cellular proliferation followed by clonal expansion (Guyton and Kensler, 1983). Although the mechanisms are complicated, tumour promoters can act through both epigenetic and genetic changes to produce clonal expansion and eventually tumour growth. Progression involves events occurring between the development of a relatively benign growth and the development of a clinically evident cancer with potential invasion to surrounding tissue and metastases to secondary sites.

Little is known about the forces driving tumour promotion and progression in humans. However, there is some suggestion that inflammatory processes play a role. One of the hallmark activities of tumour promoters in animals is their ability to recruit inflammatory cells to an application site and stimulate a respiratory burst in these cells. The cells release ROS such as superoxide anion and hydrogen peroxide, as well as lipid oxidation products.

Observational studies with animals and humans have shown increased tumour development in tissues undergoing mechanically- or chemically-induced irritation and concomitant inflammation. Examples of laboratory studies on animals include studies showing increased tumour development in areas exposed to irritant tumour promoters such as croton oil (Berenblum, 1941). Similarly, Friedwald and Rous (1944) mechanically increased tumour

development by punching holes thus stimulating inflammation and tissue regeneration in rabbit ears after treatment with a carcinogen. Other examples of studies that have shown this phenomenon include the following: tumours in chickens induced by Rous sarcoma virus appear preferentially at sites of injury and inflammation, despite systemic viral infection (Sieweke, *et al.*, 1989); tumours induced in rodents appear in sites of metallic ear tags (Waalkes *et al.*, 1987); bladder cancer appears in sites of sutures through the bladder wall of mice (Chester *et al.*, 1987); tumours induced by bovine papillomavirus genome in transgenic mice usually occur in areas of wounding and inflammation (Lacey *et al.*, 1986); in rats the instillation of inflammatory peptides into the colon induces an inflammatory process similar to ulcerative colitis and increased rates of carcinoma of the colon occur in animals co-exposed to the colon carcinogen 1,2-dimethylhydrazine (Chester *et al.*, 1985; Chester *et al.*, 1986); flat sheets of plastic placed subcutaneously in mice result in sarcomas at that site (Brand, 1982); early neoplastic lesions are seen in the rat bladder after multiple injections of *E.coli* bacteria (Davis *et al.*, 1984); and sodium saccharin induces promotion of rat bladder carcinogenesis (Hasegawa *et al.*, 1985).

Clinical observations of humans have similarly revealed increased cancer development in areas of the human body where chronic inflammation and trauma are present. Examples are tumours associated with cutaneous lesions such as burns (Neve, 1910; Lawrence, 1952); burn scars, chronic ulcers and draining sinuses (Cruickshank *et al.*, 1963); gastric cancers associated with atrophic gastritis (Correa, 1988); esophageal adenocarcinoma after reflux esophagitis and Barrett's esophagus (Dahms and Rothstein, 1984; Cameron *et al.*, 1985); gall bladder cancer associated with chronic cholecystitis and cholelithiasis (Diehl, 1983); ulcerative colitis - associated colorectal cancer (Simmonds *et al.*, 1992); gastric cancers associated with the *Helicobacter pylori*

infection (Correa, 1992); bladder cancers associated with *Schistosoma haematobium* (Chen and Mott, 1989); and bladder cancers associated with indwelling urinary catheters (Esrig *et al.*, 1992). A summary of these findings is found in table 1.

Table 1. Inflammation and cancer

Inflammatory Stimulant	**Tumour Site**	**Reference**
ANIMAL STUDIES		
Croton oil	Mouse skin	Berenblum, 1941
Mechanical irritation	Mouse skin	Brand, 1982
Mechanical incision	Rabbit ears	Friedwald and Rous, 1944
Mechanical irritation	Rat ears	Waalkes *et al.*, 1987
Mechanical irritation	Chicken wings	Sieweke *et al.*, 1989
Mechanical irritation	Areas of irritation	Lacey *et al.*, 1986
Chemotactic peptides	Mouse colon	Chester *et al.*, 1985, 1986
Mechanical irritation	Mouse bladder	Chester *et al.*, 1987
Bacterial injection	Rat bladder	Davis *et al.*, 1984
Sodium saccharin	Rat bladder	Hasegawa *et al.*, 1985
CLINICAL OBSERVATIONS		
Burn scars	Skin (thighs/ abdomen)	Lawrence, 1952; Neve, 1910
Burns scars, chronic ulcers	Skin (legs/ buttocks)	Cruickshank *et al.*, 1963
Draining sinuses	Various sites	Cruickshank *et al.*, 1963
Reflux esophagitis/Barrett's esophagus	Esophagus	Cameron *et al.*, 1985
Chronic cholecystitis and cholelithiasis	Gall bladder	Diehl, 1983
Atrophic gastritis	Stomach	Correa, 1988
Ulcerative colitis	Colon/ rectum	Simmonds *et al.*, 1992
Helicobacter pylori	Stomach	Correa, 1992
Schistosoma haematobium	Urinary Bladder	Chen and Mott, 1989
Indwelling catheters	Urinary Bladder	Esrig *et al.*, 1992

An important aspect of this thesis was to develop a better understanding of promotion in humans by focusing on the role of inflammation in cancer development. *In vivo*, there are two model populations where there is a strong association between chronic irritation, inflammation and tissue regeneration and cancer. The first are patients infected with the parasite *Schistosoma haematobium* in third world countries. Another population are patients with indwelling urinary catheterization in the western world. The following two sections will describe these populations and provide insight into the proposed

mechanisms of carcinogenesis associated with *S. haemabobium* and urinary catheterization.

1.2 *Schistosoma haematobium* and Bladder Cancer

1.2.1 Epidemiology

Schistosomiasis is a parasitic infection that currently infects an estimated 200 million people worldwide (IARC, 1994). Although 19 species of schistosoma exist, only *Schistosoma haematobium*, *Schistosoma mansoni* and *Schistosoma japonicum* are of major pathological importance in humans and this section is restricted to *S. haematobium*.

S. haematobium is endemic in many parts of Africa and the Middle East and is recognized as a major public health problem, second only in importance to malaria among the major tropical diseases (Smith and Christie, 1989). Although many complications can arise from urinary schistosomiasis, this parasite is strongly associated with metaplasia and subsequent bladder cancer (Chen and Mott, 1989). In areas with a high rate of infection by *S. haematobium*, such as Egypt, 11% - 44% of cancer cases are in the urinary bladder and these cancers are linked with infection by this parasite (Rosin, 1991). Of those studies that describe an association between *S. haematobium* and bladder cancer, there seems to be a preponderance of squamous-cell carcinoma (reviewed in IARC, 1994).

1.2.2 Etiology of schistosomiasis-associated bladder cancer

Infection by the parasite occurs when an individual is exposed to the free-swimming larval stage in contaminated water. The larvae penetrate the skin, enter the peripheral capillaries, and migrate to the liver. Here they mature into male and female worms and travel in pairs through the blood stream to the

bladder. In the venules of the bladder mucosa the female sheds her eggs. The eggs penetrate the mucosa and become trapped in the tissue or are released into the urine (Rosin *et al.*, 1994). It is the presence of the eggs in the mucosa that is associated with chronic tissue irritation, inflammation and stimulated cellular proliferation.

The etiology of bladder cancer associated with *S. haematobium* is complex and has yet to be elucidated. However, a few theories exist. The infiltration of eggs into and through the bladder stroma produces tissue trauma. This trauma is usually chronic in patients residing in endemic areas such as Egypt and stimulates cellular proliferation and chronic inflammation, both of which are associated with tumourigenesis (Gentile and Gentile, 1994; Rosin *et al.*, 1994). There are a number of mechanisms by which stimulated proliferation plays a role in carcinogenesis. For example: (1) the altered structural configuration of replicating DNA may facilitate interaction of a carcinogen with DNA; (2) the probability that DNA lesions are converted to permanent genetic changes before they can be repaired is increased when cells are actively dividing; (3) many types of genetic changes require cell division to occur which can lead to activation of oncogenes and loss of suppressor genes; (4) clonal expansion of cells will increase the number of cells in which the next genetic change can occur and increase the probability of acquiring the specific genetic alterations necessary for neoplastic conversion. Inflammation plays a role in carcinogenesis through such mechansisms as carcinogen bioactivation, ROS release and nitrosamine formation. These processes can induce DNA damage, which is a hallmark of carcinogenicity. Egg deposition in the bladder wall and urethral walls can also lead to obstruction, urinary retention and secondary bacterial infection. Mucosal damage from these processes can increase the absorption of free carcinogens in the bladder (Young *et al.*, 1973). Also,

stagnant urine (Badr *et al.*, 1958) increases the length of time that the bladder is exposed to carcinogens. Bacteria can play a role in the stimulation of the inflammatory process and the production of nitrosamines. Finally, based mainly on results from experimental animal studies, it has been suggested that urinary schistosomiasis can lead to alterations in carcinogen metabolizing enzymes such as those of the p450 superfamily and β-glucuronidases (Gentile *et al.*, 1985; Badawi *et al.*, 1993). These mechanisms are reviewed in more detail in the following sections. A general overview of the interrelationship between bladder pathology and cellular changes associated with carcinogenesis in schistosomal bladders is shown in figure 1.

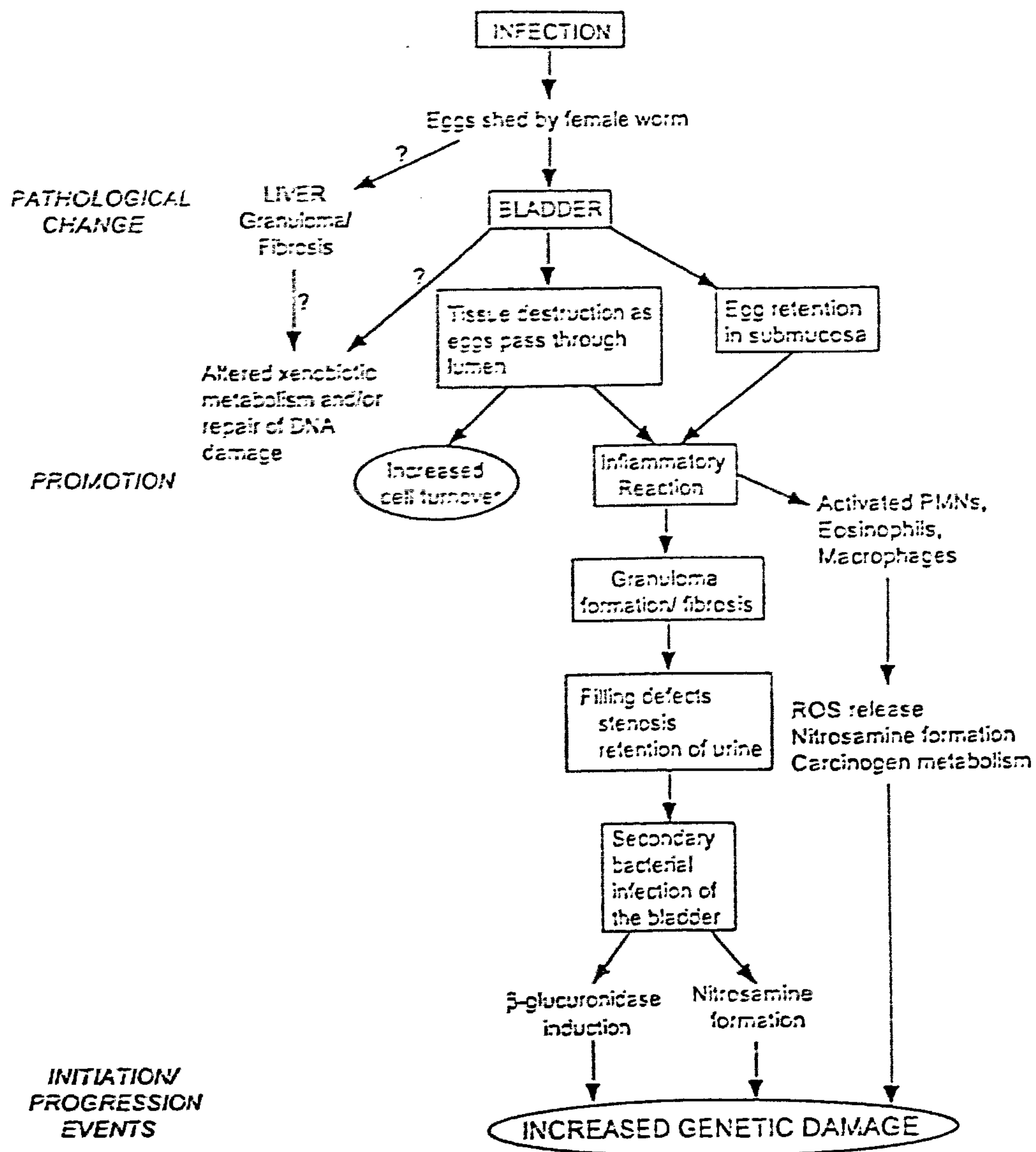


Figure 1. Interrelationship between bladder pathology and cellular changes associated with carcinogenesis in schistosomal bladders. Adapted from Rosin and Hofseth (1996).

1.3 Indwelling Urinary Catheters and Bladder Cancer

1.3.1 Epidemiology

Long-term indwelling catheterization is associated with bladder cancer (reviewed in Esrig *et al.*, 1992). Locke *et al.* (1985) reported 8% of a population with indwelling catheters for over 10 years had squamous cell carcinoma of the bladder and suggested the risk is 16 - 28 times higher in this population than a non-catheterized population. Broeker *et al.* (1981) reported that 19% of 81 patients maintained on catheter drainage for 10 or more years developed squamous metaplasia in the urinary bladder. The incidence of carcinoma of the bladder is at least 20 times higher in spinal-cord injured patients than in the general population according to another investigation (El-Masri and Fellows, 1981). This population usually uses long-term catheter drainage. Finally, La Vecchia *et al.* (1991) reported a relative risk of 4.7 for development of bladder cancer after multiple episodes of cystitis, a very common feature in patients on chronic indwelling catheterization. Although the nature of this relationship has not been elucidated, several studies have cited bladder stones, chronic urinary tract infections and the mechanical trauma from the catheter itself as predisposing factors (Kaufman *et al.*, 1977; Broecker *et al.*, 1981). These statistics necessitate an inquiry into the pathogenesis of bladder cancer and its relationship to chronic urinary catheter drainage.

1.3.2 Complications associated with catheterization

It is clear that bladder cancer is prevalent in those with indwelling urinary catheterization. Why, then, do clinicians place those who lose the ability for micturation on this type of drainage? The answer is that in the many patients, the benefits associated with this type of drainage outweigh the problems associated with other types of drainage. There are 5 methods of urinary

drainage: (1) diaper; (2) condom catheter; (3) indwelling suprapubic catheter; (4) indwelling urethral catheter, or (5) urinary diversion with an abdominal stoma or a nonrefluxing colon conduit (Esrig *et al.*, 1992). Diapers present a temporary solution; however, they are very messy and often uncomfortable to the patient. Condom catheters are useful for incontinent men without outlet obstruction and with an intact voiding reflex (Chaitow, 1981). However, its use is limited to men, requires meticulous care when skin macerations arise and easy removal by the patient leads to more frequent manipulation and increased risk for urinary tract infection (Hirsh *et al.*, 1979). Suprapubic catheters are an alternative, but they require surgical incision and fail to reduce the incidence of ascending pyelonephritis and its complications (Arego and Koch, 1986). Urinary diversion requires surgery, which is costly, and some patients simply cannot endure surgery or do not want it. For these reasons, many patients in these populations require long-term (≥30 days) indwelling urinary catheter drainage.

The indwelling urinary catheter is a Foley-type closed drainage system (see appendix B). It is a long tube that has a sampling port to take sterile samples for analysis without disturbing the system. One end inserts into the bladder through the urethra and the other end connects to sterile rubber tubing by a sterile straight connector. The rubber tubing then connects to a sterile bedside drainage bag. The end of the Foley catheter that inserts into the bladder lumen is inflatable so that after the tube is inserted, the catheter cannot be easily removed. Since the other end of the tubing is connected to a sterile bag, the system is called a 'closed drainage system'. When first developed, the system was an 'open drainage system'. However, this was very ineffective in protecting against urinary tract infections. Virtually 100% of patients with indwelling urinary catheters draining into an open system for longer that 4 days were reported to develop significant bacteriuria, defined as 100 colony forming

units/ ml in patients on indwelling catheter (Daifuku *et al.*, 1986; Kass, 1956). After the introduction of the closed system, the onset of bacteriuria was delayed. This system, however, is not fool proof against foreign bacteria. Bacteriuria and a concomitant risk for urinary tract infection still develops, albeit at a slower rate. Daily incidence of bacteriuria is about 5 to 10 per cent per day (Kunin and McCormack, 1966; Warren *et al.*, 1978) with nearly all patients developing infected urine after 4 weeks of catheterization (Jewes *et al.*, 1988).

To conclude this section, it is necessary to properly define a 'bladder infection' in a patient with an indwelling catheter. Bladder infection (or lower urinary tract infection) is a clinical term suggesting bacterial sepsis and bladder inflammation (cystitis) with the onset of symptoms such as leakage of urine around the catheter (Seiler and Stahelin, 1988), frequent obstruction (Seiler and Stahelin, 1988), suprapubic pain (Seiler and Stahelin, 1988; Warren, 1991; Harding *et al.*, 1991), encrustation of the catheter (Seiler and Stahelin, 1988), bladder spasms (Seiler and Stahelin, 1988; Lapin, M.D., personal communication), detrusor muscle spasms (Seiler and Stahelin, 1988), abdominal bloating (Lapin, M.D., personal communication), dysreflexia (Lapin, M.D., personal communication), cloudy urine (Lapin, M.D., personal communication), fever (Seiler and Stahelin, 1988) and possibly dysuria (Harding *et al.*, 1991) and frequent urination (Harding *et al.*, 1991). When a person develops cystitis symptoms, the bladder wall is inflamed. On cystoscopy, inflammatory changes include multiple petechial hemorrhages, tiny, raised, pearly mucosal lesions, disruption of the uroepithelium, and infiltration of inflammatory cells into the submucosal layers. Furthermore, there is often an increased production of prostaglandins in the inflamed mucosa (Seiler and Stahelin, 1988).

1.3.3 Etiology of catheter-associated bladder cancers

In catheterized patients, the origin of a bladder infection begins with the entrance of bacteria into the catheterized bladder by one of two routes: periurethrally or intraluminally. For most females, the former is true and for most males the latter is true (Stamm, 1991). When in the bladder, the bacteria persist and colonize, often rising in number from 100 cfu/ml (low colony count) to >100 000 cfu/ml (high colony count) in less than 48 hours (Strak and Maki, 1984). There are several reasons for such rapid multiplication. The catheter provides a welcome microenvironment for bacterial growth (Nickel *et al.*, 1985; Coz *et al.*, 1989), the catheter, being a foreign agent, decreases the ability of white cells to function properly (Zimmerli *et al.*, 1984; Lopez-Lopez *et al.*, 1991) and the bladder uroepithelial surface and urinary environment provide a natural niche for bacteria to grow.

Bacteriuria does not necessarily mean inevitable development of cystitis. Many patients remain bacteriuric but never develop cystitis symptoms (Jewes *et al.*, 1988). One reason for this is based on the species of bacteria with which the person is infected. Although all bacterial strains contain inflammatogenic substances such as lipoproteins, lipopolysacharides, outer membrane proteins and proteoglycans (de Man *et al.*, 1988), different strains may have different abilities to present these compounds to the uroepithelium. A possible reason for this is the different adherence capabilities of various strains of bacteria to diverse tissues. For example, de Man *et al.* (1988) found that attaching strains of *E. coli* appeared to be more capable of causing inflammation than were other bacteria. Daifuku *et al.* (1986) identified diverse adherence capabilities of gram-negative bacteria which played a role in the risk for urinary tract infection. Furthermore, different strains of bacteria were reported to be involved in

different disorders (eg. pyelonephritis vs. cystitis) and this may be influenced by adherence properties.

Another reason for the development of acute cystitis, in addition to bacterial invasion of the uroepithelial wall, is the mechanical trauma produced by the catheter itself. Any catheter manipulations such as bladder lavage, catheter exchange, pulling on the catheter by the agitated patient and a blocked catheter with overfilling of the bladder are primary causes of damage to the uroepithelium (Seiler and Stahelin, 1988). Some authors suggest this mechanical trauma is a necessary step toward the clinical manifestations of cystitis. They hypothesize a two-step process where bacteria do not invade the bladder mucosa until it is damaged by catheter manipulation (Lapides, 1979). This hypothesis provides another explanation why some patients acquire cystitis symptoms and some do not, even though they are both bacteriuric.

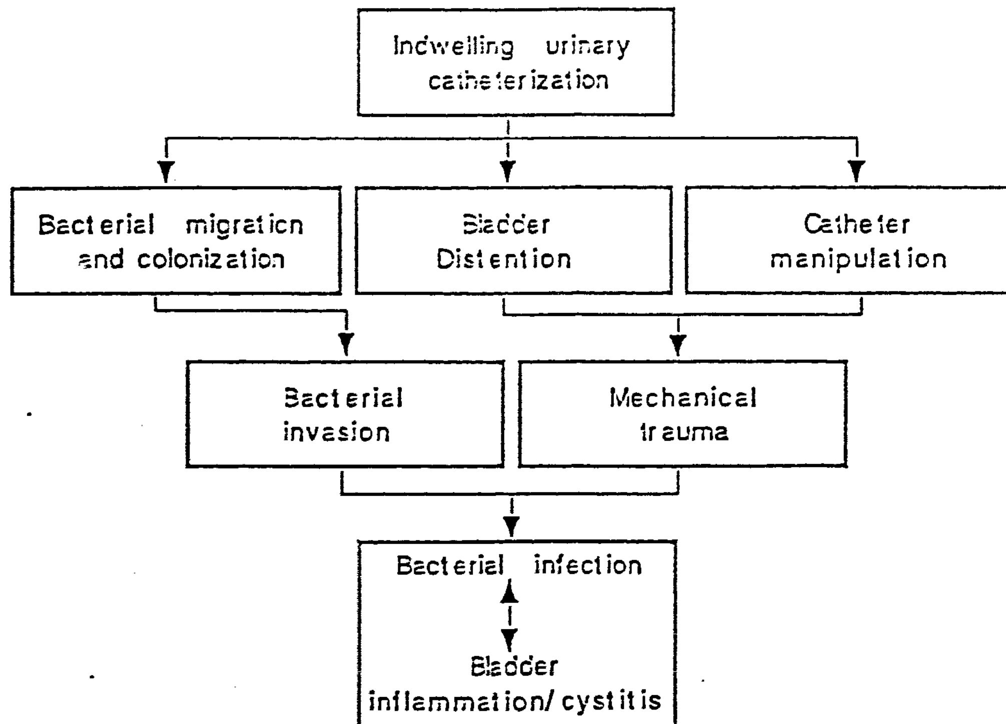


Figure 2. Etiology of a bladder infection

The association between indwelling urinary catheterization and bladder cancer is complex. It seems that bladder cancer arises from a combination of bacterial invasion into the bladder lumen and mechanical trauma associated with the bacterial invasion and the catheter rubbing against the bladder luminal surface. The mechanical trauma is associated with chronic irritation, chronic inflammation and an enhanced rate of proliferation associated with chronic tissue regeneration. Bacterial invasion is similarly associated with these phenomena and in addition play a role in the production of carcinogenic nitrosamines. A brief summary of some of the potential carcinogenic pathways associated with urinary catheterization can be found in table 2.

Table 2. Carcinogenic pathways associated with urinary catheterization

Primary Carcinogenic Stimulant	**Secondary Associated Responses**	**Tertiary Associated Responses**	**Final Carcinogenic or Tumour-Associated Products**
Bacterial invasion & Mechanical trauma	Tissue irritation	Increased tissue proliferation	Cytokines
			Growth factors
		Inflammation	Reactive oxygen species
			Arachidonic acid metabolites
			Nitrosamines
			Carcinogen bioactivation

1.4 Inflammation and Cancer

Inflammation is associated with carcinogenesis. Whether it is causal and under what conditions it actually plays a role is not known. Some of the experiments used in this thesis are designed to test whether inflammation plays a role in carcinogenesis through the induction DNA damage.

Inflammation begins with a reaction to an irritant characterized by movement of fluid and white blood cells into extravascular tissue. This is followed by tissue repair and regeneration and involves cell proliferation.

Associated with these processes are arachidonic acid metabolism (Lewis *et al.*, 1986), the generation of oxygen-derived radicals (Weitberg *et al.*, 1983; Weitzman and Gordon, 1990; Doonan, 1991; Frenkel, 1992) and carcinogen bioactivation (Isola *et al.*, 1993). The following sections review current knowledge of these processes and suggest how they may be involved in the relationship between inflammation and cancer.

1.4.1 The respiratory burst

An inflammatory reaction is a natural defense system of the body to invading foreign material/xenobiotics. Upon tissue infiltration, inflammatory cells are stimulated to produce a respiratory (oxidative) burst during which molecular oxygen is used to destroy the xenobiotic. The respiratory burst is characterized by a rapid consumption of molecular oxygen followed by the production of superoxide ($O_2\bullet^-$). Induction of this process depends on stimulation of the (phagocyte) cellular membrane by any one of a number of stimuli such as bacteria, parasites, or other opsonized particles (Marasco *et al.*, 1984; Evans *et al.*, 1989). This activates a membrane bound enzyme such as pyridine nucleotide oxidase that catalyzes the production of $O2\bullet^-$ using NADPH as an electron donor (Gordon and Weitzman, 1988). Subsequent to the production of $O2\bullet^-$, other reactive oxygen species (ROS) are formed and are capable of damaging the DNA of either the phagocytes themselves or neighboring tissue cells. Concomitant with the formation of DNA-damaging free radicals is an attack on biological membranes resulting in lipid peroxidation and arachidonic acid metabolism. This can be caused directly by foreign material (Obrien, 1988) or indirectly through free radical production (Slater, 1984). Lipid peroxidation and arachidonic acid metabolism may play a large role in carcinogenesis

through formation of intermediates and end-products and through free radical production, all capable of damaging the DNA.

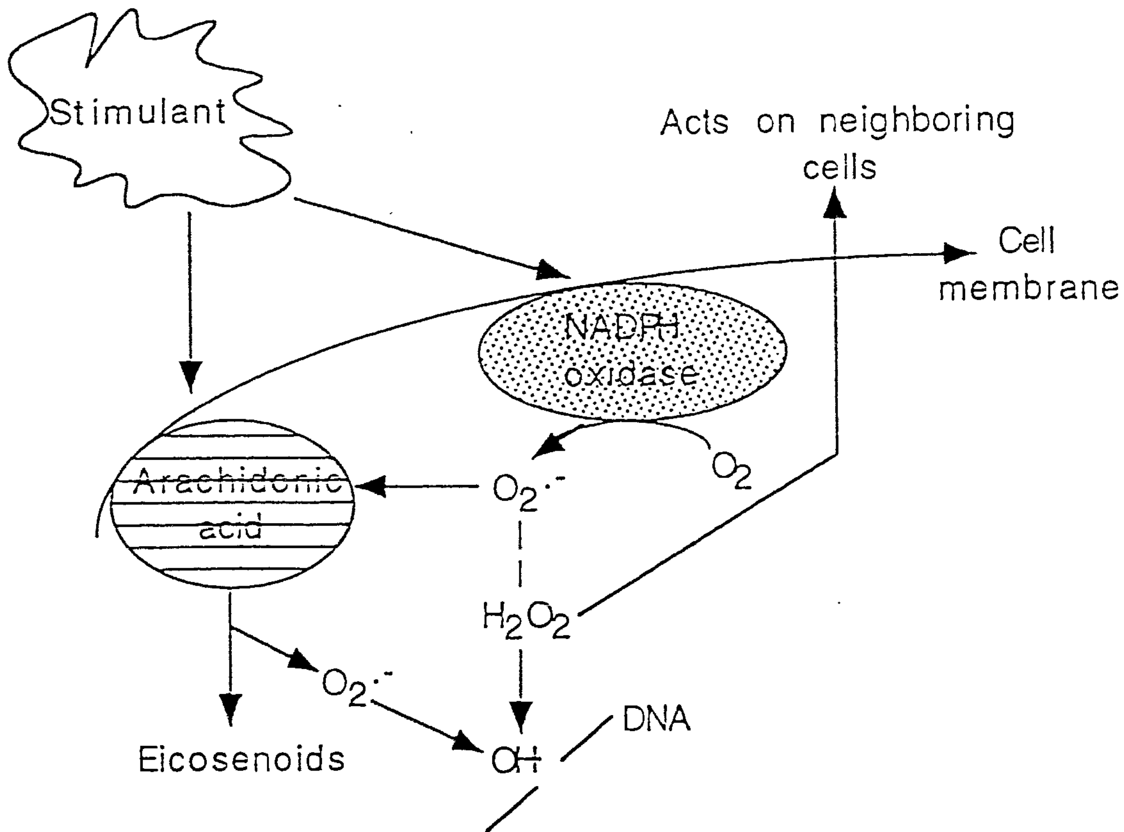


Figure 3. The respiratory burst

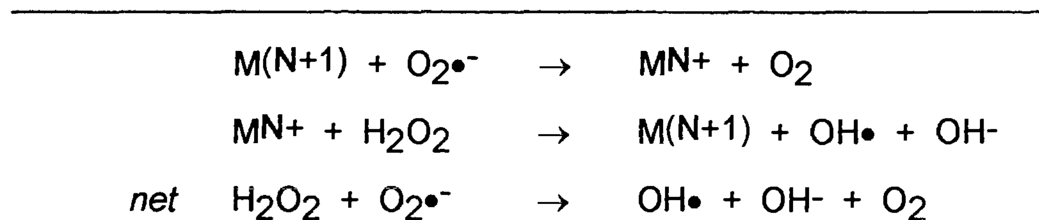
1.4.2 Reactive oxygen species (ROS)

Although oxygen is necessary to sustain life in aerobic organisms, a persistent excess may by harmful. As suggested, during the metabolism of oxygen, intermediates called reactive oxygen species (ROS) are produced that are extremely reactive to biological material such as DNA. If ROS overwhelm antioxidant defense mechanisms, cellular damage can result. ROS include both oxygen-centered free radicals such as $O_2\bullet^-$, hydroxyl radical (OH•) and nitric oxide radical (NO•) and nonradical derivatives of oxygen such as hydrogen

peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$) and peroxynitrite. These agents often attack and damage DNA and other biological molecules (Imlay and Linn, 1988; Halliwell and Aruoma, 1991), contribute to lipid peroxidation (Mead, 1976), stimulate cell proliferation (Burdon *et al.*, 1989) and are involved in various stages of neoplastic development (reviewed in Borek, 1991; Frenkel, 1992).

ROS are formed continuously in small amounts in aerobic organisms (Halliwell and Aruoma, 1991; Foegh *et al.*, 1990). Normally, ROS and in particular $O_2\bullet^-$ are produced during auto-oxidation reactions or by leakage of electrons from the electron transport chain onto oxygen (Fridovich, 1989; Foegh *et al.*, 1990; Imlay and Fridovich, 1991; Frenkel, 1992). On occasion, however, these molecules are produced in large amounts via cellular enzymes in activated phagocytes such as those involved with inflammatory reactions (Weitzman and Gordon, 1990). Superoxide dismutase (SOD), a biological enzyme important in defense against excess $O_2\bullet^-$, may convert $O_2\bullet^-$ into O_2 and H_2O_2. H_2O_2 has the ability to cross cell membranes (Halliwell and Gutteridge, 1989), enter tissue cells and act indirectly on its biological constituents.

Biological transition metal salts, such as iron, copper and nickel, promote free radical reactions by reducing H_2O_2 and generating $OH\bullet$, capable of reacting with lipids, proteins and DNA (Breen and Murphy, 1995). Generally, this (Haber-Weiss) reaction involves variable valency metal ions reducing $O_2\bullet^-$ and H_2O_2 to produce a very reactive $OH\bullet$. This reaction can be shown by the following equation (Gordon and Weitzman, 1988):



where M is a variable valency metal ion bound to a specific site in the cell. Evidence suggests that the most common ion in biological tissues is the ferrous ion (Halliwell and Gutteridge, 1989; Halliwell and Aruoma, 1991; Breen and Murphy, 1995).

Hydroxyl radical formation and its damaging action depend on such conditions as availability of antioxidant mechanisms and the area of the cell where the metal ion, and thus the reaction, occurs. For example, when this potent hydroxyl free radical is formed in close proximity to the DNA, it can cause DNA strand breakage (Halliwell and Aruoma, 1991); when it is in close proximity to membrane lipids, lipid peroxidation may occur (Dargel, 1992). The ferrous ion seems to be readily available in both sites (Halliwell and Aruoma, 1991; Dargel, 1992).

ROS play an important role in mutagenesis and carcinogenesis. Oxygen radicals may directly act as initiators by interacting with nuclear DNA or may promote lipid peroxidation, the degradation products of which have DNA-damaging potential (Marnett, 1990; Frenkel, 1992). ROS have been shown to cause mutations in *Salmonella typhimurium* (Moody and Hassan, 1982) and mammalian cells (Hsie, 1987; Frenkel, 1992). Radiation, a powerful inducer of ROS production (Frenkel, 1992), causes cancer in humans (Albert and Shore, 1986). H_2O_2 has been associated with the production of cancer in animals (Ito *et al.*, 1981). Agents such as xanthine/xanthine oxidase, a superoxide generator, have been shown to be weak carcinogens (Zimmerman and Cerutti, 1984) and capable of inducing genetic damage in human bladder cells (Ward *et*

al., 1993). Furthermore, it is believed that oxygen radical-induced genetic damage may be important for the development of neoplasms associated with chronic inflammation (Cerutti, 1985). Recently it has been shown that co-incubation of human neutrophils with human bladder cells stimulates chromosomal breakage *in vitro* (Ward *et al.*, 1993).

1.4.3 Lipid peroxidation

Lipid peroxidation is the non-enzymatic oxidative deterioration of fatty acids, particularly unsaturated fatty acids (eg. arachidonic acid), largely resulting from free radical reactions in biological membranes. In the presence of oxygen, the initial step is the removal of hydrogen from polyunsaturated fatty acids, giving rise to a lipid peroxyl radical. $OH\bullet$ and $O_2\bullet^-$ seem to play an important role in this initial step (Minotti and Aust, 1987), although the mechanism is unresolved. The formation of the lipid peroxyl radical begins a multistep process resulting in a variety of biologically reactive products. These include lipid peroxides, free radicals and non-radical products, such as carbonyl compounds (Ueda *et al.*, 1985).

Among the non-radical products formed during lipid peroxidation are fairly unreactive hydroxyalkanels which have the potential to reach distant sites, resulting in direct reactions with DNA and inhibition of DNA repair (Dargel, 1992). Another decomposition product of lipid peroxidation, malondialdehyde, has been reported to cause interstrand cross-linkage and to be mutagenic and carcinogenic (Mukai and Goldstein, 1976; Sevanian *et al.*, 1988; Marnett, 1990).

1.4.4 The arachidonic acid cascade

Epithelial cells contain arachidonic acid (Zenser *et al.*, 1990) and inflammatory cells contain unusually high concentrations of arachidonic acid (Goetzl, 1980; Aderem *et al.*, 1986). The arachidonic acid cascade represents the various events associated with the catabolism of arachidonic acid to eicosenoids (general term for the end products of the cascade). It is initiated by either the stimulus-induced activation of phospholipase A_2 or phospholipase C. Phospholipase A_2 enhances the hydrolysis of arachidonic acid from glycerol of membrane phospholipids. Phospholipase C is involved in the metabolism of phosphatidylinositol to diacylglycerol and inositol phosphates. Subsequently diacylglycerol lipase cleaves arachidonic acid from diacylglycerol.

Metabolism of arachidonic acid proceeds through two pathways: the cyclooxygenase and/or the lipoxygenase pathway. Cyclooxygenation, through the action of various enzymes such as prostaglandin synthetase, results in the formation of prostaglandins (PGs, eg. PGI_2, $PGF_{2\alpha}$, PGE_2), thromboxanes (TXs, eg. TxA_2) and prostacyclins (PCs); lipoxygenation results in the formation of several hydroperoxy fatty acids such as hydroperoxy eicosatetraenoic acid (HPETE). Further reduction, enzymatically (eg. glutathione peroxidase of PHS peroxidase) or non-enzymatically, results in the formation of hydroxy acids (HETEs) and leukotrienes (LTs) (Vane *et al.*, 1982; Krauss and Eling, 1984; Rubin and Farber, 1988). A third less characterized pathway of arachidonic acid metabolism proceeds through cytochrome p450 monooxygenases and forms biologically active epoxides (Foegh *et al.*, 1990).

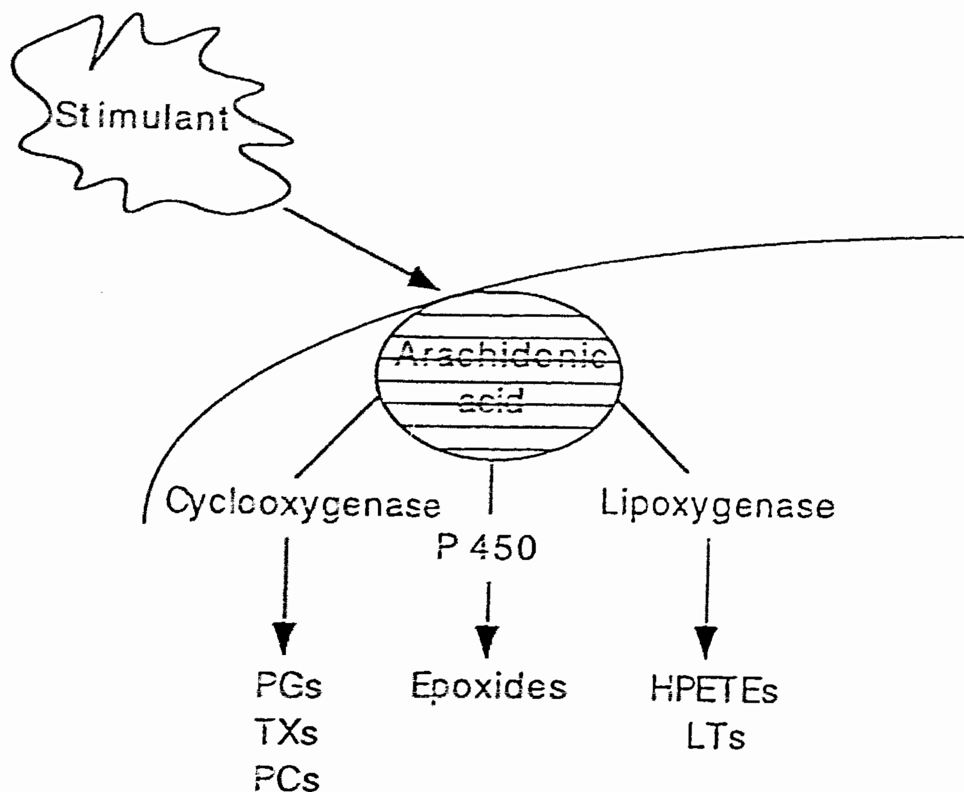


Figure 4. The arachidonic acid cascade

The products of arachidonic acid metabolism may play an important role in inflammation-associated tumourigenesis. Although the exact mechanisms have not been elucidated, evidence suggests that the many different aspects of the arachidonic acid cascade may be involved in several steps in the carcinogenic process. For example, the eicosanoids are considered to be involved (Fischer *et al.*, 1985; Kozumbo *et al.*, 1987; Honn *et al.*, 1983; Bennett, 1988; Furstenberger and Marks, 1985; Kobayashi *et al.*, 1985). Additionally, intermediates in the cascade play a role in the carcinogenic process. Evidence for this comes from the observation that the common tumour promoter phorbol-12-myristate-13-acetate (PMA) which induces inflammation and leukocyte-

mediated clastogenicity stimulates human cells to release a greater than normal amount of intermediates in the arachidonic acid cascade (Kozumbo *et al.*, 1987). The intermediates are involved in free radical production (Foegh *et al.*, 1990), bioactivation of carcinogens (Marnet, 1981; Dix and Marnett, 1983; Battista and Marnett, 1985; Yamazoe *et al.*, 1988) and direct genetic damage (Ueda *et al.*, 1985). The following is a summary of possible mechanisms by which intermediates may play a role in changing a morphologically normal cell into its cancerous progeny.

1.4.4.1 Direct DNA binding

Metabolism of arachidonic acid results in hydroperoxy intermediates. These are unstable and bind directly to DNA and induce mutations or cell transformation (Lewis *et al.*, 1986; Cerutti, 1987; Gordon and Weitzman, 1988). For example HETEs and HPETEs produced during lipid peroxidation elicit DNA strand breakage in mouse embryo fibroblast C3H/10T1/2 cells (Ochi and Cerutti, 1987).

1.4.4.2 Carcinogen bioactivation

Prostaglandin H synthase in bioactivation

Metabolism of certain carcinogens, such as aromatic amines, by the peroxidase component of prostaglandin H synthase (PHS) is proposed as a pathway for carcinogen bioactivation (Wise *et al.*, 1984). The PHS enzymes are involved in the metabolism of arachidonic acid towards the synthesis of PGs, TxA_2 and/or PCs (via the cyclooxygenase pathway) or LTs (via the lipoxygenase pathway). In the cyclooxygenase pathway, PHS catalyzes the oxygenation of arachidonic acid to prostaglandin G_2 (PGG_2). Subsequently, the peroxidase component of PHS (PHS peroxidase) catalyzes the reduction of PGG_2 to

prostaglandin H2 (PGH_2). At this step, PHS peroxidase requires reducing co-factors such as aromatic amines. PHS oxidizes an aromatic amine to its electron deficient reactive form. This process is called a co-oxidation. Although less defined, this same process appears to occur in the lipoxygenase pathway of the cascade; during the reduction (via PHS peroxidase or glutathione peroxidase) of HPETES to HETES (Krauss and Eling, 1984). However, Wise *et al.* (1984) noted that lipoxygenase activity is not demonstrated in certain tissues, such as the bladder epithelium.

Although not well established, it appears the activation of B(a)P-7,8-dihydrodiol through PHS-dependent pathways does not involve the classical co-oxidation process seen with carcinogen families such as aromatic amines. It seems to involve a 'hydroperoxide-dependent epoxidation' by PHS using molecular oxygen as a substrate (Marnett *et al.*, 1983). The epoxidizing agent, which activates the proximate carcinogen, seems to be a fatty-acid derived peroxy radical. Basically, the process involves a reduction of the hydroperoxide (eg. PGG_2) to an alkoxy radical and hydroxide radical. A percentage of the alkoxy radical cyclizes to an epoxide and carbon-centered radical which is trapped by oxygen to form a peroxy radical. The peroxy radical epoxidizes the isolated double bond of B(a)P-7,8-dihydrodiol, in the process changing the procarcinogen to its activated form.

Myeloperoxidase in bioactivation

Myeloperoxidase, an important constituent of polymorphonuclear leukocytes (PMNs), is another enzyme which has been shown to increase the concentration of carcinogenic metabolites (and DNA damage) in tissue by bioactivating carcinogens. It has been shown to mediate the formation of the ultimate carcinogenic metabolite of several carcinogens, including the polycyclic

aromatic hydrocarbon, benzo(a)pyrene, aromatic amines and nitroaromatic derivatives (Eling *et al.*, 1990; Trush and Kensler, 1991).

1.4.4.3 Free radical production during arachidonic acid metabolism

It is not quite apparent exactly where free radicals originate during arachidonic acid metabolism. Foegh *et al.* (1990) mention that both lipoxygenase and cyclooxygenase yield superoxide radicals. However, Czerniecki and Witz (1989) note that it appears oxygen radical stimulation is only mediated through the lipoxygenase pathway and not the cyclooxygenase pathway of the cascade. Irrespective, free radicals are produced concomitantly with the metabolism of arachidonic acid. Intermediates in the arachidonic acid cascade are capable of initiating or sustaining free radical reactions (Gordon and Weitzman, 1988). In turn, as suggested earlier, free radicals play a role in the carcinogenic process.

1.4.5 Nitric oxide and nitrosamines

In addition to the respiratory burst, inflammatory cells are associated with the release of *N*-nitroso compounds that have the ability to induce DNA damage and are of known mutagenic potential (Marletta, 1988; Roediger *et al.*, 1990). In stimulated inflammatory cells nitric oxide synthase catalyzes the conversion of arginine to citrulline with a concomitant generation of nitric oxide (NO•) (Harris *et al.*, 1992). When nitric oxide reacts with the superoxide anion it forms peroxynitrite, which decomposes to nitrate. Peroxynitrite can also undergo homolytic cleavage to generate the hydroxyl radical and nitrogen dioxide. Nitrogen dioxide exists in equilibrium with potent nitrosating agents such as N_2O_3 and N_2O_4 which yield nitrate and nitrite when reacted with water. If, however, the nitrosating agents react with secondary amines, *N*-nitrosamines

are formed (Leaf *et al.*, 1991; Mulligan *et al.*, 1991). DNA damage to bladder cells during chronic inflammation is associated with increased exposure to *N*-nitrosamines (El-Aaser *et al.*, 1984; Tricker *et al.*, 1989).

1.5 Micronuclei (MN) as a Quantifiable Indicator of *In Vitro* and *In Vivo* DNA Damage

Clearly inflammation and the products associated with it induce DNA damage. To evaluate DNA damage in this thesis, the micronucleus (MN) assay was used. MN are formed by damage to chromosomes or to the spindle apparatus in dividing cells. When a cell with such damage divides, the chromosomes or parts of chromosomes are excluded from the main nucleus and form their own membrane. After staining by the Feulgen reaction, they are easily seen as extranuclear bodies under a high power (1000x magnification) microscope.

MN have been used as a valid quantifiable indicator of genetic damage both *in vitro* and *in vivo*. *In vitro*, validation has come from their use as an endpoint in many studies involving both genotoxic and carcinogenic species. Cells exposed and assessed for MN frequencies in these studies include fibroblasts, hepatocytes, human lymphocytes, myeloblasts, erythroblasts and epithelial cells (Schmid, 1976; Heddle *et al.*, 1978; Ward *et al.*, 1993). *In vivo*, the MN assay serves as an endogenous dosimeter of DNA damage in the specific tissue being assessed. Cells can be isolated by biopsy or collection of exfoliated cells. Validation has come from both animal and human studies and includes such tissues as the bladder, lung, mouth, cervix, esophagus and the nasal cavity (Rosin, 1992; Hofseth *et al.*, in press).

Although the MN test does not give an indication of specific genetic changes, it does quantify the extent of widespread genetic alterations occurring

throughout a tissue. A chronic elevation in MN frequencies therefore indicates there is an increased probability of the tissue acquiring all the specific genetic changes necessary for cancer development.

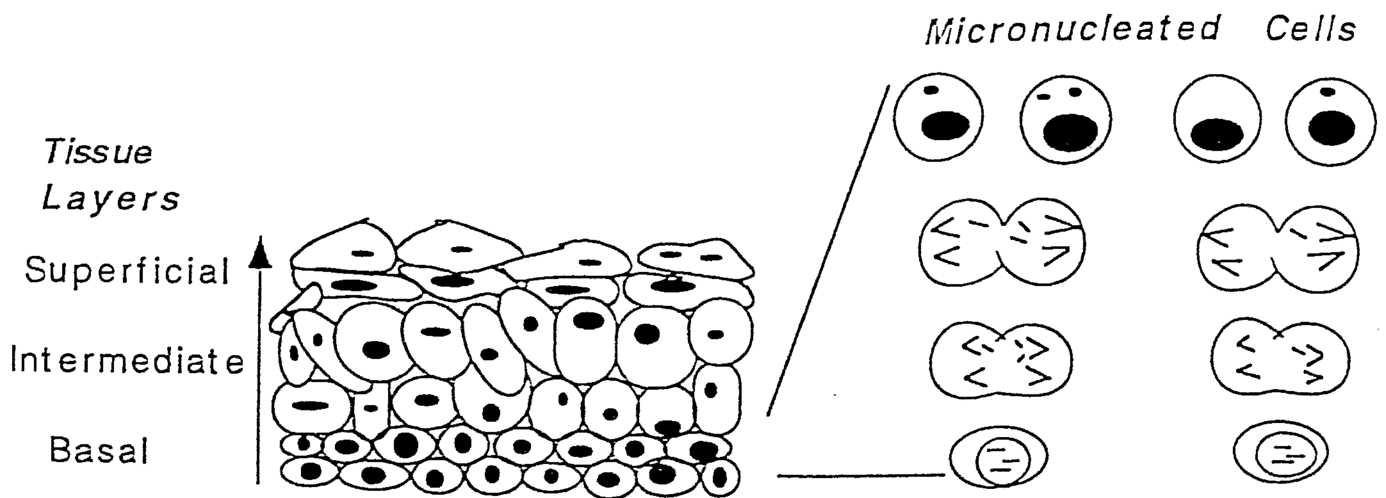


Figure 5. Micronucleus formation in epithelial tissues. *In vivo*, MN are formed in the dividing basal cells where upon differentiation, the cells migrate to the lumenal surfaces of the tissue and are exfoliated into the lumen where they can be collected.

1.6 Chromosome 11 and Bladder Cancer

MN are a validated index of DNA damage and are an important endpoint used in this thesis in the assessment of damage induced by inflammatory associated ROS. The *in vitro* portion of this thesis, however, also provides insight into the role of chromosome 11 in the protection against inflammatory related DNA damage. This section assesses specific genetic alterations associated with bladder cancer and provides insight into the rationale for using chromosome 11 in protecting cells against DNA damage.

Studies of bladder tumours indicate the involvement of multiple genetic alterations (deletions, mutations, chromosomal rearrangements) in the genesis of bladder cancer. These alterations occur throughout tumourigenesis. Some occur as early events and some occur as later events. Current evidence suggests chromosomes 9(p and q) (Knowles *et al.*, 1994; Tsai *et al.*, 1990), 11 (Knowles *et al.*, 1994; Tsai *et al.*, 1990; Presti *et al.*, 1991; Olumni *et al.*, 1990; Fearon *et al.*, 1985), and 17(p) (Knowles *et al.*, 1994; Tsai *et al.*, 1990) as the most frequently altered (deleted) chromosomes. However, to a lesser extent, other studies have suggested bladder cancer is associated with anomalies of chromosomes 1 (Vanni *et al.*, 1988), 3(p) (Presti *et al.*, 1991), 4(p) (Knowles *et al.*, 1994), 7 (Waldman *et al.*, 1991), 8(p) (Knowles *et al.*, 1994), 13(q) (Knowles *et al.*, 1994) and 18(q) (Presti *et al.*, 1991). Of the more common bladder cancer-associated chromosomal changes, a deletion of chromosome 9 seems to occur in earlier phases of bladder cancer development and chromosome 11p and 17p deletions seem to be associated with later events. A summary of these findings can be found in Table 3.

Table 3. Genetic alterations in human bladder cancer

Genetic Alteration	% Tumours	Stage/Grade most commonly associated with (Low, Mod, High)[a]	Reference
ch.1 anomalies (delet.,trans)	36.6%	NI	Vanni *et al.*, 1988
ch. 3p deletions	66.7%	late event	Presti *et al.*, 1991
ch. 4p deletions	22%	NI	Knowles *et al.*, 1994
ch. 4 deletions	46%	high grade/stage	Polascik *et al.*, 1995
ch. 7 tetrasomy	38%	NI	Waldman *et al.*, 1991
ch. 8p deletions	23%	high grade/stage	Knowles *et al.*, 1994
ch. 9 deletions	34%	low&high grade low stage	Spruck *et al.*, 1994
ch. 9 deletions	58%	low-mod stage	Spruck *et al.*, 1994
ch. 9p deletions	51%	low-mod grade/stage	Knowles *et al.*, 1994
ch. 9q deletions	57%	low-mod grade/stage	Knowles *et al.*, 1994
ch. 9q deletions	65%	low/mod/high grade	Olumi *et al.*, 1990
ch. 9q deletions	67%	high grade/stage	Tsai *et al.*, 1990
ch. 9q deletions	75%	early event	Sidransky *et al.*, 1992
ch. 9 monosomy	41%	NI	Waldman *et al.*, 1991
ch. 9 monosomy	37%	early	Hopman *et al.*, 1991
ch. 11 deletions	34%	NI	Shaw and Knowles, 1995
ch. 11p deletions	32%	NI	Knowles *et al.*, 1994
ch. 11p deletions	33.3%	late event	Presti *et al.*, 1991
ch. 11p deletions	39%	high grade	Olumi *et al.*, 1990
ch. 11p deletions	40%	high grade/stage	Tsai *et al.*, 1990
ch. 11 tetrasomy	26%	NI	Waldman *et al.*, 1991
ch. 14q deletions	47%	All grades/stages	Chang *et al.*, 1995
ch. 17p deletions	32%	NI	Knowles *et al.*, 1994
ch. 17p deletions	50%	late event	Presti *et al.*, 1991
ch. 17p deletions	63%	high grade/stage	Tsai *et al.*, 1990
ch. 17p deletions	64%	high grade	Olumi *et al.*, 1990
ch. 18q deletions	28.6%	late event	Presti *et al.*, 1991
Other			
p53 mutation	65%	late event	Spruck *et al.*, 1994
p53 mutation	51%	late event	Spruck *et al.*, 1994
Altered Rb expression		late event	Presti *et al.*, 1991

[a] NI, not indicated in reference.

It is evident that chromosome 11 is associated with tumourgenicity. *In vitro*, many studies have provided functional evidence for the presence of suppressor loci on chromosome 11 (Saxon *et al.*, 1986; Weissman *et al.*, 1987; Komatsu *et al.*, 1990; Parshad *et al.*, 1992; Ichikawa *et al.*, 1992; Koi *et al.*, 1993). For example, Weissman and co-workers (1987) showed the introduction of a normal human chromosome 11 into a (Wilms') tumour cell line induced

complete suppression of tumourigenic potential. Komatsu and co-workers (1990) reported increased resistance of radiosensitive AT cells to killing by X-irradiation after insertion of a normal copy of chromosome 11.

Although it is clear that chromosome 11 suppresses tumourgenicity in certain cancer cell lines, the mechanisms underlying this suppression remain unresolved. There is an indication that chromosome 11 plays a role in the suppression of DNA damage, such as that induced by ROS. Parshad and co-workers (1992) reported an alteration in human tumour cells that resulted in an increased sensitivity to X-irradiation, seen as an abnormally high frequency of chromatid breaks and gaps after exposure during the G_2 phase of the cell cycle. Of interest was the observation that the addition of a normal chromosome 11 by microcell fusion to these cell lines resulted in efficient repair of the radiation-induced damage to the level seen in normal cells, even though the tumours were of diverse tissue origin. Another study by Ward *et al.* (1993) demonstrated that insertion of an extra chromosome 11 into a bladder carcinoma cell line reduced chromosomal breakage induced by xanthine/ xanthine oxidase and human neutrophils. The reasons for this protection are unknown.

There are several interesting hypotheses which might explain a restoration of ROS-induced DNA damage to levels of controls by insertion of chromosome 11. One mechanism by which chromosome 11 insertion could affect the sensitivity to oxidative stress is by altering the levels of catalase in the cell, since this gene is located at 11p13 (Glaser et al, 1986). Catalase acts to reduce H_2O_2 to oxygen and water, thereby decreasing the quantity of ROS available to induce DNA damage. Another hypothesis has been that there is a defective gene on chromosome 11 in these cells that is corrected by the insertion of normal chromosome 11 and this gene might be involved in DNA

repair. The following section details the roles of catalase and repair genes in protection of cells against DNA damage induced by agents that create ROS.

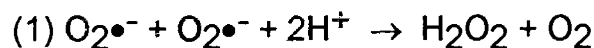
1.7 Loci on Chromosome 11 Associated with Sensitivity to ROS

1.7.1 Chromosome 11 and catalase activity

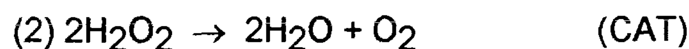
All cells have antioxidant defenses that protect them from damage caused by ROS (Davies *et al.*, 1990). These defenses include enzymes (superoxide dismutase, SOD; glutathione peroxidase, GPx; glutathione reductase, GR; and catalase, CAT) and compounds (vitamin E, β-carotene, vitamin C, uric acid, metal chelators and glutathione).

With regards to antioxidant compounds, Vitamin E and β-carotene inhibit free radical propagation in membrane lipids by reacting with the peroxyl radical and quenching singlet oxygen, thereby protecting against peroxidation. Uric acid and vitamin C act as radical scavengers in the cytoplasm and membranes. Metal chelators reduce the involvement of iron compounds and other variable valency metal ions acting in the production of the hydroxyl radical during such reactions as the Fenton reaction.

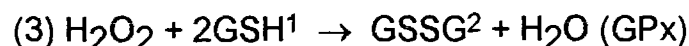
With regards to the enzymes, SOD reduces superoxide to hydrogen peroxide and oxygen (equation 1). GPx and CAT work to convert hydrogen peroxide to water and oxygen (equations 2 and 3, respectively). GR reduces glutathione in an effort to provide a substrate for GPx (equation 4).



$$(1)\ O_2\bullet^- + O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2$$

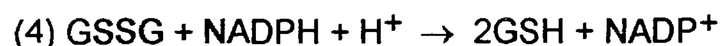


$$(2)\ 2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (CAT)$$



$$(3)\ H_2O_2 + 2GSH^1 \rightarrow GSSG^2 + H_2O\ (GPx)$$

[1]reduced glutathione
[2]oxidized glutathione



$$(4)\ GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

In the last decade, it has been established that the CAT gene resides at chromosome 11p13 (Glaser *et al.*, 1986). The presence of CAT ensures H_2O_2 is degraded to its harmless and beneficial biproducts H_2O and oxygen (equation 2 above). In animals, CAT is present in all major body organs, being especially concentrated in the liver and erythrocytes. At the cellular level, CAT is found mostly in peroxisomes and the cytosol. The usual form of CAT has a MW of about 240000 and consists of four protein subunits, each containing a heme group bound to its active site (Sun, 1990). In this thesis, a comparison of catalase activity is made between cell lines with and without an insert of an additional chromosome 11. This comparison is made in order to better delineate the mechanisms involved in the protection by chromosome 11 of DNA damage induced by ROS.

1.7.2 Chromosome 11 and DNA repair

The most common cell lines studied thus far with respect to the influence of chromosome 11 on the protection against tumourgenicity are cells derived from ataxia telangiectasia (AT) patients. The AT gene ('ataxia telangiectasia mutant', ATM) has recently been localized to 11q22-q23 (Savitsky *et al.*, 1995). AT is a genetic syndrome which shows autosomal recessive inheritance. Characteristic clinical features are early progressive development of cerebellar ataxia and oculocutaneous telangiectasia appearing somewhat later. It is also characterized by spontaneous chromosome instability and cellular sensitivity to agents that generate ROS such as X-rays.

Radiation hypersensitivity in AT cells and its relationship to the AT gene loci on chromosome 11 has been studied with the use of microcell-mediated chromosome transfer. Introducing a normal chromosome 11 into AT cells by this

method results in a normal level of chromosome breakage after X-irradiation. Hybrid AT cells with introduced chromosome 12 fail to show this reduction. This indicates that a defect on chromosome 11, probably the 11q23 region, in AT cells is responsible for the X-ray hypersensitivity to cell killing and chromosome breakage (Ejima *et al.*, 1990; Ejima *et al.*, 1991; Komatsu *et al.*, 1990; Kodama *et al.*, 1992).

AT cells are sensitive to agents other than X-irradiation. For example, AT fibroblasts are sensitive to chromosomal damage induced by hydrogen peroxide and by areca nut, a plant extract which when oxidized produces ROS (Yi *et al.*, 1990). It has been postulated that the underlying cause of spontaneous chromosome instability in AT cells may be an excess of endogenously generated ROS and a possible defect in the repair of DNA damaged by these radicals (Yi *et al.*, 1990; Pandita and Hittelman, 1992a,b; Gatti, 1993).

In most cases radiation has been the favoured agent for inducing DNA damage in an attempt to look at repair processes in AT cells. Cornforth and Bedford (1985) found that after X-irradiation, AT cells had the same initial frequency of breaks and the same initial rate of rejoining of chromosome breaks as normal cells. However, the fraction of chromosome breaks that did not rejoin was 5 to 6 times greater for the AT cells. Hittelman and co-workers (1992a,b), using λ-irradiation to treat transformed lymphoblastoid cell lines derived from AT and normal individuals, suggested that AT cells have a defect in double-strand DNA fast repair.

Several investigators have suggested that the basis of radiosensitivity in AT cells might be misrepair, rather than lack of DNA double-strand repair. By introducing a simple double strand break (dsb) into plasmid DNA with restriction enzymes, they were able to analyze rejoining at different sites with different types of DNA termini. Although there was not a difference in the extent of

rejoining, there was a reduction in the fidelity of dsb rejoining in AT cells compared with controls (Cox *et al.*, 1986; North *et al.*, 1990; Runger *et al.*, 1992; Ganesh *et al.*, 1993). This 'mis-rejoining' process may be a specific non-conservative recombination mechanism involving a loss of sequence around the break sites (Thacker *et al.*, 1992). Similarly, Meyn (1993) has shown elevated spontaneous intrachromosomal recombination in AT cells. These findings suggest that AT cells have a general disturbance in recombination processes. Figure 6 highlights one of the repair mechanisms proposed to take place in mis-rejoining of DNA double strand breaks.

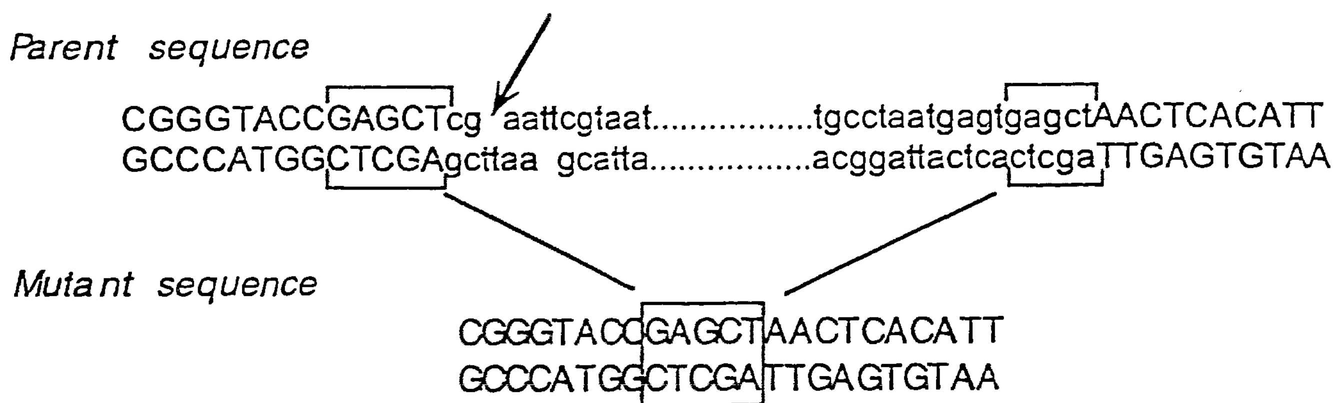


Figure 6. Strand-exposure and repair (SER) model for deletion formation at the sites of short direct repeats. It is envisaged that at the site of the initial break (arrow), the DNA strands unwind, or single strands are removed by exonuclease action, to reveal complementary short repeats in the sequence. These repeats provide sufficient homology for strands to reanneal, followed by repair synthesis and rejoining. Adapted from Thacker *et al.* (1992).

Cell lines other than those derived from AT patients have also been investigated for their repair capabilities. Parshad *et al.* (1992) demonstrated that human tumour cells, after X-irradiation during the G_2 phase of the cell cycle, had an abnormally high frequency of chromatid breakage and the addition of a single human chromosome 11 resulted in reduction of this sensitivity. They suggested that chromosome 11 (probably the long arm between the centromere and q23) carries a DNA repair gene or genes that results in efficient repair of the radiation-induced damage to the level in normal cells. Another interesting study done with bladder carcinoma cells found repair defects in radiosensitive compared to non-radiosensitive cells (Powell *et al.*, 1992). Table 4 summarizes some specific repair enzymes located on chromosome 11 that might be implicated in the protection against tumourgenicity offered by chromosome 11.

Table 4. Loci on chromosome 11 that play a role in DNA repair.

Repair Protein	Species Found In	Postulated Activity	Chromosome Location	Reference
ATM	human	Dsb repair and V(D)J recombination; Recruit DNA repair machinery or control DNA repair activity; Protein kinases that regulate cell cycle progression; Intimately linked to DNA damage and repair recognition.	11q22-q23	Keith & Schreiber 1995; Hartley, 1995
RP (Ribosomal Protein) S3	human	Endonuclease in repair of UV-induced DNA damage.	11q13.3-q13.5	Polakiewicz *et al.*, 1995
Fen-1	murine cells	Flap-structure-specific endonuclease activity; 5'-3' exonuclease activity.	11q12	Haraoka *et al.*, 1995
RAG 1	human	V(D)J recombination with RAG 2.	11p13	Oettinger *et al.*, 1992; Schwarz *et al.*, 1994
RAG 2	human	V(D)J recombination with RAG 1.	11p	Oettinger *et al.*, 1992

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CHAPTER 2

HYPOTHESES, OBJECTIVES AND APPROACHES

2.1 *In Vitro Studies*

The *in vitro* section of this thesis explores the concept that an altered sensitivity to reactive oxygen species (ROS) of cell clones in the bladder of chronically inflammed individuals could be a force that drives the development of neoplasia. This altered sensitivity could be innate or acquired during the process of carcinogenesis. These sensitive clones of cells would show an elevation in genetic instability in the presence of ROS and an increased probability of acquiring the multiple alterations to specific oncogenes or suppressor genes that are required for tumour development.

2.1.1 Hypothesis 1

During bladder carcinogenesis in individuals belonging to populations with chronic bladder inflammation, alterations to specific loci on chromosome 11 occur in some individuals and this alteration elevates the risk of genetic damage and hence cancer development.

2.1.2 Background and Approach

The choice of this chromosome for study was based on the following:

1) Allelic loss on chromosome 11 is a frequent genetic alteration in bladder carcinomas. Fine mapping studies support the possible involvement of at least two deletion targets on this chromosome (Shaw and Knowles, 1995). Although the timing at which these alterations occur during bladder tumourigenesis is as yet not established, loss of heterozygosity (LOH) on

chromosome 11 is already present in 54% of cases with carcinoma *in situ* (Rosin *et al.*, 1995). This suggests that these changes may play a role in early stages of the development of these cancers.

2) *In vitro* studies suggest that the insertion of normal chromosome 11 into tumour cells results in a reduction in their capacity to form tumours and in an altered sensitivity to ROS (see chapter 1 for summary).

Prior studies in this laboratory using a bladder tumour cell line have established that the fusion of normal chromosome 11 into these cells results in a reduced sensitivity to chromosomal breakage during treatments with human neutrophils activated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Ward *et al.*, 1993). The latter exposure is a model system for inflammation. The mechanism underlying this protection is not known.

The objective of the *in vitro* studies in this thesis was to better characterize this protective response and to test 2 hypotheses as underlying mechanisms responsible for the effect. The cell cultures used were expanded to three. They included the primary bladder carcinoma culture ('parent') and its microcell hybrid containing a normal chromosome 11 insert (termed 'hybrid'). Both of these cultures had been previously used. In addition, these studies used a third cell line derived from the 'hybrid' by spontaneous loss of the chromosome (11) insert (termed 'revertant').

The appoach used was to first characterize the sensitivity of these 3 cell lines to two ROS-generating agents H_2O_2 and X-rays. Following this, 2 hypotheses were tested.

2.1.3 Hypothesis 2

The protective response observed in the 'hybrid' is associated with an alteration to catalase activity.

2.1.4 Objective and Approach

The objective here was to determine if the insertion of chromosome 11 into the bladder carcinoma cells enhanced the activity of catalase. The approach used was to incubate 'parent', 'hybrid' and 'revertant' cells with H_2O_2 and to quantify the loss of H_2O_2 spectrophotometrically at fixed time intervals.

2.1.5 Hypothesis 3

The fidelity and/or the efficiency of double strand break rejoining is elevated in bladder carcinoma cells with a normal chromosome 11 insert.

2.1.6 Objective and Approach

The objective here was to determine if the insertion of chromosome 11 into the bladder carcinoma cells enhanced fidelity or the efficiency of DNA double strand break rejoining. To test this hypothesis, nuclear extracts from 'parent', 'hybrid' and 'revertant' cells were incubated with the plasmid pUC18 containing site-specific dsb in the *lacZ* gene created by the restriction enzymes *SalI*, *Eco*RI or *Kpn*I. The influence of nuclear extracts on dsb rejoining was assessed by gel electrophoresis and bacterial transformation using the *E. coli* strain DH5α.

2.2 *In Vivo Study*

2.2.1 Hypothesis 4

Patients on indwelling urinary catheterization have elevated levels of urinary white blood cells indicating bladder inflammation. This inflammation is associated with increased DNA damage in neighboring urothelial cells caused by the release of DNA damaging agents from the inflammatory cells.

2.2.2 Objective and Approach

The objective was to evaluate the role of chronic bladder infection and inflammation in inducing DNA damage *in vivo* in order to obtain a better understanding of tumour promotion in humans. The study populations were comprised of individuals on long-term indwelling urinary catheterization and an age-matched non-catheterized control group. Exfoliated bladder cells were collected to assess MN frequencies and a urinalysis was done to establish whether there was a relationship between chromosomal breakage and inflammation.

2.3 References

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CHAPTER 3

The influence of chromosome 11 and associated catalase levels on DNA damage induced by hydrogen peroxide

3.1 Abstract

Chromosome 11 plays an important role in determining a cell's sensitivity to DNA damage by reactive oxygen species (ROS). Although it is unclear which loci are involved in this protection, a candidate is the catalase gene which resides at 11p13. This study looked at DNA damage, assessed by micronucleus (MN) frequencies, and catalase activities in bladder carcinoma cells with and without a chromosome 11 insert. A bladder carcinoma cell culture (termed 'parent'), its microcell hybrid containing a normal chromosome 11 insert (termed 'hybrid') and a cell line derived by spontaneous loss of the extra chromosome 11 (termed 'revertant') were exposed to hydrogen peroxide (0-32.3 μM) then assessed for MN induction. The 3 cultures were also assayed for catalase activity by looking at the disappearance of hydrogen peroxide (H_2O_2) at 240 nm. We found a significant protection against H_2O_2 induced MN in the 'hybrid' culture compared to the two cell lines without the chromosome 11 insert ('parent' and 'revertant') ($p < 0.01$). Furthermore, the 'hybrid' had significantly elevated catalase levels. The extent to which the altered catalase activity is responsible for the protection against chromosomal damage in the 'hybrid' is unknown.

3.2 Introduction

Oxidative stress occurs when an enhanced rate of reactive oxygen species (ROS) generation results in a level which exceeds the antioxidant capacity of the cell, leading to an accumulation of radical damage. One of these ROS, hydrogen peroxide (H_2O_2), can freely diffuse across biological membranes and, through the Haber-Weiss reaction, produce a number of lesions in DNA by the production of a very reactive hydroxyl free radical (OH•) (Dizdaroglu, 1992; Paul *et al.*, 1989; Yi *et al.*, 1990).

Recent studies suggest that chromosome 11 may have loci that affect a cell's sensitivity to ROS. Sanford and co-workers have reported the presence of elevated chromosomal damage in tumour cells compared to normal cells after X-irradiation (Parshad *et al.*, 1992). Insertion of a normal chromosome 11 into these tumour cultures resulted in a restoration of the level of radiation-induced chromosomal damage in these cells to that observed in normal cultures. Our lab has recently shown that chromosome 11 protects against micronucleus (MN) induction by xanthine / xanthine oxidase or TPA - activated neutrophils coincubated with bladder-carcinoma cells (Ward *et al.*, 1993). The reason(s) for this protection against oxidative stress, however, remains unresolved. One mechanism by which chromosome 11 insertion could affect the sensitivity to oxidative stress is by altering the levels of catalase in the cell, since this gene is located at 11p13 (Glaser *et al.*, 1986; McAlpine *et al.*, 1988). In this hypothesis, catalase acts to reduce H_2O_2 to oxygen and water, thereby decreasing the quantity of ROS available to induce DNA damage.

In this study we have examined the influence of chromosome 11 on the sensitivity of cells to oxidative stress induced by H_2O_2. A bladder carcinoma culture (termed 'parent'), its microcell hybrid containing a normal chromosome 11 insert (termed 'hybrid') and a cell line derived by spontaneous loss of the

insert (termed 'revertant') were exposed to H_2O_2 (0 - 32.3 μM), then assessed for **MN** frequencies. Each of the 3 cultures were also assayed for catalase activity by looking at the disappearance of H_2O_2 at 240 nm.

3.3 Materials and methods

3.3.1 Cells and culture conditions

All cell lines were obtained from Dr. O. Pereira-Smith, Baylor College of Medicine, Houston, Texas. The primary bladder culture ('parent') and its microcell hybrid with the chromosome 11 insert ('hybrid') have been described previously (Ward *et al.*, 1993). The revertant clone ('revertant') was derived from the 'hybrid' cell line by spontaneous loss of the chromosome 11 insert. Results using karyotype analysis on metaphases suggest that the 'parent' and 'revertant' cell lines have 3 copies of chromosome 11, while the 'hybrid' has 4 copies. Cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Flow Laboratories, McLean, VA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY), penicillin (100 units/ml) and streptomycin (100 mg/ml) as described previously (Ward *et al.*, 1993). All stock cultures were maintained in 175cm^2 culture flasks at 37°C in incubators with a 5% CO_2/ 95% air atmosphere.

3.3.2 Micronucleus (MN) assay

Cultures were plated into 100 cm^2 square Petri dishes containing 3 glass slides and appropriate media. Cells were removed from stock culture flasks by trypsinization, put into an equal volume of media and plated at 1.6 x 10^6 cells/dish. Cells were incubated 24 hours prior to treatment at which time they were approximately 70% confluent.

Hydrogen peroxide (H_2O_2) was diluted from an 8.9M stock solution in Phosphate-Buffered Saline (PBS). Twenty-four hours after plating, media was removed, cells were washed 1x with wash media (no serum) and 1x with PBS. Cells were then exposed to H_2O_2 (in PBS) for 1 hour at 37°C. Following treatment, H_2O_2 was removed, and the cells were washed first with PBS then

with wash media. Finally, normal growth media was reapplied and the cells were allowed to recover from the damaging agent for 3 hours at 37°C. In order to identify cells that passed through one division cycle after exposure, cytochalasin B (CB, 2 μg/ml) was added to the media and the culture incubated a further 24 hours. Cells were then washed in PBS, allowed to dry, then fixed in Carnoy's fixative (1:3 glacial acetic acid:methanol) for 20 minutes before air-drying.

Cells were stained with the Feulgen reaction as described previously (Yi *et al.*, 1990). Coverslips were mounted onto the stained slides using Permount (Fisher Scientific) and allowed to dry until ready for MN assessment. This was done by coding the slides then analyzing a total of 500 binucleated cells for the presence of MN. Each experiment was repeated 3 times.

3.3.3 Catalase assay

'Parent', 'hybrid' and 'revertant' bladder carcinoma cell lines were grown to approximately 70% confluency. To harvest, media was suctioned off, cells were washed 2x in PBS then 2 ml of a catalase buffer (50 mM potassium phosphate, pH 7.8, 0.1 mM EDTA) was applied and cells scraped off each plate and placed into centrifuge tubes. The cells were pelleted by centrifugation then resuspended in catalase buffer at a final concentration of 1.5×10^6 cells/ml.

The catalase activity, corresponding to the dismutation of H_2O_2 into water and molecular oxygen, was measured after addition of H_2O_2. To initiate the reaction, 2 ml of each cell suspension were subjected to freeze-thawing for 4 x 5 minute intervals. Final reaction volume was 3.0 ml and contained 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA and 15 mM H_2O_2. Reactions were initiated by the addition of 1 ml of 45 mM H_2O_2. 100 μl reaction aliquots were removed at fixed time intervals and placed in microcentrifuge tubes containing 900 μl of 0.11 mM NaN_3. The first sample was taken at 30

seconds, the second at 1 minute then a sample was taken at 1 minute intervals for up to 12 minutes. The tubes were then centrifuged for 15 min at 13 500 g, and the amounts of H_2O_2 remaining in the supernatant fluid determined spectrophotometrically at 240 nm against a blank containing buffer and 0.1 mM NaN_3. The catalase activity was then determined by measuring the slopes of the lines in a graph representing a loss of H_2O_2 per unit time.

3.3.4 Statistical analysis

The results are expressed as the mean values and standard errors of the mean (S.E.) for each data point. Each experiment was repeated a minimum of 3 times. Tests for the effect of treatment within each cell line were performed using a randomized block design ANOVA with Bonferoni multiple comparisons, treating the values as categorical. Comparisons between different cell lines were performed using both a 2-way ANOVA and polynomial regression analysis. The p-value chosen for significance in these studies was 0.01.

3.4 Results and Discussion

Our choice of chromosome 11 for study was initially based on the observation that the introduction of this chromosome into ataxia telangiectasia (AT) cells suppressed radiosensitivity (Komatsu *et al.*, 1990). Since this observation, our lab has been examining the role of loci on chromosome 11 in protecting tumour cell lines against chromosomal damage induced by ROS. We have found that this chromosome protects against DNA damage induced by xanthine/ xanthine oxidase, activated human neutrophils and X-irradiation (Ward *et al.*, 1993; Hofseth *et al.*, unpublished data). In order to further explore this protection provided by chromosome 11 and better delineate the mechanisms by which it protects, this study examined MN induction by H_2O_2 in bladder carcinoma cells with ('hybrid') and without ('parent' and 'revertant') an extra normal chromosome 11. We also assayed these cells for catalase activity. The results suggest that an insert of chromosome 11 into bladder carcinoma cells protects against DNA damage induced by H_2O_2 (figure 7). Treatment of bladder carcinoma cells with H_2O_2 induced MN. However, this response was significantly lower in the 'hybrid' cells ($p < 0.0001$) compared to 'parent' and 'revertant' cells; the latter two cell types having similar MN frequencies ($p = 0.176$). At the highest dose, MN frequencies were approximately 5-fold greater for the 'parent' and 'revertant' than for the 'hybrid' cells (mean induced frequency $\pm$ S.E: 'parent', 17.1 ± 0.9; 'revertant', 18.6 ± 0.4; 'hybrid', 3.7 ± 0.4, $p < 0.0001$).

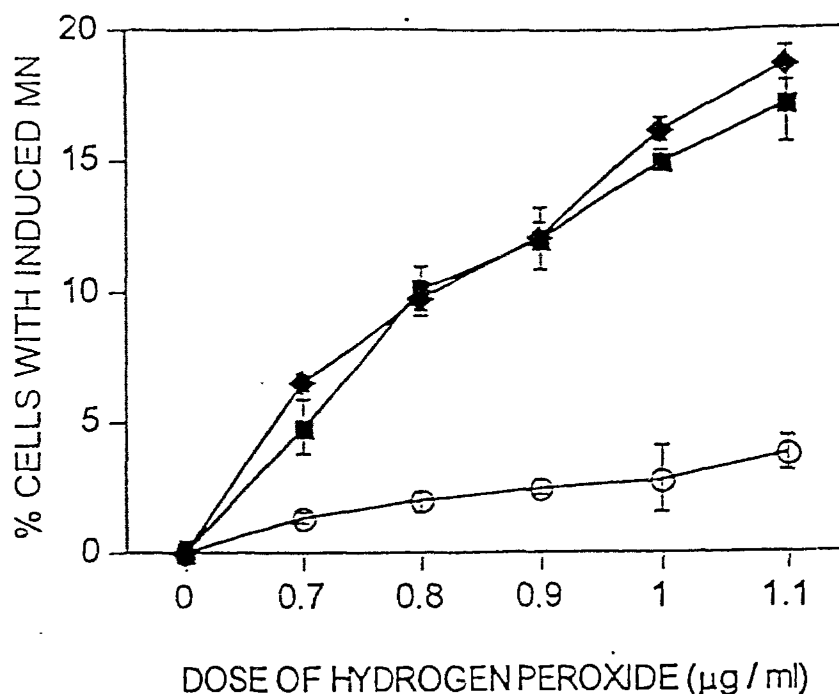

Figure 7. The effect of H_2O_2 treatment on micronucleus formation in 'parent' (■), 'hybrid' (O) and 'revertant' (◆) cultures. Spontaneous values for each culture have been subtracted to make it easier to see the effect of the treatment ('induced values').

The protein products of chromosome 11 that protect the 'hybrid' culture from H_2O_2 - induced DNA damage remain unknown. The possibility that the catalase activity is augmented is intriguing. Other studies have suggested the involvement of alterations in this enzyme as a factor that contributes to cancer risk. Catalase activities have been shown to be reduced in biopsies obtained from xeroderma pigmentosum patients compared with normal individuals (Vuillaume *et al.*, 1983, 1986) and may play a role in the sensitivity of these patients to ultraviolet light. An alteration in this enzyme has also been reported for many tumours and some pre-neoplastic lesions (Rabilloud *et al.*, 1990; Vuillaume *et al.*, 1991; Hoffschir *et al.*, 1993; Jaruga *et al.*, 1994). These observations suggest that in these tissues, the catalase locus is damaged or its regulation is altered, thus reducing a protection against DNA damaging ROS. In

the present study, we found that the catalase activity was significantly greater in the 'hybrid' cell culture ($p < 0.0001$) compared to that measured in the 'parent' and 'revertant' cells. Catalase activities in the latter two cell lines were not significantly different ($p > 0.05$) (figure 8).

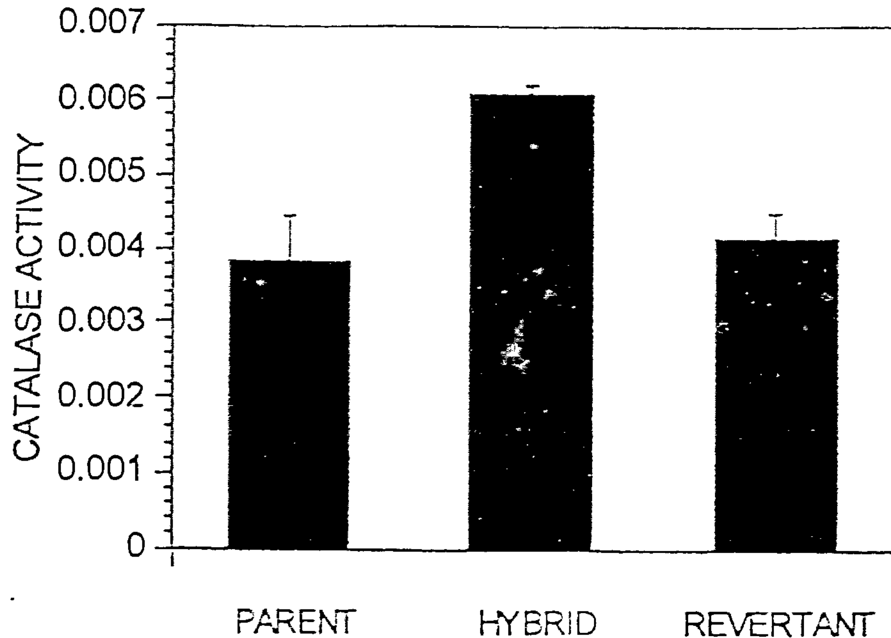

Figure 8. Catalase activity (± S.E.) in 'parent', 'hybrid' and 'revertant' cultures. Activity was expressed as a loss of H_2O_2 per minute.

There appears to be more catalase activity in the 'hybrid' culture than would be expected from a gene dosage effect. The tumour cell lines used in this study are aneuploid. There are 3 copies of chromosome 11 in the 'parent' and 'revertant' cell lines and 4 copies in the 'hybrid'. However, the 'parent' and 'revertant' cultures had 62% and 54% of the catalase activity of the 'hybrid' culture respectively and not 75%, as would be expected if all of the copies of the catalase loci were equally functional.

Since normal bladder cell lines are not commercially available, we were unable to directly determine whether the catalase activities observed in the tumour cell culture was deficient compared with a primary urothelial cell culture.

Although readily available, fibroblast or keratinocyte skin cultures from normal individuals cannot substitute for urothelial cultures since catalase activities could vary significantly with cell type and tissue source. A more direct way of approaching this question would be to measure catalase activities in fresh bladder biopsies from patients, for example, during cystectomies. Ideally one would like to have biopsies from normal urothelium, from premalignant lesions, and from tumours, and from individuals belonging to populations with and without an associated chronic bladder inflammation. Unfortunately this would be quite difficult logistically. Alternatively, it would be possible to use molecular techniques to examine DNA extracted from archival paraffin blocks of such lesions to determine whether there are mutations in the catalase gene. However, these formalin-fixed biopsies cannot be used to study alterations in gene expression or enzyme activity, so they can only partially test for catalase dysregulation in high-risk bladders.

It is of interest to note that when assaying for ROS-induced chromosomal damage by other techniques, our lab has found that the additional chromosome 11 in 'hybrid' cells is not protective. For example, we have looked at alterations to chromosome number after H_2O_2 treatment by using fluorescent *in situ* hybridization techniques with centromere-specific DNA probes to identify chromosome loss or gain (Pang, 1995). Although H_2O_2 caused significant chromosome loss and gain in 'parent' and 'hybrid' cultures, there was no significant difference between the cultures. Other co-workers have exposed the 'parent' and 'hybrid' cells to xanthine/ xanthine oxidase and measured the level of single-strand DNA breakage. Their experimental outcome indicated that the amount of single-strand DNA damage in the 'parent' and 'hybrid' was not significantly different (Ward *et al.*, 1993). These results suggest that the additional catalase present in the 'hybrid' cells is insufficient to prevent

numerical chromosome loss/ gain or protect against single-strand breakage in the 'hybrid' cells. The difference in results observed for these different endpoints is not due to the use of different concentrations of H_2O_2 in the aforementioned studies, since the same concentrations of peroxide were used to induce both chromosome loss and gain and MN.

In conclusion, the data obtained in this study suggest that an elevation in catalase activity is responsible for at least some of the protection of the 'hybrid' cells against hydrogen peroxide. However, other factors may also be playing a role. This possibility is supported by other studies in the literature. For example, Cantoni and co-workers (1994) suggested that an acquired resistance of certain CHO cells to oxidative stress is not entirely dependent on catalase activity. A similar finding was reported by Alcorn and co-workers (1994) using different strains of *Neisseria gonorrhoeae*. One such possibility, that an alteration in the processing of DNA damage is occurring in the 'hybrid' culture, will be explored in the following section.

3.5 References

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CHAPTER 4

Rejoining of DNA double strand breaks after the introduction of chromosome 11 into a radiosensitive bladder carcinoma cell line

4.1 Abstract

Insertion of a normal chromosome 11 into tumour cell lines can protect against a sensitivity to irradiation and oxidative stress. A possible mechanism underlying this effect is that there is a correction of a defect in the rejoining of double strand breaks (dsb) by the chromosome insertion. In order to explore this hypothesis, three cell lines were evaluated for their ability to rejoin dsb: 1) a bladder carcinoma cell line ('parent') previously shown to be sensitive to irradiation and radical generating species, 2) a derivative of this cell line into which a normal chromosome 11 had been inserted by microcell fusion ('hybrid') showing corrected radiosensitivity, and 3) a 'revertant' cell line that had spontaneously lost the insert and reverted to the radiosensitive phenotype. Nuclear extracts from the 3 lines were isolated and evaluated for their capacity to rejoin plasmid (pUC18) DNA broken at defined restriction sites (*Sal*I, *Eco*RI, *Kpn*I) in the *lacZ* gene. The extent of rejoining was determined by gel electrophoresis and the fidelity of rejoining determined by expression of the *lacZ* gene in *E.coli* DH5α bacteria. Results suggest there is no difference between the 'parent', 'hybrid' and 'revertant' nuclear extracts in the fidelity and the total extent of rejoining, regardless of the type of break. However, there is an alteration in the distribution of rejoined products. Nuclear extracts from 'hybrid' cells tend to rejoin linear DNA into circular monomers with a greater efficiency than extracts from both 'parent' and 'revertant' cells. The results suggest that loci on chromosome 11 are involved in the rejoining of dsb, affecting the relative amount of the different rejoined products. Whether this alteration plays a role in the 'parent' cell's radiosensitivity is yet to be determined.

4.2 Introduction

DNA repair deficiencies have been described for many tumour cell lines. In 1992, Parshad and co-workers (1992) reported an alteration in human tumour cells that resulted in an increased sensitivity to X-irradiation, seen as an abnormally high frequency of chromatid breaks and gaps after exposure during the G_2 phase of the cell cycle. Of interest was the observation that the addition of a normal chromosome 11 by microcell fusion to these cell lines resulted in efficient repair of the radiation-induced damage to the level seen in normal cells, even though the tumours were of diverse tissue origin. These data suggest the possibility of a common genetic defect in tumours associated with this chromosome that could lead to genomic instability and thus could play a critical role in tumour progression. There has recently been a renewed interest in studying the involvement of loci on this chromosome in DNA damage recognition due to the localization of the putative gene for ataxia telangiectasia, the ATM gene, on chromosome 11 (Savitsky *et al.*, 1995). Ataxia telangiectasia (AT) cell cultures show a similar pattern of chromosomal radiosensitivity that is corrected by insertion of normal human chromosome 11 into AT cells (Komatsu *et al.*, 1990; Kodama *et al.*,, 1992).

In order to further explore the involvement of chromosome 11 in DNA damage in tumour cell lines, we have begun working with a bladder carcinoma cell line (termed 'parent'), a microcell hybrid of this line containing normal chromosome 11 (termed 'hybrid') and a third cell line derived from the 'hybrid' by spontaneous loss of this insert (termed 'revertant'). The 'parent' cell line has an elevated sensitivity to chromosome breakage induced by radical-generating agents, including X-irradiation, hydrogen peroxide, and xanthine/xanthine oxidase mixtures (Yi *et al.*,1990; Ward *et al.*, 1993; Hofseth and Rosin, unpublished data). Insertion of chromosome 11 resulted in a correction of this

defect, with breakage decreasing to levels observed in normal fibroblast and epithelial cultures. This protection was lost in the 'revertant' culture (figure 9).

The objective of the present study was to explore the possibility that the chromosome 11 insertion corrects a defect in the rejoining of double strand breaks (dsb). A model system often used to study defective repair in mammalian cells is to examine rejoining of restriction endonuclease-broken DNA by these cells (Thacker, 1989). Several studies have used this approach and found repair defects in radiosensitive cells such as those derived from AT patients or bladder carcinoma cells (North *et al.*, 1990; Powell *et al.*, 1992). In this study, nuclear extracts from 'parent', 'hybrid', and 'revertant' cells were incubated with DNA of the plasmid pUC18, broken at defined restriction sites in the *lacZ* gene. Dsb were introduced into the *lacZ* gene of this plasmid with either the *Eco*RI, *Sal*I, or *Kpn*I restriction enzymes. These restriction endonucleases were selected because previous studies suggested dsb induced by these enzymes were rejoined with an alteration in fidelity in radiosensitive cell lines (North *et al.*, 1990; Powell *et al.*, 1992). Following incubation, dsb rejoining of the plasmid DNA was monitored using Southern analysis after gel separation and the fidelity of rejoining assessed by expression of the *lacZ* gene after bacterial transformation with the treated plasmid. The results show that chromosome 11 insertion does not alter the extent or fidelity of dsb rejoining. Instead, a greater portion of the plasmid DNA was rejoined into circular monomers during incubation with 'hybrid' extracts whereas mainly linear multimers were produced by 'parent' and 'revertant' extracts.

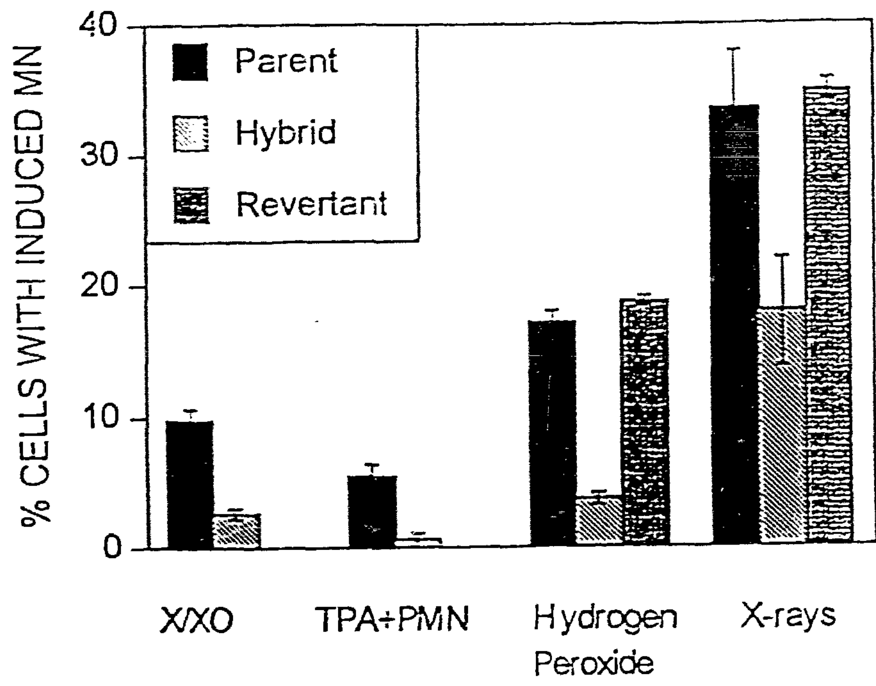


Figure 9. Micronucleus (MN) induction in 'parent', 'hybrid' and 'revertant' bladder carcinoma cells after exposure to xanthine/xanthine oxidase (X/XO), TPA-stimulated neutrophils, hydrogen peroxide (H_2O_2) and X-rays. Data for X/XO and TPA-stimulated neutrophils were obtained from Ward et al (1993). Data for H_2O_2 and X-rays were obtained by the author in this thesis.

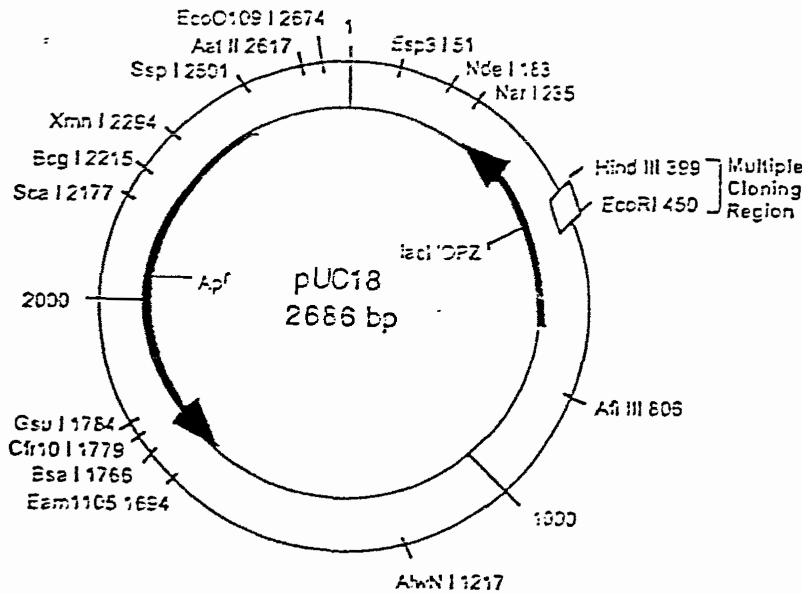
4.3 Materials and methods

4.3.1 Cells and culture conditions

'Parent', 'hybrid' and 'revertant' cell lines were obtained from Dr. O. Pereira-Smith, Baylor College of Medicine, Houston, Texas. The 'parent' culture (A1698) is a bladder carcinoma cell line. The 'hybrid' clone is derived from the 'parent' culture and contains an intact der(11), which consists of a derivative chromosome 11 (der11) from a normal donor that has a balanced X;11 translocation [der(11)t(X;11) (q25;q23)]. The der11 is composed of most of chromosome 11 with a small piece of distal Xq containing the HPRT locus attached (Scott *et al.*, 1979; Ning, 1991). The 'revertant' clone is derived from the 'hybrid' cell line by spontaneous loss of the chromosome 11 insert. All cultures were grown in Dulbecco's modified Eagle's media as described by Ward et al. (1993) and in chapter 4.

4.3.2 Description of plasmid pUC18 DNA

The pUC18 plasmid (Gibco) used in this thesis was a *E. coli* cloning vector containing the *Pvu II/EcoRI* fragment of pBR322 that carries the β-lactamase gene (ampicillin resistance) and the origin of replication. A *Hae*II fragment (position 239-684) contains the α-peptide of the *lacZ* (β-galactosidase) gene and a multiple cloning site of one of the M13mp vectors. Insertion of DNA or breaking the DNA at the multiple cloning site results in interruption of the α-peptide, producing colorless, rather than blue, colonies on medium containing ampicillin and X-gal. Figure 10 shows the plasmid pUC18 DNA and the multiple cloning region where breaks are made.



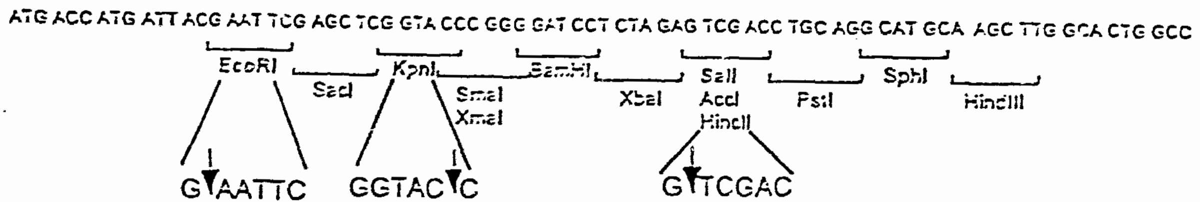


Figure 10. Plasmid pUC18 DNA

4.3.3 Preparation of plasmid and nuclear extracts

LacZ^{+} pUC18 DNA (Gibco) was grown in bulk in *E.coli* DH5α. This was followed by alkali lysis and purification by ultracentrifugation in a CsCl/ethidium bromide density gradient. The plasmid was characterized by restriction endonucleases (data not given) and frozen as a stock at $^{-}20^{o}$C. Prior to coincubation, the plasmid was linearized with the restriction enzymes *Eco*RI, *Sal*I, or *Kpn*I (Gibco). Digestion was monitored by agarose gel electrophoresis. The linearized DNA was then purified by phenol/chloroform extraction and ethanol precipitation.

Nuclear extracts were prepared from bladder cell cultures that were 70-80% confluent on 3 separate occasions. The cells were harvested and nuclear cell extracts were prepared from approximately 10^8 cells using methods described by North *et al.* (1990). Extracts were stored at -70°C where they were stable for over 8 months.

4.3.4 Plasmid rejoining assay

The assay followed conditions described by North *et al.* (1990) with some modifications. Protein extracts and linearized plasmid DNA were mixed in 50 µl reactions containing Tris-HCl (pH 7.5, 52.4 mM), $MgSO_4$ (10 mM), ATP (3 mM), EDTA (0.1 mM), 2-mercaptoethanol, (5 mM), PMSF (phenylmethylsulfonyl fluoride, 0.1 mM), glycerol (10%), and linearized plasmid DNA (2 µg of 40 ng/µl stock). Nuclear extract protein (0 - 200 µg/ml) or T4 ligase (Gibco, 2.0 U) was added to start the reactions and incubation was carried out at 17°C for 1 h. Reactions were terminated by addition of an equal volume of 100 mM EDTA/1% SDS and the DNA purified by digestion with Proteinase K (Sigma, 2 mg/ml) and Ribonuclease A (Sigma, 1 mg/ml). The volume was adjusted to 200 µl with TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) and the sample purified by phenol/chloroform extraction followed by ethanol precipitation. Trace amounts of ethanol were removed from the DNA pellet by vacuum centrifugation. The pellet was then dissolved in 40 µl double-distilled H_2O and stored at -20°C.

4.3.5 Gel electrophoresis and Southern analysis

Each DNA sample was heated at 65°C for 10 min, diluted to 5 ng/µl and a 10 µl aliquot (50 ng DNA/well) was run on a 1.4% agarose gel in TAE (0.04 M Tris-acetate, 0.001 M EDTA) for 4 h at 2.25 V/cm. For Southern analysis, the gel was blotted onto a hybond N+ membrane (Amersham), and probed with the

pUC18 plasmid, labelled by the ECL random primer labelling system according to the instructions of the supplier (Amersham).

4.3.6 Quantification of DNA bands in Southern blots

Immediately after labelling, Southern blots were autoradiographed for 30 sec - 5 min. The blots were then exposed for 1 h to an imaging screen for chemiluminescence and the screen scanned by a Molecular Imager™ System (Model GS-250, Bio-Rad Laboratories). The image was preserved and DNA bands later quantified using Phosphor Analyst/PC Image Analysis Software for Microsoft Windows version 3.1. Bands corresponding to circular DNA products, linear monomers and linear multimers (dimers, trimers and tetramers) (figure 11) were carefully identified by comparing the migration patterns to a 1 kb ladder and by use of controls which marked the positions of circular and linear bands. The densities of these bands were determined (figure 12) and the sum of the densities was calulated for each lane. The percentage DNA rejoined into circular products and into linear multimers as well as the total amount of rejoining was then determined.

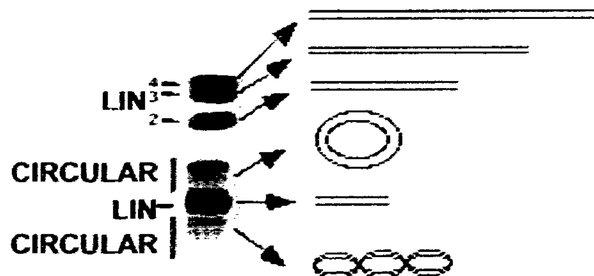


Figure 11. Example of the bands that were examined after nuclear extracts were incubated with linearized pUC18 DNA. Linear DNA (LIN) was rejoined either into circular products (including relaxed forms and various forms of supercoiling) or linear multimers, including dimers (LIN^2), trimers (LIN^3) and tetramers (LIN^4).

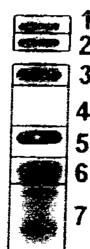


Figure 12. Quantification of rejoined pUC18 DNA after incubation with nuclear extracts. Each band was quantified individually in each lane and the area quantified was consistent from lane to lane. The area of each DNA band was carefully identified and the sum of the phosphor counts within all of the pixels in that area was determined as the 'density' of the band. 1, linear tetramer; 2, linear trimer; 3, linear dimer; 4, lane background; 5, circular products; 6, linear monomer (non-rejoined product); 7, circular products.

4.3.7 Bacterial transformation and rejoin fidelity

Bacterial transformations were carried out using the *E.coli* DH5α strain. Briefly, 5 μl of each sample (200 ng DNA) was added to 10 μl of competent bacteria and allowed to sit on ice for 30 min. The samples were incubated at 37°C for 30 min, diluted 10-fold with SOC, then further incubated for 1 h at 37°C. Samples were plated on LB plates supplemented with ampicillin (100 μ g/ml) and X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 40 μg/ml) and incubated overnight at 37°C. Bacterial viability was assessed by plating sample aliquots on plates without ampicillin. Transformation frequencies were expressed as transformants per viable cell to allow comparison between different experimental samples.

4.3.8 Statistical analysis

Differences between 'parent', 'hybrid' and 'revertant' cell extracts in terms of the total amount of rejoining were examined using a two-way analysis of variance (ANOVA). Differences between extracts or restriction enzyme break-types in terms of circular rejoining, linear rejoining, and transformation frequencies were examined using a one-way ANOVA with Scheffe's multiple comparison test. The p-value chosen for significance in this study was 0.05.

4.4 Results

4.4.1 Rejoining of *SalI*-induced dsb by 'parent' extracts

Initial studies were performed by incubating *SalI* broken pUC18 DNA with nuclear extracts (25 - 200 μg/ml) of the bladder carcinoma cell line ('parent'). Rejoining was analyzed by autoradiography after separation of the reaction products by gel electrophoresis. As shown in figure 13, the plasmid DNA without treatment by *SalI* restriction endonucleases migrated mainly as closed circular and supercoiled bands. After *SalI* digestion, a single linear (LIN) band was identified. When the broken plasmid DNA was incubated with either T4 ligase or the nuclear extract, the plasmid was rejoined into circular forms, produced by intrastrand rejoining, as well as linear multimers (LIN^2, LIN^3, LIN^4), produced by interstrand rejoining. The formation of these products was dependent on the concentration of the nuclear extract present in the reaction. This rejoining was absent when the nuclear extract was boiled (10 mins, 100 ug/ml extract) prior to incubation with the broken plasmid DNA.

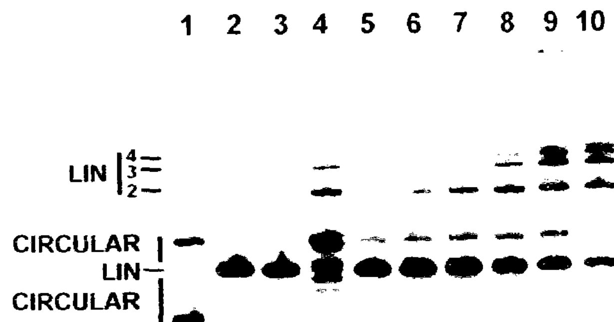


Figure 13. Autoradiogram showing rejoining of linearized (LIN) pUC18 DNA. Linear monomers were rejoined into either circular products or linear multimers, including dimers (LIN^2) , trimers (LIN^3), and tetramers (LIN^4). Lane 1, uncut pUC18 DNA. Lanes 2 - 12, pUC18 DNA linearized with *SalI* then incubated with: lane 2, boiled 'parent' extracts (100 μg/ml); lane 3, no extract; lane 4, T4 ligase (2.0 U); lanes 5 - 10: 25, 50, 75,100, 150, and 200 μg/ml of 'parent' nuclear extract, respectively.

4.4.2 Effect of insertion of chromosome 11 into bladder carcinoma cell line

Since it was previously determined that insertion of chromosome 11 into the 'parent' cell line corrected radiosensitivity, we examined the possibility that loci on this chromosome were altering the efficiency of rejoining of dsb. Nuclear extracts were isolated from 'parent', 'hybrid', and 'revertant' cells and incubated with *Sal*I digested DNA. As shown in figure 14, DNA dsb were rejoined by extracts from all 3 cell lines. However, the distribution of rejoined products was different. 'Hybrid' nuclear extracts produced more circular products and less linear multimers than either the 'parent' or 'revertant' extracts. In order to better characterize this rejoining, we used a phosphoimager to quantify each of the reaction products. Figure 15a shows the percentage of the *Sal*I-broken plasmid DNA that is rejoined by increasing concentrations of extract from the 3 cell lines. There was no statistical difference in the efficiency of rejoining by extracts from these cell lines ($p = 0.12$). In each case, about 40 - 50% of the plasmid DNA was rejoined. Approximately 80% of this rejoining occurred with an extract concentration of 100 μg/ml. Doubling the protein concentration resulted in only an additional 20% increase in rejoining.

In order to compare the distribution of rejoined products generated by the different nuclear extracts, we quantified the fraction of *Sal*I-digested DNA that rejoined into circular and linear products. As shown in figure 16a, nuclear extracts from 'hybrid' cells rejoined this type of break into circular forms with a higher efficiency than that of 'parent' and 'revertant' extracts (% of rejoined products that are circular with 200 μg/ml extract: 'hybrid', 62%; 'parent', 26%; 'revertant', 28%; $p < 0.05$). In contrast, the same concentrations of nuclear extracts from 'parent' and 'revertant' cells resulted in a preferential rejoining of

the broken plasmid DNA into linear multimers (fig. 16b). Furthermore, the distribution of rejoined products was not statistically different between the 'parent' and 'revertant' extracts ($p > 0.05$).

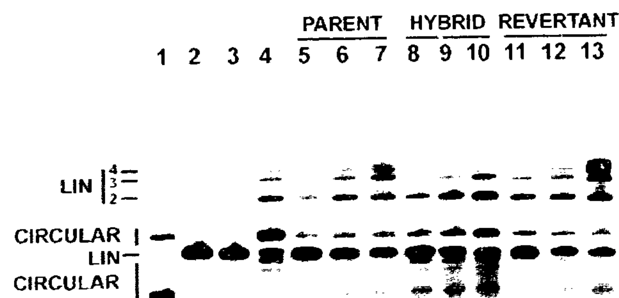


Figure 14. Autoradiogram comparing the distribution of rejoining by 'parent', 'hybrid', and 'revertant' nuclear extracts. Linear monomers were rejoined into either circular products or linear multimers. Lane 1, uncut pUC18 DNA. Lanes 2 - 13, pUC18 DNA linearized with *SalI* then incubated with: lane 2, boiled 'parent' extracts (100 μg/ml); lane 3, no extract; lane 4, T4 ligase (2.0 U); lanes 5, 6, and 7: 50, 100 and 150 μg/ml of 'parent' nuclear extract, respectively; lanes 8, 9, and 10: 50, 100 and 150 μg/ml of 'hybrid' nuclear extract, respectively; lanes 11, 12, and 13: 50, 100 and 150 μg/ml of 'revertant' nuclear extract, respectively.

4.4.3 Rejoining of dsb induced by other restriction enzymes

In order to determine whether differences in dsb end-structure would affect the rejoining characteristics by the different cell lines, we examined the rejoining of dsb created at other restriction sites in the pUC18 DNA multicloning region. *Eco*RI and *SalI* both generate a 5' 4-base overhang (but with different temini) whereas *KpnI* induces a 3' 4-base overhang. Similar to the results observed with *SalI*, all 3 cell lines showed a comparable capacity to rejoin dsb induced by *Eco*RI ($p = 0.86$) and *KpnI* ($p = 0.40$) (figure 15). However, a larger

percentage of the *Sal*I-broken plasmid DNA is rejoined (40 - 50%) than *Eco*RI- and *Kpn*I-broken plasmids (20 -30%) ($p < 0.05$). Finally, as in *Sal*I, about 80% of the rejoining reaction occurs with extract concentrations of 100 µg/ml when incubated with pUC18 DNA broken with *Eco*RI and *Kpn*I and doubling the concentration produces only an additional 20% rejoining.

In terms of the distribution of rejoined products, *Eco*RI- and *Kpn*I-induced dsb were also rejoined by 'hybrid' nuclear extracts into predominantly circular products, while the 'parent' and 'revertant' extracts preferentially rejoined DNA into linear multimers (figure 16c,d,e,f). At the highest concentration of extract used, 65% of the rejoined products after *Eco*RI digestion were circular in mixtures containing 'hybrid' extract, compared with 18% for both 'parent' and 'revertant' extracts. For *Kpn*I-digested plasmid, the percentage of rejoined products that was circular was 70% for the 'hybrid' extract, compared to 15% - 20% for the 'revertant' and 'parent' extracts respectively. These differences between the 'hybrid' extracts compared to the 'parent' and 'revertant' extracts were significant ($p < 0.05$).

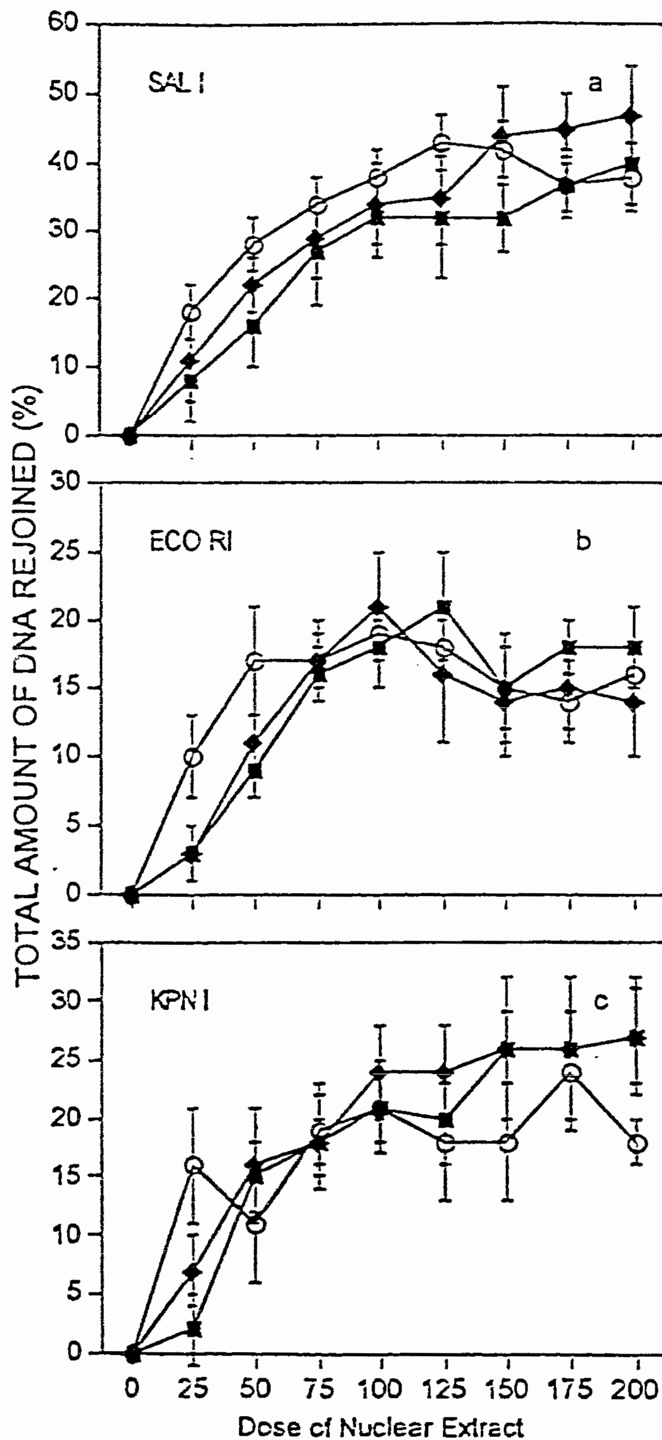


Figure 15. The effect of nuclear extracts from 'parent'(■), 'hybrid'(O), and 'revertant'(◆) bladder carcinoma cells on the total amount of rejoining of (a) *Sal*I, (b) *Eco*RI-, and (c) *Kpn*I-induced dsb.

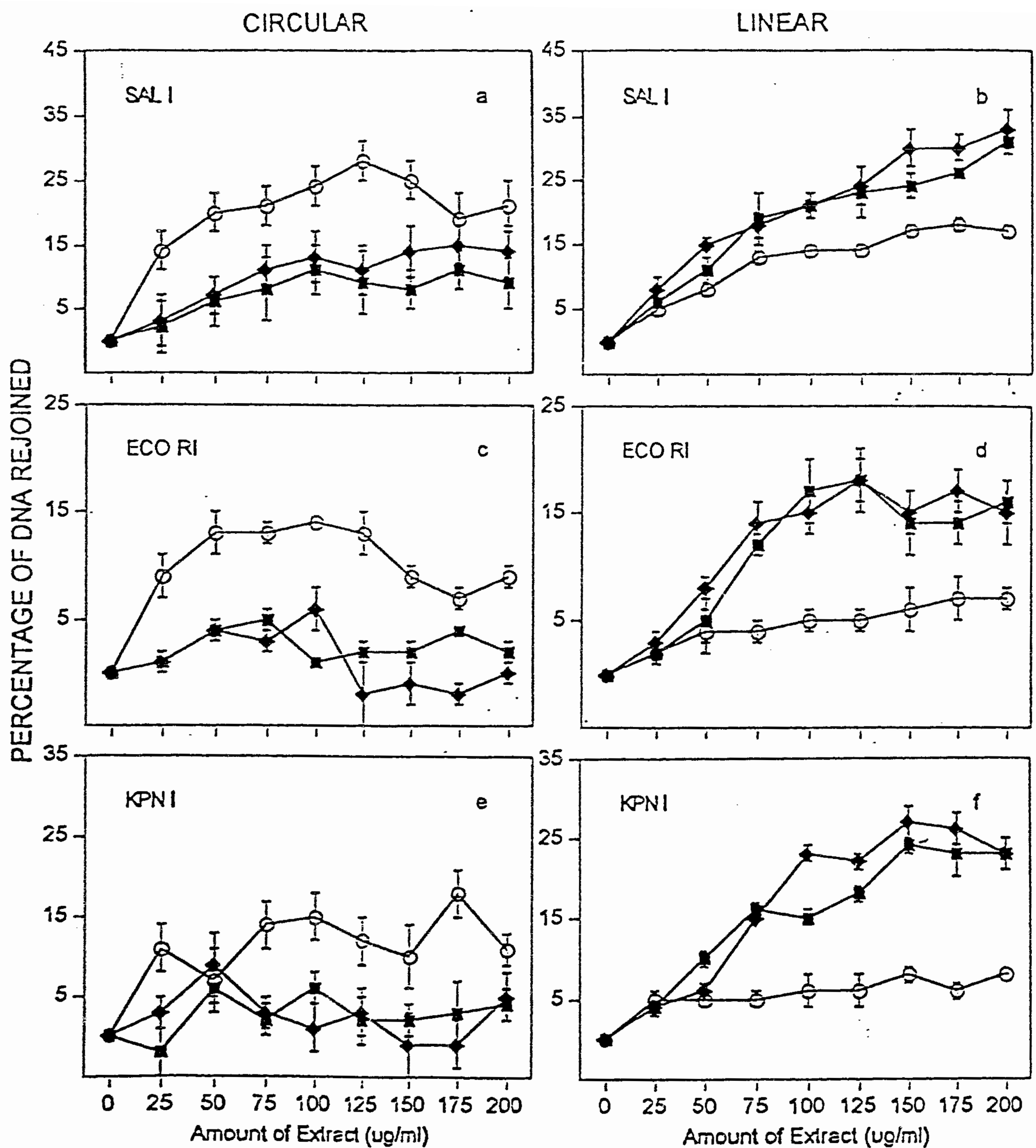


Figure 16. The effect of nuclear extracts from 'parent'(■), 'hybrid'(O), and 'revertant'(◆) bladder carcinoma cells on the rejoining of linearized DNA into circular products (a, c, e) or linear products (b, d, f). pUC18 DNA was linearized with either *Sal*I, *Eco*RI, and *Kpn*I restriction endonucleases as labelled.

4.4.4 Transformation frequencies and fidelity of rejoining of DNA dsb

In order to determine whether the fidelity of rejoining was similar for the aformentioned nuclear extracts, large-scale transformation experiments were performed. While linearizing the DNA with restriction enzymes reduced the transformation frequency by up to 300 fold, treatment of the linearized plasmid DNA with any of the nuclear extracts increased this frequency significantly. In all cases, incubation with 'hybrid' extracts produced higher transformation frequencies than 'parent' and 'revertant' extracts (table 5). Frequencies were similar for 'parent' and 'revertant' extracts ($p > 0.05$). Finally, for all 3 cell lines, higher frequencies were observed when bacteria was transfected with rejoined DNA after *Sal*I digestion compared to *Eco*RI or *Kpn*I digestion.

Table 5. Transformation frequencies of *Eco*RI, *Sal*I, and *Kpn*I linearized pUC18 DNA after incubation with 'parent', 'hybrid', and 'revertant' nuclear extracts.

Dsb type	Transformation frequency (±S.E.) per 10^7 viable bacteria[a]				
	Neg. controls	T4 ligase	Parent	Hybrid	Revertant
*Eco*RI	11 ± 2[b]	1948 ± 123[b]	50 ± 18[b,c]	133 ± 25	39 ± 9[b,c]
*Sal*I	7 ± 2[b]	1269 ± 610[b]	139 ± 32	300 ± 99	160 ± 36
*Kpn*I	10 ± 3[b]	856 ± 1[b]	55 ± 7[c]	81 ± 13[c]	46 ± 6[b,c]

[a]Each data point represents the mean transformation frequency from 3 experiments. Bacteria were transfected by pUC18 that was incubated with a range of protein concentrations (25 - 100 μg/ml). Negative controls were a boiled nuclear extract and a buffer. Mean transformation frequency ± S.E. for native pUC18 DNA, 2131 ± 470.
[b]Significant difference from 'hybrid' values ($p < 0.05$); there was no significant difference between 'parent' and 'revertant' values.
[c]Significant difference from *Sal*I break-type values ($p < 0.05$); there was no significant difference between *Eco*RI and *Kpn*I.

The same experiments were used to evaluate the fraction of pUC18 plasmid DNA producing colorless colonies and blue colonies. If plasmid DNA

was rejoined correctly, the *lacZ* gene was functional, producing blue colonies on the medium containing ampicillin and X-gal. If the plasmid DNA was 'mis-rejoined', the *lacZ* gene was not functional and produced colorless colonies. As shown in table 6, transfection experiments produced mostly blue colonies with extremely low levels of colorless colonies in all of the controls and treatments. All 3 nuclear extracts showed a similar fidelity of dsb rejoining irrespective of the type of damage.

Table 6. Fidelity of rejoining of pUC18 by human nuclear extracts from 'parent', 'hybrid', and 'revertant' cells.

Dsb type	Treatment	Total number of colonies[a]		% colorless[b]
		blue	colorless	
*Sal*I	T4 ligase	15825	0	0
	Parent	12060	2	0.02
	Hybrid	25716	2	0.01
	Revertant	10884	2	0.02
*Eco*RI	T4 ligase	24150	1	0.004
	Parent	3267	5	0.15
	Hybrid	11973	8	0.07
	Revertant	3429	5	0.15
*Kpn*I	T4 ligase	7530	0	0
	Parent	3924	2	0.05
	Hybrid	6354	3	0.05
	Revertant	4416	3	0.17
	Neg. controls	627	0	0
	Native pUC18	78450	0	0

[a]Total number of colonies calculated from the range of doses used in 3 separate experiments (0 - 200 μg/ml nuclear extract).
[b]Results are comparable to control values from other studies (North *et al.*, 1990).

4.5 Discussion

Several independent studies support the involvement of loci on chromosome 11 in correcting chromosomal radiosensitivity; yet, the mechanism underlying this response remains unknown (Parshad *et al.*, 1992; Komatsu *et al.*, 1990; Kodamu *et al.*, 1992; Ward *et al.*, 1993; Hofseth and Rosin, unpublished data). Since DNA dsb are an important event mediating such chromosomal damage, an attractive hypothesis is that radiosensitive cells have a defect in their repair, and that this defect is corrected by proteins produced by loci present on chromosome 11. The present study used a cell-free system to examine such a possibility. The results show that the altered sensitivity to radical damage observed in the 'hybrid' cell line (containing the normal chromosome 11 insert) is not associated with an altered capacity to rejoin dsb induced by restriction enzymes. Nuclear extracts from the 'parent', 'hybrid', and 'revertant' cell lines showed a similar ability to rejoin linearized plasmid DNA, irrespective of whether the plasmid was broken by *Sal*I, *Eco*RI, or *Kpn*I.

Although some radiosensitive cell lines display an alteration in the efficiency of dsb rejoining, others exhibit apparently normal rejoining (Bryand and Liu, 1994). It has been suggested that in the latter case the defect may involve an alteration in the fidelity of this repair (North *et al.*, 1990; Powell *et al.*, 1992; Cox *et al.*, 1986; Thacker *et al.*, 1992; Meyn, 1993; Ganesh *et al.*, 1993). For example, Powell and co-workers (1992) examined the ability of another bladder carcinoma cell line (MGH-U1) and its radiosensitive clone (S40b) to repair X-ray-induced dsb and to rejoin breaks induced in a plasmid broken by *Kpn*I. The study found no difference in the extent or rate of rejoining of dsb by the 2 lines; however, the amount of 'mis-rejoining' of *Kpn*I-induced dsb was significantly greater in the radiosensitive clone. In a second study that used procedures similar to those described in our study, North and co-workers (1990)

evaluated nuclear extracts from a transformed AT and a normal cell culture for the capacity to rejoin restriction enzyme-induced dsb. Similar efficiencies of rejoining were found for the 2 lines; however, the fidelity of rejoining was much lower for the AT extracts. This 'mis-rejoining' was dependent on the type of break; present after rejoining of *Eco*RI-, but not *Sal*I-induced dsb. In contrast to these 2 studies, we did not observe any difference in the fidelity of rejoining of plasmids by nuclear extracts of the 'parent', 'hybrid', and 'revertant' cell lines, regardless of which restriction enzyme was used. These data suggest that the protection resulting from the chromosome 11 insertion is not due to altered fidelity. It should be noted that transformation frequencies and the fidelity of rejoining observed with these 3 cell lines were similar to those previously reported for extracts from normal cells (North *et al.*, 1990).

A novel observation made in the present study is that the insertion of chromosome 11 into our bladder carcinoma cells alters the distribution of rejoined products. At this point, it is only possible to speculate on the mechanisms underlying this observation. Previous reports have suggested that multiple proteins are involved in dsb rejoining and some of these, such as 'alignment proteins', may stimulate linear rejoining (Fairman *et al.*, 1992). Our data suggests that loci on chromosome 11 produce proteins that either stimulate circular rejoining (and thus participate in intrastrand rejoining) directly or act indirectly by altering the transcription of other proteins. Alternatively, these proteins may directly or indirectly inhibit linear rejoining. Whether the change in the distribution of rejoined products plays a role in the altered sensitivity of the 'hybrid' culture to radiation is yet to be resolved. It is possible that the effect of chromosome 11 on radiosensitivity may be further upstream, and involve some aspect of the 'damage surveillance network' (Meyn, 1993) that leads to the induction of new proteins in response to stress. In order to assay for such an

effect, it would be necessary to challenge cell lines by pre-treating them with X-rays or other radical generators prior to preparing nuclear extracts for study. Such a study is in progress.

It is of interest to speculate on the loci on chromosome 11 that might be involved in the radioprotective effect. Loss of heterozygosity for regions on chromosome 11 is a common occurrence in many tumour types, quite often involving 11q22-23, the location of the ATM gene (Savitsky *et al.*, 1995; Ambrose *et al.*, 1994). However, a recent fine mapping study of bladder carcinomas has identified 2 regions of loss, one at 11q13-q23.2 and the other at 11p15.1-15.5 (Shaw and Knowles, 1995). It is possible that one of these regions of loss could represent an alteration to the critical controlling loci being studied in this paper. From the standpoint of known genes involved in repair processes that are located on this chromosome, the list includes not only the ATM gene but also several others: the Ribosomal Protein S3 (11q13.3-q13.5), involved in endonuclease repair of UV damage (Polakiewicz *et al.*, 1995); the RAG1 and RAG2 (11p), implicated in V(D)J recombination (Oettinger *et al.*, 1992); and the FEN1 locus (11q12), implicated in flap structure specific endonuclease activity and 5'-3' exonuclease activity (Hiraoka *et al.*, 1995). Further studies are required to better define the location of the active loci on chromosome 11 and to determine the mechanism by which it exerts its protective effect.

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CHAPTER 5

Micronucleus frequencies in urothelial cells of catheterized patients with chronic bladder inflammation

5.1 Abstract

Epidemiological studies suggest an association between chronic inflammation and increased risk for cancer, although the mechanism underlying this relationship is unresolved. In the present study, we test the hypothesis that DNA damage is induced in the epithelium of tissues during such inflammation by products of activated inflammatory cells. Individuals on long-term indwelling urinary catheterization were used as a study population. These individuals have chronic bladder inflammation and, as a population, an increase in risk for bladder cancer. Urine of 29 patients and 26 age-matched non-catheterized controls was collected and micronucleus (MN) frequencies were determined in exfoliated urothelial cells in the urinary sediments. The urine from the catheterized group had large numbers of white blood cells (mean count, 26.6 ± 3.6 cells per high-power field), indicating the presence of a chronic bladder infection and an inflammatory reaction. In contrast, white blood cells were not present in urine from individuals in the control group. There was no significant difference in MN frequencies in the 2 groups (mean frequencies, controls: 0.098 ± 0.030%; catheterized: 0.140 ± 0.025%, p = 0.13). These data imply that chromosomal damage does not always occur during chronic inflammation. Although the reasons for this observation are yet to be determined, possible explanations include the pathophysiology of the inflammatory reaction and the influence of vitamins, non-steroidal anti-inflammatory drugs and the catheter itself in protection against inflammatory cell - mediated DNA damage.

5.2 Introduction

There is a current interest in exploring the role of inflammation in cancer development, largely because of accumulating evidence that suggests inflammatory cells have the capacity to induce DNA damage in neighboring cells (Chong *et al.*, 1989; Ward *et al.*, 1993). This damage may result directly from the release of reactive oxygen species (ROS) during the respiratory burst of inflammatory cells (Weitzman and Gordon, 1990), or be induced indirectly by an involvement of the cells in carcinogen bioactivation (Trush *et al.*, 1990; Isola *et al.*, 1993) or the formation of nitrosamines (Iyengar *et al.*, 1987; Roediger *et al.*, 1990; Grisham *et al.*, 1992).

The specific role of inflammation in the development of urinary bladder cancer is unclear. Several animal models suggest bladder tumourigenesis is enhanced by the induction of inflammation in the bladder (Kawai *et al.*, 1993; Martins-Green *et al.*, 1994). Epidemiological studies have shown that populations undergoing chronic bladder infection and associated inflammation have an elevated risk of bladder cancer. For example, it has been suggested that an association between recurrent cystitis and bladder cancer exists in Western populations (La Vecchia *et al.*, 1991). A more convincing association involves patients infected with the water-born parasite *Schistosoma haematobium*. Such individuals have extensive damage and inflammation in the bladder urothelium and are at elevated risk for cancer at this site (El-Bolkainy *et al.*, 1981; Al-Shukri *et al.*, 1987). Although the nature of this relationship is unresolved, there is some suggestion that *S. haematobium* patients have si[illegible]cantly elevated levels of DNA damage in their bladder. We have previously shown that micronucleus (MN) frequencies are elevated 9.1 - fold in urothelial cells with an accompanying elevation in urinary white blood cell levels. Treatment with praziquantel, which kills the parasite, results in a reduction of MN

frequencies to the level of non-infected controls with a concomitant reduction in urinary white cell levels. These data support the possibility that inflammatory cells may be involved in the chromosomal damage (Rosin and Anwar, 1992; Rosin *et al.*, 1994).

The present study was designed to further explore the role of inflammatory cells in inducing DNA damage in urothelial cells. This investigation examined patients on continual long-term ($\geq$ 30 days) urinary catheterization who have recurrent bacterial infections and chronic bladder inflammation. Such populations have been reported to have a 20-fold increase in bladder cancer risk (El-Masri and Fellows, 1981). Exfoliated urothelial cells were isolated from the urine of catheterized patients and non-catheterized controls and analyzed for MN frequencies. At the same time, the urine was screened for the presence of urinary white blood cells.

5.3 Materials and methods

5.3.1 Populations

This study was conducted on a group of patients in long-term care facilities in Vancouver, British Columbia: 29 patients (10 males, 19 females) with continual long-term ($\geq$ 30 days) indwelling urinary catheter drainage (Foley-type) and 26 age-matched, non-catheterized controls (all males). Females were not evaluated in the control population since cells in their urine sediments are mainly from the vulva and lower genital tract. The selection of which cells to score in these sediments is somewhat subjective and can create a large bias in a study (Rosin, 1992). In contrast, for catheterized populations, either male or female, virtually all of the cells come from the bladder. Furthermore, historically no difference in MN frequencies has been observed for males and females in control populations. The study was performed during clinically quiescent periods of bladder inflammation (ie. in the absence of acute cystitis symptoms such as bladder spasms, abdominal bloating, dysreflexia, cloudy urine) and no subjects had kidney stones during collection. Information on diet, supplements, medication, tobacco usage, and medical histories were obtained from questionnaires and hospital records.

5.3.2 Sample collection

Urine was collected from each subject on three separate occasions at one week intervals. For the catheterized group, urine was collected into 500 ml polypropylene bottles through the flutter valve of the catheter's drainage bag. Urine was collected from individuals in the control group by having them void directly into 500 ml polypropylene bottles.

5.3.3 Sample processing

In preliminary experiments, it was difficult to examine exfoliated cells in urine samples from catheterized patients because of the presence of large amounts of contaminating bacteria in the majority of samples. An attempt was made to separate bacteria from exfoliated cells and from occasional urinary sediment crystals by density gradient centrifugation using polysucrose/ sodium diatrizoate solutions, normally used for the isolation of white blood cells (eg. Histopaque, Sigma Chemicals). These trials failed, as exfoliated bladder mucosa cells were not buoyant even in the presence of high concentrations of such density gradient materials. While morphologically intact, mucosal cells from urinary sediments were non-viable by Trypan Blue staining. It was hypothesized that in such non-viable cells, the density gradient material could penetrate into the cell in the same manner as does Trypan Blue, negating the possibility of buoyancy. Exfoliated bladder mucosa cells, however, were found to be buoyant in density gradients prepared with Percoll (Pharmacia), a micro-particulate material which cannot diffuse through holes of molecular dimensions. Flotation in 20% Percoll proved to be a simple and efficient way to isolate exfoliated bladder mucosa cells for microscopic analysis.

Urine samples were processed within 2 hours of collection. Each sample was transferred into 250 ml polypropylene centrifuge tubes, topped up with 0.9% sodium chloride (NaCl) and centrifuged (10 min at 1000 g). The pellet was washed with 0.9% NaCl by centrifugation (10 min at 1000 g). The pellet isolated from individuals in the control group was washed a second time with 0.9% NaCl and fixed in 80% methanol (MeOH). For the catheterized group, the pellet was resuspended in a mixture of 20 parts Percoll (Pharmacia) and 80 parts distilled water and transferred to 16 x 100 mm glass disposable culture tubes. After carefully layering 1 - 2 ml 0.9% NaCl on the Percoll mixture, the tubes were

again centrifuged (10 min at 1000 g). A band formed between the Percoll and 0.9% NaCl layers containing the urothelial cells was removed, washed with 0.9% NaCl and fixed in 80% MeOH. All samples were stored at 4°C in a 1.5 ml polypropylene microcentrifuge tube. To prepare for analysis, cells were dropped onto slides, air-dried, stained with Schiff reagent, counterstained with Fast Green and mounted with coverslips as described previously (Rosin *et al.*, 1994).

5.3.4 Sample analysis

5.3.4.1 Assessment of Urinary White Blood Cells (WBC)

At the time of collection for MN determination, an extra 12 ml urine was assayed for the presence of urinary white blood cells. For the catheterized group, this was done by aseptically puncturing the sampling port of the Foley apparatus with a sterile plastic-tipped syringe and needle. For the control group, 12 ml urine was removed directly from the collection bottle. Urinary white blood cells were quantified by microscopic examination as described previously (Peterson and Roth, 1989). 12 ml urine sample was centrifuged (5 min at 700 g), the supernatant was decanted and the remaining pellet and urine (approximately 1 ml) was resuspended. A drop of this suspension was placed on a glass slide, covered with a cover slip and 10 - 12 fields (400x magnification) were examined. The amount of urinary white blood cells per high power field (hpf) was then placed into one of five categories: 0-5 cells/hpf, 6-20 cells/hpf, 21-40 cells/hpf, 41-100 cells/hpf and >100 cells/hpf. A prototype scoresheet used for the urinalysis can be found in appendix C.

5.3.4.2 Assessment of MN frequencies

Cells were examined for MN using 1000x magnification under light microscopy followed by phase contrast. A minimum of 500 well-preserved

urothelial cells were analyzed per sample. In order to ensure a minimum of 500 scorable urothelial cells for each individual, it was necessary to combine 3 samples taken from each subject. Samples were coded prior to scoring and each potential micronucleated cell was reassessed with a second observer. The criteria for identifying MN have been described previously (Rosin, 1992; Warner *et al.*, 1994). A prototype scoresheet used for the micronucleus assessment can be found in appendix C.

5.3.5 Effect of urinary catheters on release of ROS from human neutrophils

20 ml blood was collected in heparinized tubes from 9 subjects and neutrophils isolated by Histopaque gradient centrifugation. The concentration of neutrophils was assessed using a haemacytometer and adjusted to 2×10^6 cell/ml. 3 separate 1 ml samples of each preparation were placed into 1.5 ml polypropylene microcentrifuge tubes and centrifuged at 12 000 rpm for 30 sec to isolate a pellet. For the 'control' sample, the supernatant was replaced with 1 ml PBS. For the 'eluate' sample, the supernatant was replaced with 1 ml of an eluate prepared by preincubating 30- 1.0 cm segments of siliconized latex two-way Foley catheters in 10 ml of PBS for 24 - 48 hrs at 37^oC. For the 'catheter' sample, the supernatant was replaced with 1 ml PBS containing a 1 cm segment of catheter that was preincubated for 1 hr in 10 ml PBS at 37^oC. The 3 samples were further incubated for 30 min at 37^oC. After incubation, 75 µl of each sample was added to mixtures of 75 µl luminol (Sigma, St. Louis, MO, 0.1 mg/ml) and 100 µl 12-O-tetradecanoyl-phorbol-13-acetate (TPA, Sigma, St. Louis, MO, 25 ng/ml) then the luminol-amplified chemiluminescence was immediately quantified in a Packard Model 6100 (Pico-lite) Luminometer. 3 separate assessments of chemiluminescence were taken for each sample and ROS released from neutrophils were expressed as counts per second.

5.3.6 Statistical analysis

Differences between the catheterized and control groups were examined using the Mann-Whitney U-test. Differences in chemiluminescence in control, eluate and catheter samples were examined using Analysis of Variance (ANOVA) with repeated measures. The p-value chosen for significance in this study was 0.01.

5.4 Results

5.4.1 Population Profiles

For the catheterized group, average time on indwelling catheterization was 7.2 years (range, 1 - 38 years). Catheterization was secondary to various conditions including multiple sclerosis, quadriplegia, cerebral palsy and dysfunctions resulting from various brain infarctions (table 7). Urogenital anomalies associated with the catheterized group included neurogenic bladders, bladder calculi, urosepsis, pancytopenia, intermittent hematuria, urethral cutaneous fistula, benign prostatic hypertrophy, recurrent hydronephrosis, recurrent urolithiasis and recurrent urinary tract infections (range, 0 - 7 infections over the 2 - year period prior to the present investigation; most common species seen in table 8).

Table 7. General data for catheterized patients acquired from hospital records and questionnaires

Primary Illnesses[a]			Average No. Lower UTI's (2 years prior to sampling)	% with Neurogenic Bladders	Avg. Time Between Catheter Changes
MS	Quadriplegic	Other[b]			
62%	21%	17%	1.4/yr	97%	3 weeks

[a]Numbers are presented as a percentage of the total sample population (n=29).
[b]Other primary illnesses include progressive CNS degeneration, cerebral palsy and a subarachnoid hemorrhage.

Table 8. Number of positive bacteria cultures for catheterized patients taken during 2 years prior to sample collection (December, 1990 - December, 1992)

Genus	Number of Positive Cultures
Streptococcus	33
Providencia	30
Coliforms	27
Staphylcoccus	12
Proteus	8
Citrobacter	6
Pseudomonas	4

Table 9 provides a comparison of the two groups used in this study in terms of age, smoking habits, daily vitamin intake and daily NSAID intake. These characteristics were chosen for comparison because they could act as potential confounders in this study. The smoking could elevate MN frequencies (Rosin, 1992; Reali *et al.*, 1987; Fontham *et al.*, 1986) while vitamins and NSAIDs could act as protective agents (Maffei-Facino *et al.*, 1993; Emerit *et al.*, 1983; Rosin, 1993; Sies *et al.*, 1992). There was no significant difference in age and smoking habits for the 2 groups. In contrast, the catheterized population was consuming significantly more vitamins and non-steroidal anti-inflammatory drugs (NSAIDs). Most of the catheterized patients (86%) were taking at least one vitamin or NSAID daily and just below half (44%) had more than one of these supplements each day. On the other hand, only 18% of the control group was taking one vitamin and only one control subject took an NSAID (Acetylsalicylic Acid) daily.

Table 9. Selected characteristics of catheterized patients and non-catheterized controls

Characteristic	Controls	Catheterized
Mean age in years (range)	54.0 (25 - 94)	54.8 (37 - 77)
Smoking habits		
No. of smokers	7	5
Avg. cigs/day (range)	12.6 (5 - 40)	9.1 (5 - 20)
Daily vitamin intake		
% of patients	18%	69%[a,c]
Daily NSAID intake		
% of patients	4%	34%[b,c]
Vitamins or NSAIDs		
% of patients	23%	86%[c]

[a]23/29 cases used either vitamin C or vitamin E or a multivitamin; Average intake was 1156 mg, 400 IU and 1 tablet, respectively.
[b]NSAIDs: Non-Steroidal Anti-Inflammatory Drugs; All cases used either Acetylsalicylic Acid (ASA), Ibuprofen or Diclofenac; Average intake was 557 mg, 1000 mg, and 75 mg, respectively.
[c]Significantly different from control values ($p < 0.01$).

5.4.2 Presence of inflammatory cells in urine from catheterized and control groups

All control samples were consistently void of urinary white blood cells. In contrast, urinary white cell counts were significantly elevated ($p < 0.0001$) in the catheterized group (Table 10). 66% of the subjects had >20 cells/hpf, 38% had >40 cells/hpf and 4 subjects (14%) had >100 cells/hpf in at least one out of the three samples taken. This suggests significant inflammation in the catheterized population during the sample period. Compared to controls, preliminary experiments also suggested elevated bacteria and nitrite levels in the catheterized population. None of the control samples had bacterial or nitrite contamination while 84% of samples in the catheterized group had moderate or heavy bacterial contamination and 82% of the samples were positive for nitrites (tables 11 and 12).

Table 10. Urinary white blood cells and MN frequencies in catheterized and control subjects

	Number of urinary white blood cells / hpf[a]					Mean MN frequency[b]
	0-5	6-20	21-40	41-100	>100	(%)
Controls	100%	0%	0%	0%	0%	0.098 ± 0.030
Catheterized	25%	35%	21%	13%	6%	0.140 ± 0.025

[a]Each sample was placed into one five categories; the percentage of control and catheterized samples belonging to each category was then calculated.
[b]Comparison of catheterized and control groups, p=0.13.

Table 11. Density of bacteria in catheterized and control samples

	Density of Bacteria[a]		
	Sparse	Moderate	Dense
Controls	100%	0%	0%
Catheterized	16%	35%	49%

[a]Each sample was placed into one of three categories; the percentage of control (n=26) and catheterized (n=29) samples belonging to each category was then calculated.

Table 12. Nitrite contamination in catheterized and control samples[a]

	Nitrite Contamination[a,b]	
	Negative	Positive
Controls	100%	0%
Catheterized	18%	82%

[a]To screen urine samples for nitrite contamination, Multistix Reagent Strips (Fisher Scientific, sensitivity: 13 - 22 μmol/l nitrite ion) were used by directly dipping the strips into the urine sample.
[b]Each sample was either positive or negative for nitrites; the percentage of control (n=26) and catheterized (n=29) samples belonging to each category was then calculated.

5.4.3 MN frequencies in catheterized and control groups

Figure 17 shows the distribution of MN frequencies in urothelial cells from the catheterized and control populations. In the control group, 46% of subjects had at least one micronucleated cell, compared with 65% of subjects for the catheterized group. This difference, however, was not significant ($p = 0.12$). Table 10 includes data on mean MN frequencies in urothelial cells. Although the catheterized group ($0.140 \pm 0.025\%$) had slightly higher levels of micronuclei than controls ($0.098 \pm 0.030\%$), this difference was not significant ($p = 0.13$). There was also no significant difference between MN frequencies in males and females in the catheterized group (mean frequencies, males, $0.143 \pm 0.047\%$; females, $0.138 \pm 0.030\%$, $p > 0.05$). Similarly, there was no correlation between MN frequencies and vitamin/ NSAID intake or the length of catheterization. MN values for the catheterized and control groups lie within the historical values for MN frequencies from control populations studied in this laboratory (Rosin and Ochs, 1986; Rosin and Anwar, 1992).

Figure 17. Frequency of micronucleated urothelial cells in samples from long-term urinary catheterized patients (n=29) and non-catheterized controls (n=26). *, indicates historical range of MN frequencies for controls in the laboratory.

5.4.4 Effect of a siliconized latex urinary catheter on human neutrophil activity

The results of MN frequencies found in the catheterized population were a sharp contrast to populations with chronic bladder inflammation from parasitic infections (Rosin and Anwar, 1992). A previous report in the literature suggested that siliconized latex urinary catheters could inhibit superoxide production from inflammatory cells (Lopez-Lopez *et al.*, 1991). Based on this finding, we hypothesized that the type of catheters used by subjects in this population may have played a role in the lack of chromosomal damage in the catheterized population. In the present study, neutrophils were incubated for 30 min with either catheters or eluates prepared by submersing pieces of catheters in PBS for 24 - 48 hrs. TPA was then used to stimulate the release of ROS and luminol-amplified chemiluminescense was used to measure production of ROS. Table 13 shows that the presence of the catheters or the substances eluted from them significantly impaired ROS release from neutrophils compared to controls (average counts per second ± S.E.: control, 85 877 ± 10 369; catheter, 36 153 ± 4 564; eluate, 43 278 ± 5 403 photons/ sec; N = 27, $p < 0.0001$). Both the catheter and the eluate seemed to impair the ROS release to a similar extent as there was no significant difference between these two treatment groups ($p = 0.18$). Similar results were obtained with a second eluate created by draining the original eluate, then incubating the catheter segments with PBS for an additional 24 hrs (data not shown).

Table 13. Effects of segments of siliconized latex urinary catheters or eluates obtained from them on reactive oxygen species release from neutrophils

	Chemiluminescence (± S.E.)[a] (counts/second)
Control	85 877 ± 10 369[b]
Catheter	36 153 ± 4 564[b,c]
Eluate	43 278 ± 5 403[b,c]

[a]Mean value for neutrophil isolates from 9 individuals
[b]A significant difference existed between control vs. catheter and control vs. eluate groups ($p < 0.0001$).
[c]No significant difference between catheter and eluate groups ($p = 0.18$).

5.5 Discussion

This study examined the extent of chromosomal damage in exfoliated bladder cells collected from individuals on continuous long-term indwelling urinary catheterization. Although pyuria was found in >75% of urine samples from individuals with catheters and none of the control samples, chromosomal damage was similar in the two groups. In contrast, an Egyptian population with *S. haematobium* infections had elevated MN frequencies during inflammation (mean frequencies, 0.97% for the infected group and 0.12% for controls). These frequencies decreased concomitant with a reduction in bladder inflammation after treatment with an anti-schistosomal drug, suggesting an involvement of inflammation in the production of chromosomal damage (Rosin and Anwar, 1992; Rosin *et al.*, 1994).

Although both catheterized patients and individuals infected with *S. haematobium* have an increased risk of bladder cancer, the pathological changes in the bladders of individuals in these two groups and the inflammatory reactions are different. *S. haematobium* is associated with massive egg deposition throughout the bladder urothelium, extending into the detrusor muscle. This results in inflammation throughout the urothelium, the formation of granulomas, large polypoid masses (Smith *et al.*, 1977), tissue ulceration (Smith *et al.*, 1977), epithelial erosion, and urothelial hyperplasia (Smith *et al.*, 1974). During this process, inflammatory cells release ROS which can act on otherwise healthy dividing neighboring cells in the urothelium producing chromosomal damage and MN formation. On the other hand, chronic urinary catheterization is associated with polypoid cystitis, a condition characterized by edematous fingerlike, broad based papillae with prominent inflammation in the stroma and blood vessels rather than the epithelium (Eklund and Johansson, 1979). Uroepithelial tissue damage created by trauma from the catheter, bacterial

infiltration into the bladder wall, and the release of inflammatogenic substances by bacteria (de Man *et al.*, 1988) seems to be less extensive than that observed in the *S. haematobium* patient. The type of white blood cells involved in the inflammatory infiltrate may also play a role in the difference between catheterized patients and those infected with *S. haematobium*. The latter is associated with a distinct presence of primarily eosinophils in the inflammatory infiltrate (Eltoum *et al.*, 1989) in addition to some polymorphonuclear leukocytes (Rosin *et al.*, 1994). In long-term catheterization, however, the inflammatory infiltrate consists mostly of lymphocytes with a smaller presence of plasma cells and neutrophils (Norlen *et al.*, 1988). Neutrophils and eosinophils, not lymphocytes, are the primary source of reactive oxygen species when stimulated.

Another difference between the two populations is the extent of vitamin and NSAID intake. The present study found over 85% of the catheterized patients taking at least one vitamin or NSAID daily during the time of data collection. Neither vitamin supplementation nor long-term use of NSAIDs is common in the Egyptian population infected with *S. haematobium* (M. Rosin, unpublished data). With certain antioxidants, such as vitamin C, the average daily intake in some catheterized patients from this study (48%) is estimated at 30 - 40x above that required for adult Canadians and 10x above that obtained through dietary sources (Health and Welfare Canada, 1990). This suggests a high intake of agents that have historically been shown to reduce DNA damage both *in vitro* and *in vivo* (Emerit *et al.*, 1983; Rosin, 1993). These agents can inhibit chromosomal damage induced by ROS. For example, vitamin E is the major peroxyl radical scavenger in biological lipid phases such as membranes and vitamin C has extensively been shown to scavenge free radicals (reviewed in Sies *et al.*, 1992). NSAIDs can inhibit mutagenesis by preventing the

generation of malondialdehyde, a direct acting mutagen, and by blocking the prostaglandin - dependent co-oxidation of many xenobiotics to form carcinogens (Paganini-Hill, 1994). Furthermore, diclofenac, examined in this study, has been shown to exhibit free radical scavenging activities (Maffei-Facino *et al.*, 1993). In addition to a potential inhibitory role of vitamins and NSAIDs on MN formation in catheterized patients, the catheter itself may have inhibitory properties. The current results indicated that catheters or substances eluted from them impaired the release of ROS by neutrophils. This result is similar to the finding that siliconized latex two-way urinary catheters significantly inhibit superoxide production from neutrophils (Lopez-Lopez *et al.*, 1991).

It should be noted that the management of catheterized patients has altered significantly during the last few decades. The impact of these changes on cancer risk for these patients has yet to be determined. The low MN frequencies could reflect a reduced risk either through the impact of vitamin/ NSAID supplementation or better control of the infection. From this standpoint it would be of interest to evaluate other markers of genotoxicity in these patients such as the presence of O^6-methyldeoxyguanosine adducts in bladder tissue or of *N*-nitroso compounds in urine, both of which have been reported to be elevated in individuals with bladder infections (Hicks, 1982; Badawi *et al.*, 1992; Tricker *et al.*, 1991).

In summary, the present study suggests chronic inflammation is not always associated with an elevation in tissue DNA damage, as measured by MN frequencies. We have suggested several possible explanations for these results, including the pathogenesis of the inflammatory reaction and the possibility that vitamins and NSAIDs could protect against MN formation. One approach that could be used to examine the influence of vitamins and NSAIDs on the formation of micronuclei in further studies would be to determine whether

these agents would reduce micronuclei induction in exfoliated bladder cells of *S. haematobium* patients. Future work that better delineates critical molecular events underlying the development of bladder cancer in these two high risk populations might provide significant insight into the relative roles of irritation, inflammation and cell proliferation in carcinogenesis.

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CHAPTER 6

DISCUSSION

6.1 Review of the research

The experiments used in this thesis were designed to study the role of inflammation in DNA damage and carcinogenesis in the bladder. Our rationale for examining inflammation as an etiological factor was based on the large number of clinical studies in which these two processes had been reported to occur concurrently in humans as well as the accumulating number of results obtained using animal models. However, inflammation is a normal tissue response to a variety of stimuli and as such is the body's main protection against disease. As inflammation is not always associated with the development of cancer, there must be specific conditions under which it becomes a risk. A primary requirement may be that the inflammation is chronic, occurring repeatedly or continuously over prolonged periods of time. This is true for many of the clinical conditions in which cancer is associated with inflammation. A well-established example is the involvement of the schistosomiasis infections with bladder cancer in Egypt, a condition that often involves a life-long inflammatory reaction in the urinary bladder. A population in the Western world similarly exposed to chronic inflammation in their urinary bladders are patients with indwelling catheters. This being the case, in this thesis, we looked at the influence of inflammation on DNA damage in urinary catheterized patients from Vancouver, Canada. This study and the techniques used in it were based on a previous study that found schistosomiasis patients had high levels of inflammation in their bladders and concurrently showed DNA damage (assessed by MN) in bladder urothelial cells (Rosin and Anwar, 1992). DNA damage and MN were used as an endpoint because (a) genetic damage is a hallmark of carcinogenicity and is implicated in both early and late stages of the disease; and (b) chronic elevations in MN frequencies indicate an increased rate of

genetic change in a tissue and hence a greater probability of the acquisition of the specific chromosome changes associated with neoplastic transformation.

At present it is difficult to conclude whether inflammation plays a role in bladder DNA damage in urinary catheterized patients. There are two main explanations for our results. It is possible that inflammation does not cause DNA damage in this population and that the enhanced cancer risk is due to some other factor. Alternatively, our results may be confounded by the high intake of vitamin and NSAID intake in this population. Ideally, to test this, it would be necessary to terminate the supplementation. This would be unethical. Another approach would be to supplement the schistosomiasis patients in Egypt and measure MN frequencies after supplementation. If the protection was shown in the latter population, it could provide a valuable adjunctive therapy in the treatment of populations where chronic inflammation is associated with cancer, such as people suffering from chronic *Schistosoma haematobium* infections, *Helicobacter pylori* infection and ulcerative colitis. Worldwide, this would have an impact on millions of people who are affected by these conditions. In the two former cases, it has not been possible to eradicate the parasite or bacteria causing the infection, mainly due to the high rate of re-infection that occurs after treatment.

The impact of the inflammation on bladder cancer risk may also depend on the capacity of cells in the tissue to prevent or repair damage induced by products released by inflammatory cells. Since chromosome 11 is lost in bladder cancer (usually at an intermediate stage) and other studies have suggested a reduction in tumourigenesis and a sensitivity of tumour cells to ROS by chromosome 11 insertion (Ward *et al.*, 1993), it was of interest to determine whether this chromosome could reduce the sensitivity of bladder cells to other radical generating agents. Data from this thesis suggest insertion of

chromosome 11 into our bladder carcinoma cells reduces a sensitivity to agents such as X-rays and H_2O_2.

The reasons why chromosome 11 corrects a sensitivity to irradiation and other radical generating agents are unclear. Experiments in this thesis were designed to look at two possibilities. The first was to determine whether there is an augmentation of catalase activities. These experiments suggested there was an increase in catalase activities after insertion of chromosome 11 into bladder carcinoma cells. However, it is not yet clear to what extent this increase is responsible for the reduction of MN frequencies observed in the 'hybrid' culture. The second possibility was that there was an alteration in DNA repair. The results suggested that chromosome 11 did not affect the total extent or the fidelity of dsb rejoining. However, there may be loci on chromosome 11 that produce proteins involved in altering the types of products formed in dsb rejoining. It remains to be determined whether this alteration plays a direct role in the sensitivity of these cells to oxidative stress induced by irradiation and other radical generating species.

6.2 Future studies

In order to better delineate the loci on chromosome 11 which decrease a sensitivity to ROS and protect against DNA damage, several *in vitro* approaches can be envisaged using current molecular techniques. One approach would be to create microcell hybrids containing recombinant chromosomes covering different regions of chromosome 11. Such an approach has already been used successfully to localize the region of radioprotection of AT cell to the q arm (Ejima *et al.*, 1991). Similarly, yeast artificial chromosomes (YACs) composed of smaller regions from chromosome 11 could be inserted into the sensitive cell

lines. The researcher could then better localize the chromosomal region associated with this protection.

Another logical extension of our *in vitro* model would be to directly test for alterations in genes known to be on chromosome 11. One of the more promising candidates is the ATM gene (Savitsky *et al.*, 1995). If this gene is altered in these cells it would explain the sensitivity of the 'parent' and 'revertant' cell lines to irradiation and radical generating agents (Ward *et al.*, 1993; Hofseth, unpublished data). Finally, in order to test for a more general phenomenon, it would be desirable to extend these studies to a larger number of both bladder carcinoma and normal epithelial cell lines.

Using these studies, we may find that multiple regions on chromosome 11 play a role in a sensitivity to ROS. Recently, Shaw and Knowles (1995) published results that suggest that several regions of chromosome 11 are deleted in bladder tumours, and that these regions are on both the p and q arms. Similarly, Iizuka and co-workers (1995) determined multiple regions of loss of this chromosome in lung cancer. It would be interesting to determine whether the same regions of loss for this chromosome occur (and at the same frequencies) in tissues derived from patients with chronic bladder inflammation. These tissues should not only include those derived from cancerous lesions, but also those derived from adjacent normal and dysplastic lesions.

6.3 Conclusions

The *in vivo* study described in this thesis has provided information concerning populations where inflammation is thought to play a role in cancer development. In particular, it has generated an additional hypothesis. It is possible that cancer risk is associated not only with chronic inflammation, but also with an absence of sufficient protection against ROS. This lack of

protection may be partially offset through the use of chemopreventive regimens. Further studies to explore these possibilities could be crucial to the development of preventative measures that would suppress tumourigenesis in humans.

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APPENDICES

APPENDIX A

Methods Development

1. *In vitro* studies

1.1 Evolution of the double strand break repair model

In developing the assay for analysis of double strand break rejoining by nuclear extracts from 'parent', 'hybrid' and 'revertant' bladder cancer cells, we encountered a few technical problems. The following sections review these problems.

1.1.1 Preparation of nuclear extracts

When first extracting protein and other constituents from the cells' nuclei, we obtained only about 1/1000th of the quantity of nuclear material anticipated from references (North *et al.*, 1990; Fairman *et al.*, 1992) was being isolated. By confering with various investigators, including Dr. Micaela Fairman (Medical Research Council Radiobiology Unit, Chilton, Didcot, Oxon OX11 ORD, United Kingdom), it was discovered that the procedure for precipitating the nuclear protein was inadequate. It was suggested that we increase the amount of ammonium sulphate to at least 70% saturation (0.436 g/ml) in order to isolate satisfactory amounts of protein. After this procedure was rectified, adequate amounts of protein were obtained.

To ensure all procedures during isolation of extracts were being carried out correctly, aliquots were isolated during each step and analyzed as described here. Briefly, the procedure was as follows: (a) cells were scraped into PBS using a 'rubber policeman' and washed 3 times in PBS with centrifugation (aliquot taken and analyzed); (b) the cell pellet was resuspendend in 10 ml buffer A (Tris-HCl, pH 7.5, 20 mM; MgCl2, 0.5 mM; dithiothreitol, 0.5 mM; KCl, 0.5 mM; $CaCl_2$, 2mM) contining 250 mM sucrose, and held for 15 min on ice (aliquot taken and analyzed); (c) after centrifugation, the cells were resuspended in buffer A without sucrose but with phenylmethylsulphonyl fluoride

(PMSF, 1 mM) and homogenized (40-50 strokes) in a hand-held homogenizer to release intact nuclei (aliquot taken and analyzed); (d) nuclei were pelletted (2000g, 1 min) and washed 4 times in buffer A with 0.5% triton X-100 (aliquot taken and analyzed) and 3 times without tiron but with PMSF (aliquot taken and analyzed); (e) nuclei were resuspended in 2 ml buffer B (NaCl, 500 mM; EDTA, 10 mM; PMSF, 0.1 mM) and sonicated for 4 x 15 sec bursts (aliquot taken and analyzed); (f) debris was pelletted in microtubes (12 000 rpm for 30 min) (aliquot taken and analyzed) then protein was precipitated from the supernatant by adding ammonium sulphate and stirring for 2 hrs (aliquot taken and analyzed); (g) the precipitate was pelletted at 15 000 g for 20 min (aliquot taken and analyzed); (h) the precipitate was dissolved and dialysed overnight in buffer C (Tris-HCl, pH 7.5; 50 mM; EDTA, 0.1 mM; 2-mercaptoethanol, 10 mM; PMSF, 0.1 mM; glycerol, 10%) before use or storage at -70°C.

1.1.2 Correction of plasmid heterogeneity

After obtaining the pUC18 plasmid from Gibco distributors, it was discovered that when transfecting DH5α bacteria with the plasmid, all colonies were not blue, as would be expected. Specifically, only approximately 1/3 were blue. Various attempts were made to try to correct this problem. First, two different batches of pUC18 DNA (bought from the distributor on two separate occasions) were compared. Second, different amounts of X-gal as a substrate were used on each plate. Third, serial dilutions of transfected bacteria were used. The latter two attempts were made since it was suspected that the amount of X-gal used as a substrate may have been too small. Last, although it is suggested that DH5α bacteria does not require IPTG (isopropylthio-β-galactoside) for *lacZ* expression, this was applied to the plate anyway in order to

induce β-galactosidase activity in the bacteria. All attempts failed to significantly improve the poor ratio of blue colonies to white colonies.

Analysis of the *lacZ* gene from certain commercially available pUC18 plasmids sometimes reveals a deletion resulting in a decrease in enzymatic activity by a factor of 20 to 50 (Lobet *et al.*, 1989). Thus, it was speculated that some of the *lacZ* genes of the pUC18 plasmids may have had a reduced enzymatic activity. This speculation was correct. To fix this problem, the pUC18 plasmid obtained commercially was transfected into DH5α bacteria and a blue colony was isolated after plating bacteria onto LB plates supplemented with ampicillin (100μg/ml) and X-gal (40 μg/ml). Following inoculation of LB media supplemented with ampicillin (40 μg/ml) with the first clone, bacteria were plated onto ampicillin/ X-gal supplemented LB plates and a second blue clone was isolated. The DNA of this clone was amplified by standard large-scale preparation procedures and the final DNA product was frozen as a stock solution in 10 mM Tris/ 0.1 mM EDTA. Figure 19 shows results of transfection of DH5α bacteria with the pUC18 DNA.

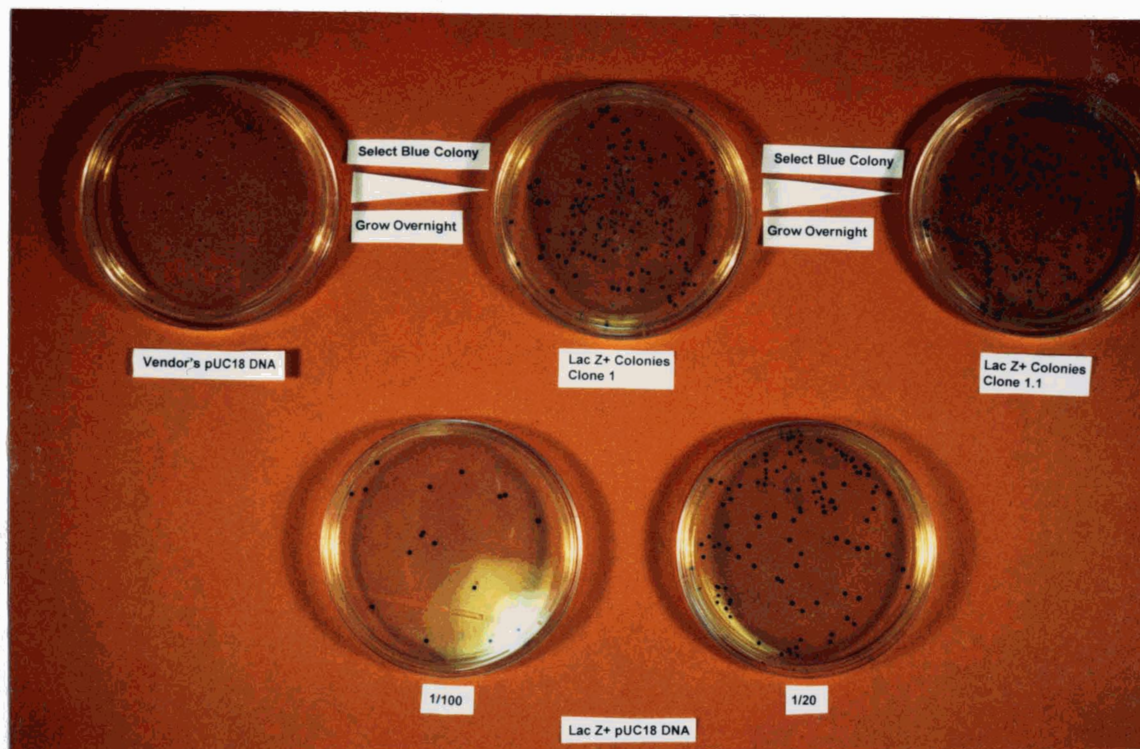


Figure 18. Results of transfection of DH5α bacteria with pUC18 DNA. First, pUC18 obtained commercially was transfected into bacteria. Second, a blue colony arising from transfection was isolated, cloned and spread onto LB plates supplemented with ampicillin and X-gal (clone 1). Finally, a blue colony from clone 1 was isolated, cloned and again spread onto supplemented LB plates (clone 1.1). A blue colony from this clone was grown in bulk culture, the DNA was isolated and used for plasmid rejoining experiments.

1.1.3 Optimizing transfection conditions

For optimum transfection efficiencies, both the amount of DNA used to transfect bacteria and the heat-shock temperatures were modified according to preliminary experimental results (data not shown). The data suggested that 0.2 μg DNA was sufficient for transfection and the heat shock temperature should be

set at 37°C for 30 min. In addition, in accordance with pilot studies, each of the bacterial samples transfected with uncut DNA or T_4 ligated samples was diluted 50x with SOC prior to plating. All other samples were diluted 10x in SOC. This ensured optimum concentrations of bacteria were spread onto each of the plates. Separate experiments were also performed in order to determine whether other constituents in the DNA samples used in these experiments inhibited transfection. 10 mM Tris/ 0.1 mM EDTA inhibited transfection significantly (data not shown). Thus, all experimental DNA samples were stored in ddH_2O.

1.1.4 Conditions used for labelling DNA

Originally, we used ethidium bromide to label DNA for analysis of rejoined products. However, it was determined that this procedure was not sensitive enough to allow analysis of all rejoined products, especially circular forms. Labelling the DNA with a fluorescein labelled probe combined with enhanced chemiluminescence detection corrected this problem.

Conditions used for Southern analysis were set by following the protocol outlined in the directions from the ECL™ random prime labelling and detection system (Amersham). All procedures were carried out according to these directions. Labelling, however, was not satisfactory for analysis of each band of DNA. This problem was carefully examined over a period of time and was finally rectified by adding more denatured DNA when preparing the labelled probe. The amount sufficient for labelling purposes turned out to be 2 ng/µl denatured DNA. 50 ul of the probe was then used to label each blot. Figure 20 shows a typical autoradiogram seen after labelling pUC18 DNA isolated after incubation with nuclear extracts.

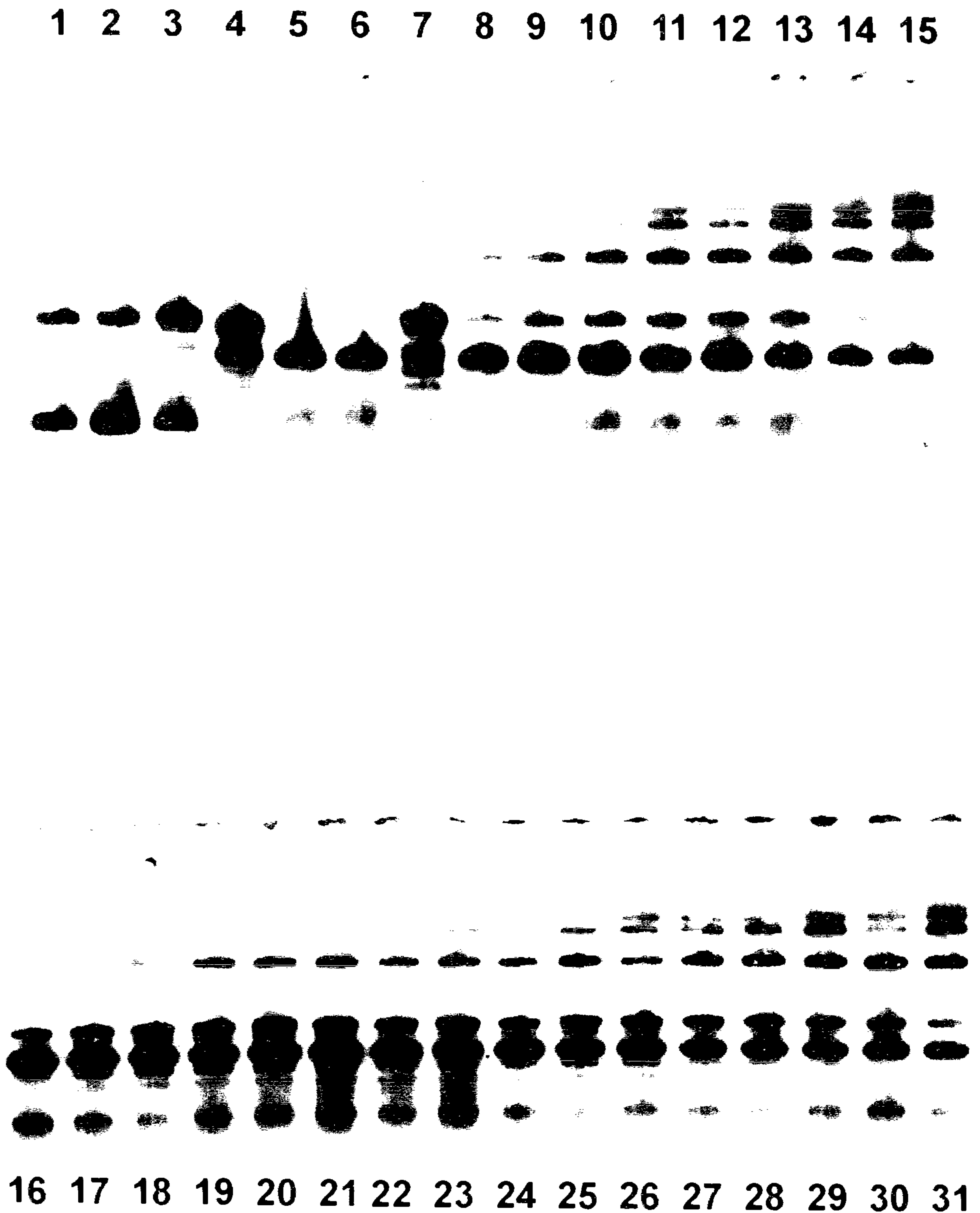


Figure 19. A typical Southern blot showing dsb rejoining characteristics after 'parent', 'hybrid' and 'revertant' nuclear extracts were incubated with *Sal*I-broken plasmid pUC18. Lane 1-4, unbroken plasmid pUC18 with: no extract, T_4 ligase (2.0U), topoisomerase and H_2O_2. Lane 5-31, *Sal*I-broken pUC18 incubated with: lane 5, no extract; lane 6, boiled extract; lane 7, T_4 ligase (2.0U); lanes 8-15, 'parent' nuclear extract (25, 50, 75, 100, 125, 150, 175 and 200 μg/ml); lanes 16-23, 'hybrid' nuclear extract (25, 50, 75, 100, 125, 150, 175 and 200 μg/ml); lanes 24-31, 'revertant' nuclear extract (25, 50, 75, 100, 125, 150, 175 and 200 μ g/ml).

2. *In vivo* study

It was not possible to directly assess MN frequencies in urothelial cells of catheterized patients because of the presence of large amounts of bacteria. The use of Percoll gradients partially corrected this problem. The rational behind the use of this procedure is given in chapter 3. Several pilot studies had to be completed prior to a decision to use 20% Percoll. We isolated urine from three urinary catheterized patients using standard procedures (see chapter 3), then mixed the urine sediment with different concentrations of Percoll (15%, 25%, 30% and 45%). After carefully layering 0.9% NaCl on the Percoll, the mixture was centrifuged (10 min at 1000g). We then isolated (a) the 'band' between the Percoll and 0.9% NaCl layer and (b) the 'pellet' (figure 18) and analyzed these fractions for exfoliated bladder cells and bacteria. Table 14 shows that in order to collect the most cells with the least contamination of bacteria from the 'band' between the Percoll and 0.9% NaCl, the optimal concentration of Percoll to use was around 15 - 25%. Thus, we decided on a 20% Percoll concentration to be used for subsequent experiments.

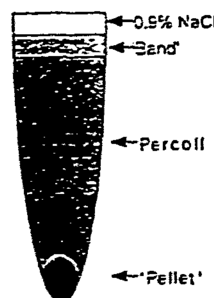


Figure 20. Labelled diagram of products observed after urinary sediments from catheterized samples were centrifuged with Percoll.

Table 14. Determination of optimal Percoll concentration to use in collection of urothelial cells from patients on indwelling urinary catheterization.

% Percoll used[a]	Analysis of water/Percoll interface and pellet[b]	Number of cells[c] and density of bacteria[d]	Sample Number[e] 1	2	3
	Layer	Cells	4.6	9.9	0
15%		Bacteria	0	0	0
	Pellet	Cells	0.6	5.6	0.2
		Bacteria	2	3	2
	Layer	Cells	1.2	35.5	0.5
25%		Bacteria	0	2	1
	Pellet	Cells	0.1	0.7	0.2
		Bacteria	1	3.5	3
	Layer	Cells	4.5	25.1	0.9
35%		Bacteria	2	2	1
	Pellet	Cells	0.4	0	0
		Bacteria	3	3.5	3.5
45%	Layer	Cells	2.7	33.5	0.6
		Bacteria	2	4	2
	Pellet	Cells	0	0	0
		Bacteria	3	2.5	3.5

[a]For dilution, Percoll was mixed with double-distilled H_2O (ddH_2O).
[b]The cell pellet was mixed with the given dilution of Percoll. 0.9% NaCl was layered on this dilution and the tube was centrifuged. Following centrifugation, material could be seen in one of two areas: an area between the Percoll and ddH_2O ('layer') and at the bottom of the centrifuge tube ('pellet'). The relative amounts of cells and bacteria were then scored in each area.
[c]The numbers of cells under high power magnification (400x) were evaluated in 10 fields and this number was averaged.
[d]The density of bacteria was also evaluated under high power magnification (400x) and subjectively placed into one of five categories ranging from 0 (no bacteria) to 5 (heavy contamination).
[e]3 separate samples from 3 different subjects were evaluated for this pilot study.

References

Lobet, Y., Peacock, M.G. and Cieplak, W. Frame-shift mutation in the lacZ gene of certain commercially available pUC18 plasmids. Nucleic Acids Research, 17 (12), 4897 (1989).

APPENDIX B

Administrative forms required for the organization of the In Vivo study outlined in Chapter 5

Proposal to staff at George Pearson Medical Centre requesting access to catheterized patients for urine collection

GENETIC DAMAGE IN CATHETERIZED PATIENTS WITH BLADDER INFECTIONS: THE ASSESSMENT OF MICRONUCLEUS FREQUENCIES IN UROTHELIAL CELLS

A proposal to Dr. Michael Lapin and the medical staff at the British Columbia Rehabilitation Society: Pearson Centre

Lorne Hofseth

Ph.D. Student

Simon Fraser University

British Columbia Cancer Research Centre

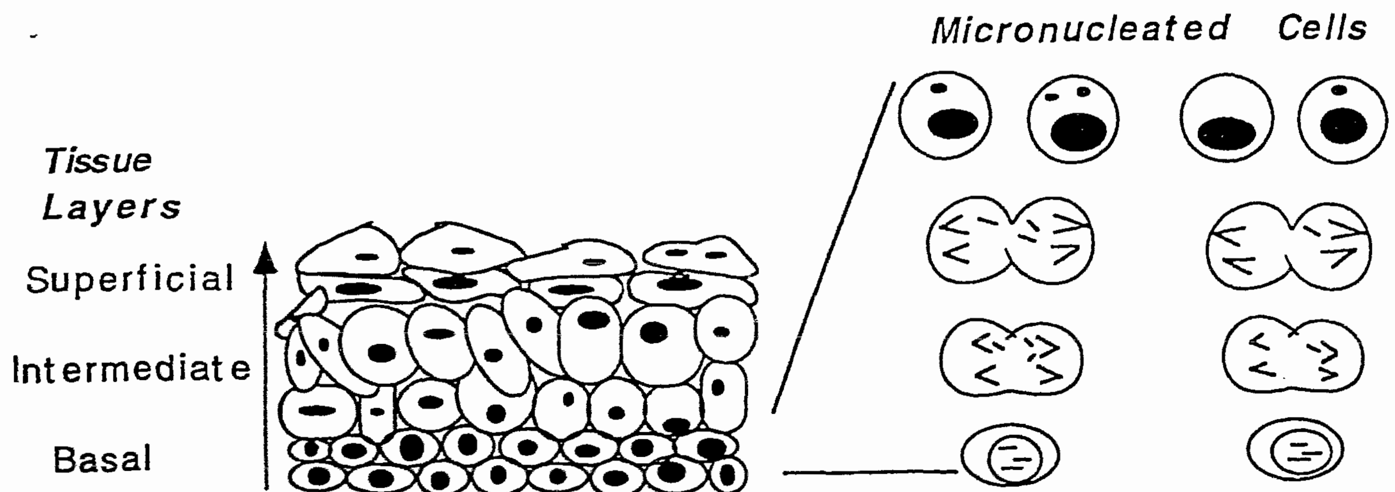
OBJECTIVE

To determine whether inflammatory reactions occurring in catheterized patients during symptomatic bladder infections result in elevated chromosomal breakage of urothelial cells.

RELEVANCE

1. Catheterized patients have been reported to have an elevated risk for urinary bladder cancer.

2. Chromosome breakage is associated with an increased probability of accumulating all the genetic changes necessary for cancer development. The assay for measuring chromosome breakage is micronucleus (MN) induction. Generally micronuclei are formed by chromosomal damage in the basal cells of the epithelium. When these cells divide, chromosomal fragments (or entire chromosomes which lack attachment to the spindle apparatus) lag behind and are excluded from the main nuclei in the daughter cells. These (pieces of) chromosomes are then identified as feulgen-positive extranuclear bodies within the cytoplasm of the cells (fig 1) (Rosin, M.P. Mutation Research, 267(1992)265-276).



Eventually, the basal cells mature and are exfoliated into the urine, allowing the relatively simple, noninvasive task of collection and analysis of micronuclei. This method of assessing chromosomal damage has been used on a variety of tissues. A summary table, adopted from Rosin, M.P. (Mutation Research, 267 (1992) 265-276) is provided:

Table 1
MICRONUCLEUS FREQUENCIES IN TISSUES AT ELEVATED RISK FOR CANCER

Risk factor	Population assessed	Sample site
Lifestyle	Betal quid chewers	Buccal mucosa
	Snuff users	Gingival groove
	Khaini tobacco users	Inner lip
	Nass users	Floor of mouth
	Cigarette smokers	Buccal mucosa
		Lung
		Cervix
		Esophagus
		Bladder
	Cigarette smokers/alcohol drinkers	Buccal mucosa
	Reverse smokers	Tongue, palate
	Heavy consumers of curried foods	Buccal mucosa
Occupation	Orchard sprayers (pesticide exposure)	Bladder
	Hospital workers (ethylene oxide exposure)	Nasal cavity
Genetics	Ataxia-telangiectasia patients	Oral mucosa, bladder
	Blooms syndrome patients	Oral mucosa, bladder
	Xeroderma pigmentosum patients	Tongue
Infections	Individuals with *S. haematobium* infection	Bladder
Premalignant lesions	Individuals with esophagitis	Esophagus
	Patients with metaplasia	Lung
	Patients with hyperplasia or dysplasia	Oral mucosa
	Patients with dysplasia	Cervix

APPROACH

The following steps are necessary for proper collection of statistically solid data. All information provided will be strictly confidential.

1. Accumulate baseline values of MN frequencies in catheterized patients.

We respectfully request:

•20 patients

•an informed consent to be filled out by each patient (researchers will provide the informed consent)

•a questionnaire to be filled out by each patient (researchers will provide the questionnaire)

•to collect samples (2-3) of urine directly from the catheter connecting tube with a regular sterile (30cc or 60cc) syringe

•urine samples (2-3) from holding bags

2. Accumulate data on MN frequencies during symptomatic bladder infection. Symptomatic infection has been defined as acute symptomatic cystitis (bladder spasms, abdominal bloating, dysreflexia, cloudy urine).

We respectfully request:

•a photocopy of all microbiology reports

•to collect a sample of urine directly from the catheter connecting tube with a regular sterile (30cc or 60cc) syringe as soon as possible after the onset of symptomatic bladder infection

•to start collection of urine from holding bags on the same day or as soon as possible after the onset of symptomatic bladder infection

•to collect every 3 days after for a total of 7 samples (collection period = 3 weeks)

THE PHONE NUMBER WHERE Dr.MIRIAM ROSIN and/or LORNE HOFSETH CAN BE REACHED UPON THE ONSET OF SYMPTOMATIC INFECTION IS:

877 - 6010 (local 5115)

**if the automated teleservice answers, press '1' then enter the local (5115)

**All sample regimens are user friendly so a minimum of interference with hospital protocol occurs. Absolutely no task outside normal hospital protocol will be asked of the patient or the professional.

Ethical approval for collection of urine from catheterized patients

SIMON FRASER UNIVERSITY

SCHOOL OF KINESIOLOGY

BURNABY, BRITISH COLUMBIA V5A 1S6
Telephone: (604) 291-3573
Fax: (604) 291-3040

July 21, 1992

Dear Dr. Hahn,

In regards to Lorne Hofseth's proposal to the British Columbia Rehabilitation Society: Pearson Centre, entitled 'Genetic damage in catheterized patients with Bladder Infections: the assessment of micronucleus frequencies in urothelial cells', I have granted ethical approval. Formal University Ethics Committee approval is unnecessary in the present case.

Sincerely,

J.A. Hoffer, Ph.D.
Director, School of
Kinesiology

Formal consent regarding collection of urine from catheterized patients

British Columbia
Rehabilitation Society

George Pearson Centre
700 West 57th Ave
Vancouver
British Columbia
Canada

Tel (604) 321-3231
Fax (604) 321-7833

September 24, 1992

Dr. Miriam P. Rosin
Division of Epidemiology,
Biometry & Occupational Oncology
British Columbia Cancer Agency
600 West 10th Ave
Vancouver, B.C.
V5Z4E6

Dear Dr. Rosin:

I am writing to confirm the participation of George Pearson Medical Centre in your laboratory's study on the involvement of inflammatory reactions in long-term catheterized patients. We understand your need to obtain urine specimens before and after episodes of cystitis in order to evaluate changes in chromosome breakage in exfoliated urothelial cells. This project has been discussed among the physicians and nursing staff of the centre and both groups have agreed that you will be informed as these cases occur. Both groups are aware of the importance of timely collection of specimens.

We have discussed these concerns with Mr. Lorne Hofseth of your laboratory and have agreed upon the procedures necessary for the successful conclusion of this study, which should contribute to a better understanding of the impact of catheterization on bladder pathology.

Sincerely yours

Dr. Michael Lapin, MD, FRCP(C)
Associate Medical Director

Example of the informed consent form each catheterized patient signed before urine was collected

SIMON FRASER UNIVΓRSITY
INFORMED CONSENT BY SUBJECTS TO PARTICIPATE IN A RESEARCH PROJECT OR EXPERIMENT

The University and those conducting this project subscribe to the ethical conduct of research and to the protection at all times of the interests, comfort, and safety of subjects. This form and the information it contains are given to you for your own protection and full understanding of the procedures, risks and benefits involved. Your signature on this form will signify that you have received the document described below regarding this project, that you have received an adequate opportunity to consider the information in the document, and that you voluntarily agree to participate in the project.

Having been asked by **LORNE JOHN HOFSETH** of the **KINESIOLOGY** Faculty/School/Department of Simon Fraser University to participate in a research project experiment. I have read the procedures specified in the document entitled: **GENETIC DAMAGE IN CATHETERIZED PATIENTS WITH BLADDER INFECTIONS: THE ASSESSMENT OF MICRONUCLEUS FREQUENCIES IN UROTHELIAL CELLS**. I understand the procedures to be used on this experiment and the personal risks to me in taking part.

I understand that I may withdraw my participation in this experiment at ant time.

I also understand that I may register any complaint I might have about the experiment with the chief researcher named above or with **DR. J.A. HOFFER**, Director of **KINESIOLOGY** Simon Fraser University.

Copies of the results of this study, upon its completion, may be obtained by contacting: **LORNE HOFSETH** or **DR. MIRIAM ROSIN**.

I agree to participate by **OFFERING VOIDED URINE TO THE RESEARCHER WHO WILL COLLECT THIS FROM THE CATHETER COLLECTING BAG AND SAMPLE PORT** as described in the document referred to above, during the period: **SEPTEMBER, 1992 - SEPTEMBER, 1993 AT GEORGE PEARSON MEDICAL CENTRE.**

NAME (please print)____________________________________
ADDRESS: ____
SIGNATURE:____________________ WITNESS:_______________
DATE:____________________

Once signed, a copy of this consent form and a subject feedback form should be provided to you.

Example of one of the notices handed out to staff at George Pearson Centre during the study.

Date: September 28, 1992

To: All staff at George Pearson Medical Centre

From: Lorne Hofseth
Ph.D. candidate
Simon Fraser University

Re: Research at George Pearson Medical Centre Topic: "Genetic Damage in Catheterized Patients with Inflammation of the Bladder: the Assessment of Micronucleus Frequencies in Urothelial Cells"

As you are all aware, I (with the extraordinary help of Delia Magallanes) have been busy coordinating a study that will, hopefully, give us a better understanding regarding bladder pathology of patients with indwelling catheters. I would like to express my sincere gratitude to all staff who cooperated with the administrative activities (patient records, informed consents, etc.) necessary to initiate this study.

I am now ready to start collecting urine for analysis of baseline data on genetic damage in urothelial cells. To remind you, this urine will be collected from the sampling port of the indwelling catheter (sterile collection for urinalysis) and the flutter valve of the catheter drainage bag (for collection of urothelial cells). Delia Magallanes has gone through the procedure of sterile collection and will assist me in collecting the first few samples of urine.

I will collect the urine between 9:00am and noon on each sampling day. If the catheter needs to be clamped for collection from the sampling port, I will clamp the hose (as Delia showed me) and post a sign beside the bed: "CATHETER CLAMPED". I will keep a close eye on the patient while the catheter is clamped.

The first days of collection will be on Thursday, October 1 and Friday, October 2, 1992. I will collect from one person on each ward during these days (approximately 5 persons per day). The purpose of collection on these days is to brush up on the proper technique for urine collection and analysis with minimum disruption of normal hospital protocol.

If otherwise suggested, on Monday, October 5, 1992, I will start collecting urine for complete analysis of baseline data from those patients who have so far consented to take part in the study. I hope to collect from the following wards on the following days:

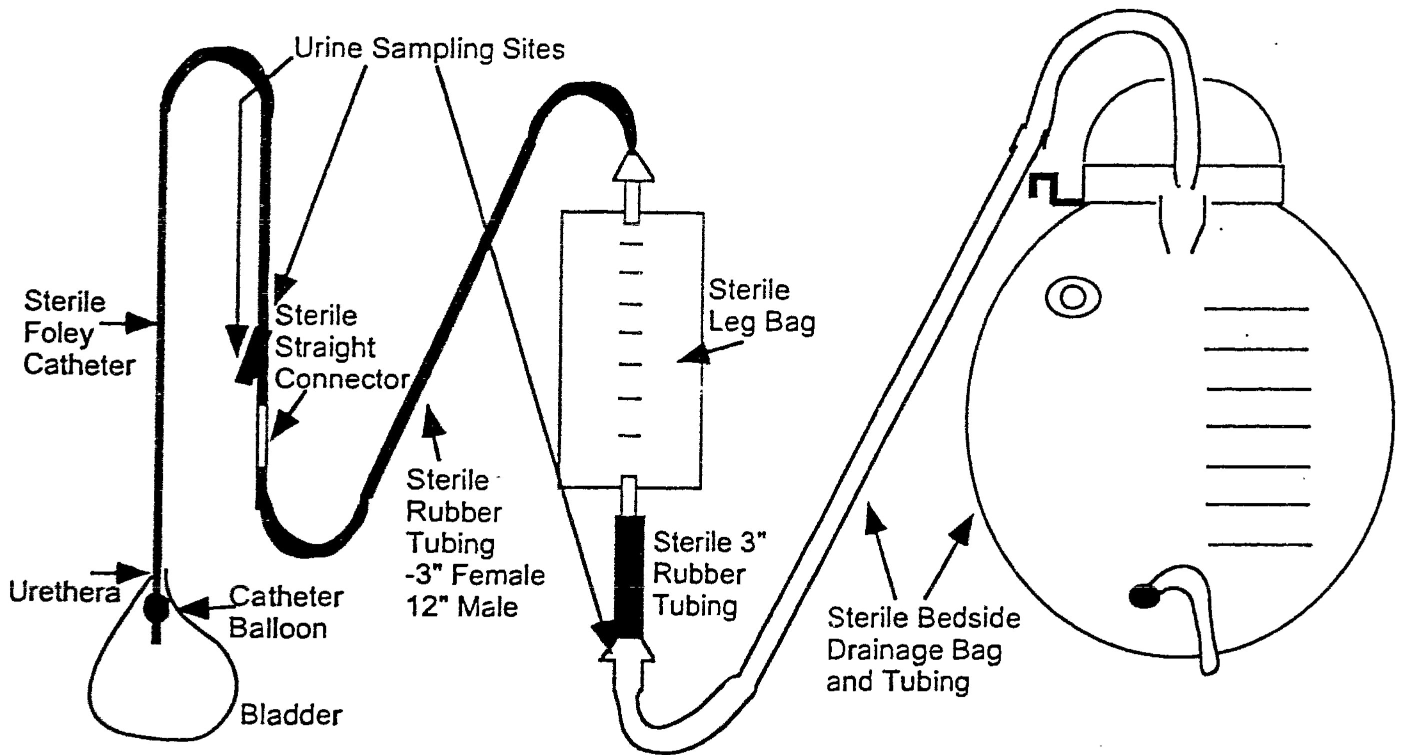
Mondays	Wards 1 and 7
Tuesdays	Ward 2
Wednesdays	Ward 3
Thursdays	Ward 5
Fridays	Collect from those patients whom I missed on the first 4 days

Please note that I am NOT collecting urine during periods of cystitis at this time. I will let you know when I am collecting urine for this purpose. At that time, I will post a letter regarding which patients are participating in collection, and the phone number I can be reached at.

Thank You,
Sincerely,

Lorne Hofseth
Ph.D. candidate

Diagram of the catheter system used by patients at George Pearson Medical Centre



CLOSED URINARY DRAINAGE SYSTEM

Example of the information sheet used when collecting information from the patient files at George Pearson Medical Centre

CATHETERIZED PATIENT QUESTIONNAIRE

Date:

Name ______________________
Birthdate ______________________
Sex ______________________
Study code ______________________

MS Patient? Quadriplegic? Paraplegic?

Date of last catheter change:

Frequency of catheter change over last year:

How many infections in the last two years?

Date of last infection:

Most predominant bacterial species in last infection:

Additional comments:

1. Have you ever smoked cigarettes regularly?

    If yes,

    How old were you when you first started regular cigarette smoking?

    On average of the entire time you smoked, how many cigarettes did you smoke per day?

    Do you smoke cigarettes now?

    If yes to the previous question, how many cigarettes do you smoke per day now?

    What brand of cigarettes do you smoke?

    Do you or did you inhale the cigarette smoke (check one):

    Slightly
    Moderately
    Deeply

2. In the last year, about how often have you taken at least one drink of beer, wine, liquor, or any other alcoholic beverage? (consider one drink roughly equivalent to one bottle of beer, one ounce of liquor or one glass of wine). Check one.

    10 or more times per day
    5-9 times per day
    2-4 times per day
    1 time per day
    4-6 times per week
    2-3 times per week
    1 time per week
    2-3 times per month
    1 time per month
    Less often than once a month
    Not at all in the last year

3. What was your occupation(s) during your time in the work force?

4. Have you ever been diagnosed with any form of cancer in the last year?

    If yes,

    Have you received any chemotherapy in the last year?

    Have you received any radiation therapy in the last year?

5. How often do you eat fresh or fresh frozen vegetables? (check one)

   Every day
   At least twice a week
   Less than twice a week

6. How often do you eat fresh fruit or fruit juice?

   Every day
   At least twice a week
   Less than twice a week

7. Are you taking any medications at present? If yes, please specify:

8. Are you taking any regular vitamin supplements?
   If yes, please specify:

Questionnaire filled out by non-catheterized controls

NON-CATHETERIZED CONTROL QUESTIONNAIRE

Date:

Name ______________________
Birthdate ______________________
Sex ______________________
Study code ______________________

1. Have you ever smoked cigarettes regularly?

   If yes,

   How old were you when you first started regular cigarette smoking?

   On average of the entire time you smoked, how many cigarettes did you smoke per day?

   Do you smoke cigarettes now?

   If yes to the previous question, how many cigarettes do you smoke per day now?

   What brand of cigarettes do you smoke?

   Do you or did you inhale the cigarette smoke (check one):

   Slightly
   Moderately
   Deeply

2. In the last year, about how often have you taken at least one drink of beer, wine, liquor, or any other alcoholic beverage? (consider one drink roughly equivalent to one bottle of beer, one ounce of liquor or one glass of wine). Check one.

   10 or more times per day
   5-9 times per day
   2-4 times per day
   1 time per day
   4-6 times per week
   2-3 times per week
   1 time per week
   2-3 times per month
   1 time per month
   Less often than once a month
   Not at all in the last year

3. What was your occupation(s) during your time in the work force?

4. Have you ever been diagnosed with any form of cancer in the last year?
   If yes,

   Have you received any chemotherapy in the last year?

   Have you received any radiation therapy in the last year?

5. How often do you eat fresh or fresh frozen vegetables? (check one)

   Every day
   At least twice a week
   Less than twice a week

6. How often do you eat fresh fruit or fruit juice?

   Every day
   At least twice a week
   Less than twice a week

7. Are you taking any medications at present? If yes, please specify:

8. Are you taking any regular vitamin supplements?

   If yes, please specify:

APPENDIX C

Scoresheets

Scoresheet for MN determination in exfoliated urothelial cells from catheterized patients at George Pearson Medical Centre

NUCLEAR ANOMALIES IN EXFOLIATED CELLS

STUDY CODE:
INVESTIGATOR:
DATE:

NORMALS		NORMAL NUMBER

MICRONUCLEUS SKETCH	LOCATION:	LOCATION:

COMMENTS:

MN FREQUENCY:

Scoresheet for analysis of urine from catheterized patients at George Pearson Medical Centre

Pearson Hospital Data Sheet for Baseline Values

Name	ID	Urine pH	Hemastix (red blood) Reading	Nitrite Dip Reading	Leukocyte Dip Reading	Urinary White Cell Count	Urinary Red Cell Count	Bacteria Count	Bladder Cells	Renal Cells	Crystals

Scoresheet for MN determination in cultured bladder cells

SAMPLE ID: TREATMENT: DATE SCORED: DATE OF EXPT:

TOTALS:				> 3 MN	TOTAL SINGLE CELLS:
TOTALS:				> 3 MN	TOTAL DOUBLE CELLS:
TOTALS:		> 3 NUCLEI	> 3 NUCLEI WITH MN		TOTAL CELLS:
TELOPHASE TOTALS:	TELOPHASE WITH BRIDGE	METAPHASE	OTHER COMMENTS:		TOTAL ALL CELLS:

CALCULATIONS: N.D.I. =
% SINGLE CELLS WITH MN =
% DOUBLE CELLS WITH MN =